From the Department of Physiology and Pharmacology
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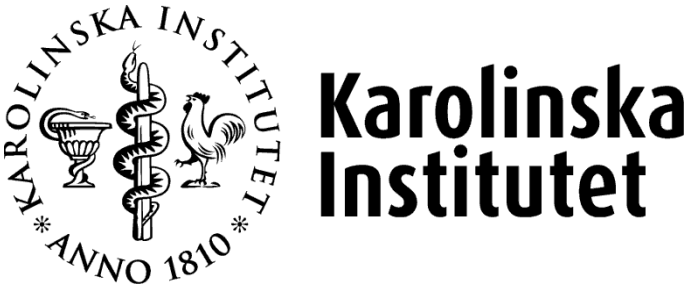
SUMOylation is a post-translational modification regulating skeletal muscle pathophysiology

Gabriel Antonio Heras Arribas



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SUMOylation is a post-translational modification regulating skeletal muscle pathophysiology

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my wife Esther, thank you for pushing me to reach for the stars.

To my parents Maria Pilar Arribas and Jose Antonio Heras, for being there every step of the way.

“Do. Or do not. There is no try”.

ABSTRACT

The research of this thesis is focused to investigate the role of SUMOylation, a protein post-translational modification reaction, implemented to the skeletal muscle pathophysiology area. SUMOylation is regulated by an enzymatic cascade of coordinated events capable of the reversible attachment of the Small Ubiquitin-like Modifier (SUMO) on to the targeted proteins. This reaction is highly susceptible to intra- or extracellular stimuli and responds immediately by altering the expression of its enzymes and the final SUMOylated products as an adaptation to the new status.

Skeletal muscle is a complex organ and it is unfortunately affected by severe diseases, which represent widespread pathologies affecting millions of people every year. Until now, despite many studies performed on the field, there is still a lack of information about the cellular and molecular mechanisms predicting or describing the early events of human muscle pathologies. We investigated different processes occurring among the SUMO network and the skeletal muscle functions and related them to the normal muscle activities or muscle alterations, from rodent models of muscle pathologies to human muscle biopsies. We described a tight correlation between the abundance of SUMO conjugated proteins and the different skeletal muscle fiber types. This association was quickly altered as a consequence of muscle activity changes or early events in acquired muscular disorders. We provided also a new skeletal muscle embryological classification based exclusively on the diverse abundances and distribution of the SUMO enzymes.

A combination of innovative techniques allowed us to identify and validate new SUMO skeletal muscle targets and determine the modulation of the SUMO enzymes abundances during myogenesis and the progression of acquired muscle diseases. These results assigned to some SUMO components a potential biomarker function to predict skeletal muscle dysfunctions.

Thus, the investigation on skeletal muscle disuse allowed us to discover a new transcriptional regulation mechanism of the E2 SUMO enzyme, Ubc9, mediated by the transcriptor factor PAX6 in soleus muscles under unloaded conditions.

Finally, we proved that targeting the SUMO pathway using chemical drugs as BGP-15 or anacardic acid have a positive effect on the treatment of myopathies and improving myogenesis under hyperglycemic conditions.

LIST OF SCIENTIFIC PAPERS

I. A Proteomic Approach to Identify Alterations in the Small Ubiquitin-like Modifier (SUMO) Network during Controlled Mechanical Ventilation in Rat Diaphragm Muscle. Namuduri AV, **Heras G**, Mi J, Cacciani N, Hörnaeus K, Konzer A, Lind SB, Larsson L, Gastaldello S.Mol Cell Proteomics. 2017 June 16.

II. Muscle RING-finger protein-1 (MuRF1) functions and cellular localization are regulated by SUMO1 post-translational modification. **Heras G***, Namuduri AV*, Traini L, Shevchenko G, Falk A, Bergström Lind S, Mi J, Tian G, Gastaldello S. J Mol Cell Biol. 2018 June 4.
(*Contributed equally to this study)

III. Expression of SUMO enzymes is fiber type dependent in skeletal muscles and is dysregulated in muscle disuse. Namuduri AV, **Heras G**, Lauschke MV, Maurizio Vitadello, Traini L, Cacciani N, Gorza L, Gastaldello S. FASEB J. 2019 December 16

IV. High glucose-induced oxidative stress accelerates C2C12 myogenesis by altering SUMO reactions. Liu X*, **Heras G***, Lauschke MV, Mi J, Geng T, Gastaldello S. *Manuscript*
(*Contributed equally to this study)

Additional publications (not included in the thesis)
"Circadian regulation of phosphodiesterase 6 genes in zebrafish differs between cones and rods: Implications for photopic and scotopic vision". Abalo XM, Lagman D, **Heras G**, del Pozo A, Eggerta J, Larhammar D. Vision Research. 2019 December 16.

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ADSIM	Acidic domain-containing SIM
ANOVA	Analysis of Variance
AP	Action potential
AP-1	Activator protein 1
ATF	Activating transcription factor family
ATG	Autophagy-related protein
ATP	Adenosine triphosphate
A.U.	Arbitrary units
BGP-15	3-Pyridinecarboximidamide, N-(2-hydroxy-3-(1-piperidinyl)propoxy)-, hydrochloride (1:2)
CMV	Controlled mechanical ventilation
CRIPS	Clustered regularly interspaced short palindromic repeats,
CSA	Cross-section area
Desi	DeSumoylating Isopeptidase
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DOC	Dodecyl sulfate
DTT	Dithiothreitol
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FGFs	Fibroblast growth factors
GLUT	Glucose transporters
HCM	Hypertrophic cardiomyopathy
HG	High glucose
HSP	Heat shock protein
HTF	HER2 Transcription Factor
ICU	Intensive care unit
IKK	IκB kinase
ISG	Interferon-Stimulated Gene
KLF5	Krüppel-like transcription factor 5

LB	Lysogeny broth
MAPKs	Mitogen-activated protein kinases
Mef2	Myocyte enhancer factor 2
MHC	Myosin heavy chain
MRFs	Myogenic regulatory factors
MuRF1	Muscle RING-finger protein-1
Myf5	Myogenic factor 5
MYH	Myosin heavy chain gene
MYO	Myosin gene
NADPH	Nicotinamide adenine dinucleotide phosphate
NEDD8	Neural-precursor-cell-expressed developmentally deep regulated 8
NEM	N-ethylmaleimide
NEMO	NF-κB essential modulator
NG	Normal glucose
NP-40	IGEPAL CA-630
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMs	Protein aggregate myopathies
PAX	Paired Box
PBS	Phosphate-Buffered Saline
PIAS	Protein inhibitor of activated STAT
PGC1α	Peroxisome proliferator-activated receptor-gamma coactivator 1 α
PML	Promyelocytic leukemia protein
PTMs	Posttranslational modifications
P38	Mitogen-activated protein kinase
qPCR	Quantitative polymerase chain reaction
RanBP2	RAN Binding Protein 2
RanGAP1	Ran GTPase-activating protein
RIM1 α	Rab3-interacting molecule 1α
RING	Really Interesting New Gene finger domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature

SAE1/2	SUMO activating enzyme subunit
SAP	SAF-A/B, Acinus and PIAS motif
SDS	Sodium dodecyl sulfate
SENPs	Sentrin-specific proteases
SERCA	Sarco/endoplasmic reticulum Ca2+-ATPase
SET7/9	Lysine Methyltransferase SETD7
SIM	SUMO interacting motif
SR	Sarcoplasmic reticulum
STUbLs	SUMO-targeted ubiquitin ligases
SUMO	Small Ubiquitin-like Modifier
TDG	Thymine-DNA glycosylase
TOPORS	Topoisomerase I binding, arginine/serine-rich
TPM	Tropomyosin gene
Ubc9	Ubiquitin-conjugating enzyme 9
UbL	Ubiquitin-like modifier
UPS	Ubiquitin proteasome system
USPL1	Ubiquitin specific peptidase like 1
VIDD	Ventilator-induced diaphragm dysfunction
wt	wild type

INTRODUCTION

Skeletal muscle is a developed composite structure important for our life. The complexity of this organ is maintained by regular body training that promotes the good health of the muscle. Unfortunately, modifications in the lifestyle or mutations in the human genome promotes muscle disorders. To understand how these changes contributed to altering the ordinary skeletal muscle functions, I present in this thesis a new concept focused to investigate the role of the SUMO protein posttranslational modification in muscle pathophysiology.

1. MUSCLE

Muscle is one of the main soft tissues present in the animal kingdom. It is in charge of generating motion, maintaining body posture, and temperature homeostasis. Muscle cells also produce and secrete specific cytokines called myokines[1,2]. Muscle is categorized into three different types according to cell structure and function: cardiac, skeletal, and smooth (**Figure 1**).

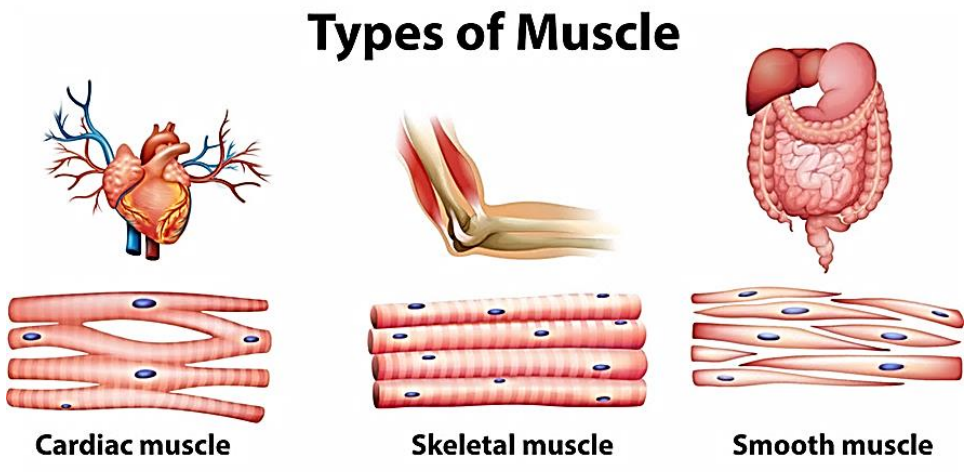

Figure 1: Types of muscle. The three types of muscles in the body, cardiac, skeletal, and smooth with their cellular organization. Blueringmedia, Vectorstock[3].

Cardiac muscle is formed by single striated cells with multiple branches and forms the major contractile tissue of the heart. Skeletal muscles are formed by multinucleated striated cells with a linear organization and gather the majority of the limbs, diaphragm, and neck-head muscles. Smooth muscle cells lack striation and form part of the inner structures of blood vessels, the uterine system, and the respiratory and digestive tracks.

Muscle cells contract in response to signals sent by the nervous system. Skeletal muscle cells receive the signal from the central nervous system causing a voluntary contraction in response to conscious orders, while cardiac and smooth cells receive the signal from the autonomic nervous system and contract involuntarily[4].

1.1. Skeletal muscle

Skeletal muscle forms the 30-50% of total human body weight and stores about 50-75% of all human body carbohydrates and amino acids. These compounds are released into the blood to maintain constant glucose levels during starvation since the release of amino acids stimulates the secretion of insulin and glucagon[5,6]. The skeletal muscle consists of different integrated components: blood vessels, nerve fibers, connective tissue, and muscle fibers. The blood vessels provide nutrients, oxygen, and remove the metabolic residues. Nerve fibers send the contraction signals to the muscle. The connective tissue helps the muscle to maintain its structure creating different organized structural levels of myofibers, muscle fascicle, and skeletal muscle (**Figure 2**).

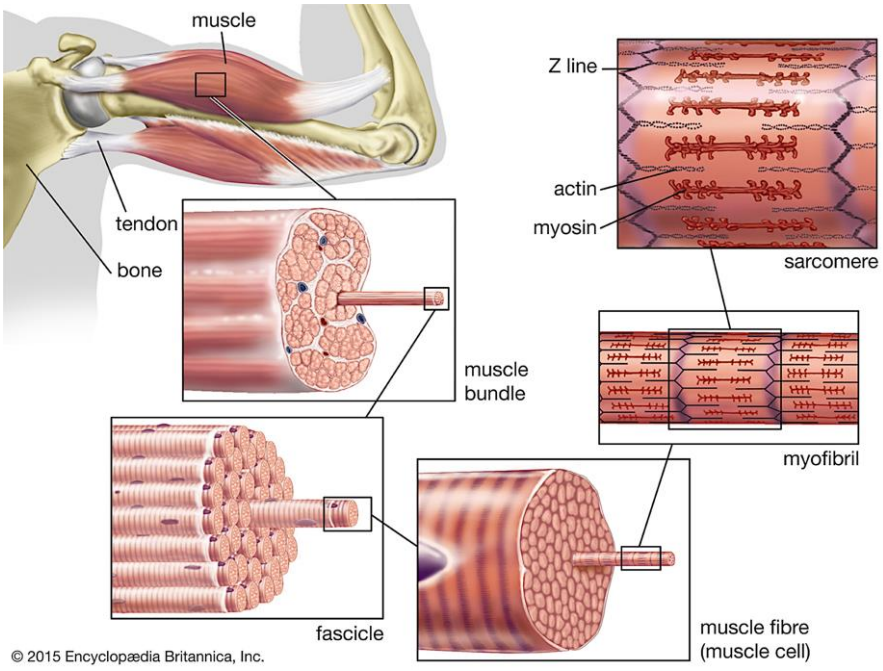

Figure 2: Striated muscle; human biceps muscle. Structural levels of the muscle organization, from muscle to the sarcomere. Encyclopædia Britannica[7].

Myofibers are long and cylindrical cells with multiple flattened nuclei in the periphery and surrounded by the myofiber cell membrane, the sarcolemma, that is enveloped by the basal lamina and connective tissue. Sarcolemma acts as a barrier between the extra- and intracellular spaces, allows the nutrients transport through different channels, maintains

the cell architecture and force transmission, and conducts the action-potential stimuli through the T-tubes. The T-tubes are membrane invaginations that permit a faster transmission of the action-potential into the muscular cells and help in the intracellular calcium regulation. Sarcoplasm, the muscle cell cytoplasm, contains myofibrils, glycogen for energy supply, and hemoglobin for gas exchange. More, sarcoplasm has a great abundance of mitochondria to fulfill high energy demand, and it is traversed by the sarcoplasmic reticulum, which forms a tight network storing calcium ions for the muscle contraction.

The myofibrils constitute the contractile structures through the repetition of sections called sarcomeres that give the striated appearance to the cardiac and skeletal muscles. Sarcomeres possess a highly organized arrangement of the contractile filaments, actin and myosin, and the regulatory proteins troponin and tropomyosin[8]. The sarcomere anatomy is described according to the observations using electron microscopy (**Figure 3**).

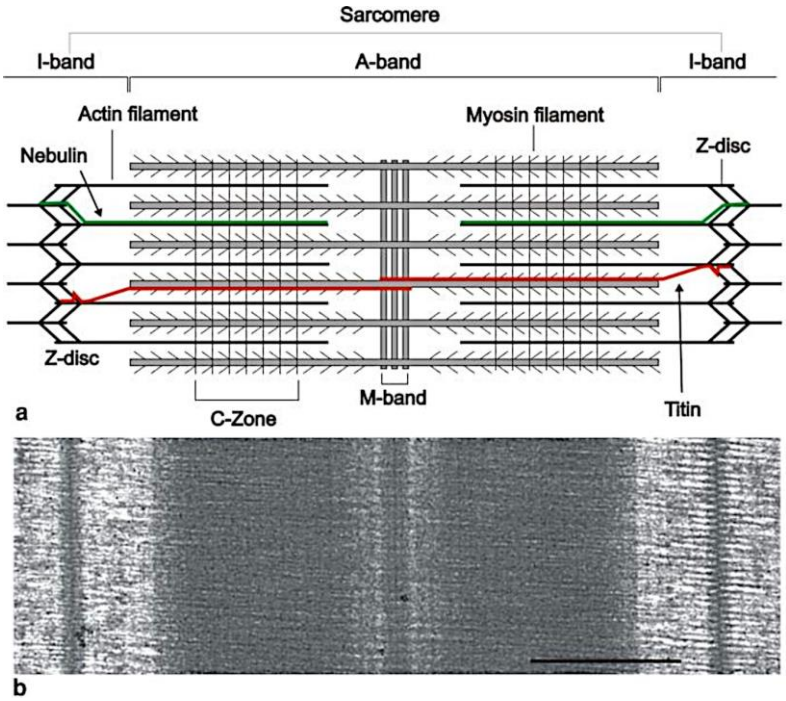

Figure 3: Striated muscle sarcomere. Schematic diagram. A-band of myosin filaments crosslinked with thin actin-containing filaments that end at the Z-disc (**a**). Nebulin (green) and titin (red) contribute to the structure. Electron micrograph of a longitudinal section of fish white (fast) muscle (**b**). Scale bar = 500 nm. Pradeep K Luther, J. Muscle Res. Cell Motil, 2009[10].

Indeed, the muscle fiber shows a dark middle band framed by two light zones. The dark zone, name as A-bands, is caused by high protein density corresponding to thick filaments overlapping with the thin filaments. A different zone, the M-line, localized in

the middle possess connective tissue merged with the thick filaments to maintain the structure. The lighter zones (I-bands) have less protein density and have a dense band (Z-disk) in the middle with connective tissue merged with the thin filaments[9]. The region contained between Z-disks is defined as a sarcomere.

1.1.1. Myosin

Myosins (500 kDa) are a big superfamily of motor proteins and the human genome encode more than 40 *MYO* genes belonging to 11 clases[11]. They interact with actin to produce muscle contraction and are involved in other activities like cell movement, transport of organelles, and mitosis. Myosins are divided into 3 regions, an N-terminus head with ATPase activity for the binding and movement over the actin filaments, a middle region, and the C-terminus tail[12]. Myosin II is the most common class, it is responsible for muscle contraction, and can be found in striated and smooth muscle cells and forming stress fibers in non-muscular tissues[13]. It is composed of two heavy chains forming the double head and the coiled-coil tails and four light chains forming the middle regions and binding the heavy chains[14,15].

1.1.2. Actin

Actin (42 kDa) is a highly expressed protein in the cells and can be found in two different states, monomeric (G-actin), or combined in a polymeric filamentous chain (F-actin). Actin can perform protein-protein interaction with a vast amount of actin-binding proteins, making it a key player in multiple functions within the cell[16]. There are 5 actin isoforms, 3 α-isoforms expressed in each of the three muscle types (smooth, skeletal, and cardiac), and a β- and γ-isoforms in all the cells, with slight amino acid differences among them mainly close to the N-terminus. The combination of two F-actin filaments forming a helicoidal structure is essential for the contractile force production.

1.1.3. Troponin

Troponin is a specific protein complex of skeletal and cardiac muscles. There are three regulatory units in the complex: troponin T (34 kDa), troponin I (23 kDa), and troponin C (18 kDa), with different functions. Troponin T anchors the troponin complex to tropomyosin. Troponin I binds the troponin-tropomyosin complex to the actin filament and blocks the actin-myosin interaction in the absence of calcium. Troponin C possesses

calcium-binding sites and induces the conformational change of troponin I to start the contraction[17].

1.1.4. Tropomyosin

Tropomyosin (70 kDa) has four tropomyosins (*TPM*) genes that generate 40 isoforms in mammals. Tropomyosin is localized in the myosin-binding sites of the actin proteins, blocking them. When the calcium levels rise and the contraction process begins, troponin rotates tropomyosin position freeing the myosin-binding sites on actin[18].

1.2. Myogenesis

Myogenesis is the process that promotes skeletal muscle formation during embryonic development but also in any other circumstances when muscle regeneration or reparation is required, like injuries or muscle remodeling. In embryonic development, a high abundance of fibroblast growth factors (FGFs) are produced inducing the myoblast proliferation process, consequently, when the FGFs levels decrease, the myoblast division is arrested and the myogenesis program begins. During muscle regeneration, activated satellite stem cells respond to the cytokines released from the immune cells as inflammatory signals to repair the damaged myofibers[19]. The satellite stem cells will follow an asymmetric division with one of the daughter cells remaining as stem cell and the other starting the differentiation into the myogenic pathway to provide enough cells to heal the fiber[20]. When muscle reparation occurs due to a severe injury condition, muscle regeneration is associated with fibrosis events that contributed to repair the area, replacing the damaged myofibers with connective tissue.

Myogenesis is a highly regulated event. Indeed, the gene expression of myogenic regulatory factors (MRFs) such as MyoD, Myf5, and Mrf4 are indispensable for skeletal muscle development. Satellite stem cells expressing high levels of Myf5 and Pax7 will follow the myogenic pathway[21]. Pax is a family of transcription factors with an important role in tissue specification and organ development[22]. An increase of MyoD gene expression regulated by Pax7 will induce the proliferation, differentiation, and muscle regeneration. Myogenin is also part of the MRF family with a later role in the differentiation of myoblasts into skeletal muscle fibers[23](**Figure 4**).

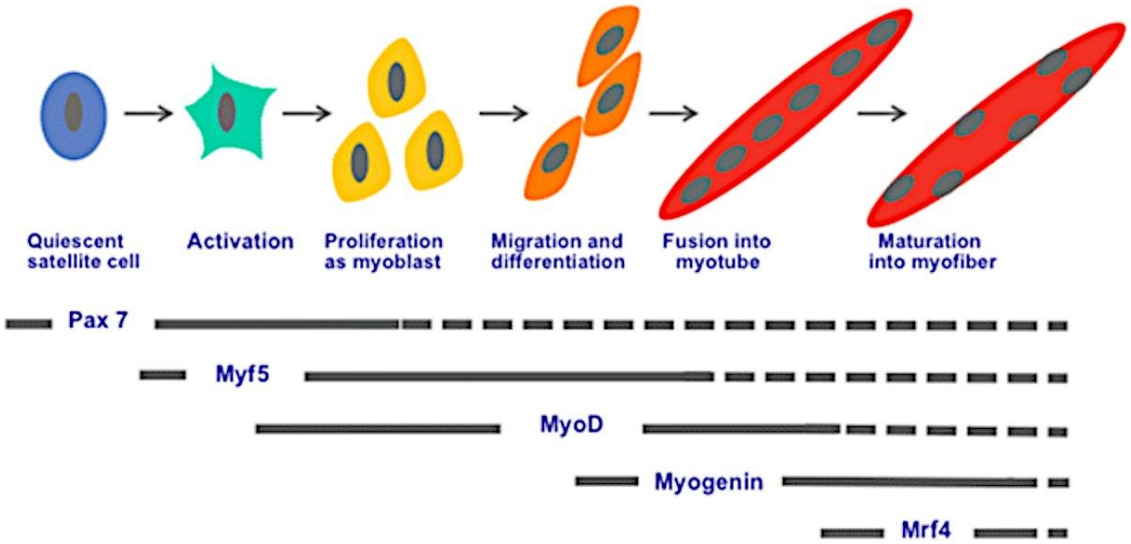

Figure 4: The myogenic regulatory factors pathway during skeletal muscle regeneration. Myogenesis steps from quiescent cells to mature myofibers. Expression of the different MRFs and Pax7 during the different stages. Nadège Zanou et al., Cellular and Molecular Life Sciences, 2013[24].

Other important players in the myogenesis pathway are the reactive oxygen species (ROS). ROS are oxygen-containing species that are chemically reactive and includes hydrogen peroxide (H_2O_2), superoxide ($\bullet O_2^-$) and hydroxyl radical (•OH). ROS origin can be mitochondrial, produced as waste when the electrons leaking from complex I and III react with oxygen, or cellular, generated by other cellular sources including NADPH oxidases, lipoxygenases, and cyclooxygenases with a different purpose like synthesis, signaling, or defense mechanism. ROS levels reduce the nuclear binding activity of the transcription factor AP-1, allowing the activation of the myogenin promoter for its expression. The activation of the satellite stem cells is mediated by mitogen-activated protein kinases (MAPKs) as a response to oxidative stress, inducing the expression of MyoD[25]. Lately, ROS have been found playing an important role as secondary messengers for many biological pathways such as healing process[26], cancer metastasis[27] and accelerating the myogenesis[28]

1.3. Skeletal muscle contraction

The voluntary contraction of the skeletal muscle is induced by signals from the central nervous system[29]. A motor unit is formed by a motor neuron that goes from the spinal cord to the muscle fibers that innervates. The neuron transmits the action potential (AP) into the muscle fibers through the branches of its axon. The AP induces the secretion of the neurotransmitter acetylcholine (ACh) activating its receptors in the muscle fiber, causing the sarcolemma depolarization and generates a new AP. The AP spreads radially,

traveling into the cell via the T-tubes to reach the sarcoplasmic reticulum (SR) releasing the calcium contained inside into the cytoplasm. Calcium interacts with troponin changing its conformation and initiating the contraction. When the depolarization is over, calcium is reabsorbed by the SR through the SERCA (SR calcium-ATPase) pumps, and the contraction finishes leading to the relaxation of the muscle[30]. The myosin-actin cycling produces the contraction through the sliding of the actin and myosin filaments one over the other. This movement requires several steps and the hydrolysis of ATP as an energy source. In the first step, ATP binds the myosin's head inducing its release from actin. In the next step, ATP hydrolyzes into ADP+P_i and the energy released induced the elongation of the myosin head and the binding in a new myosin-binding site in the actin. Finally, ADP+P_i is released and the head of the myosin bends producing the movement[31] (**Figure 5**).

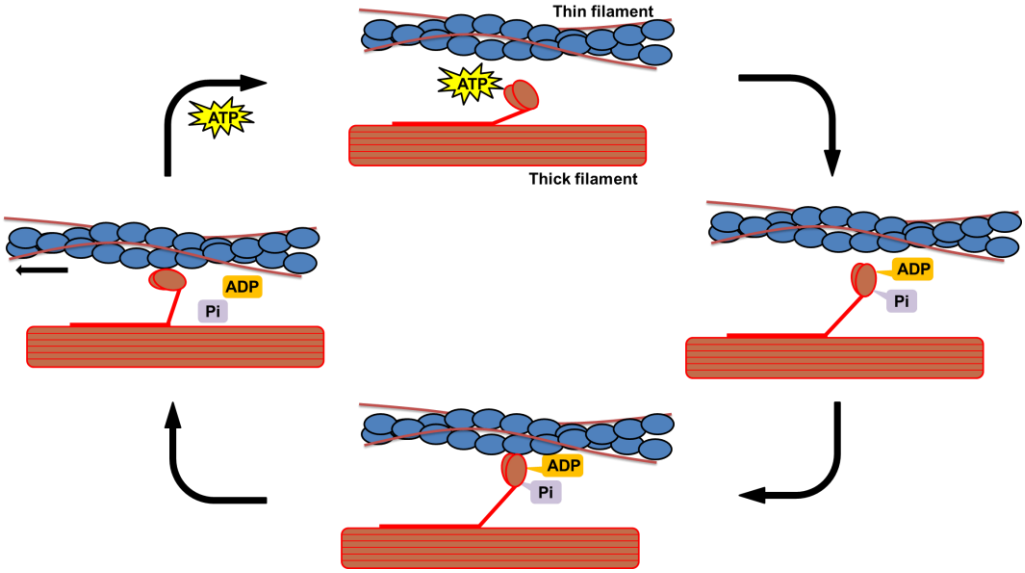

Figure 5: Skeletal muscle contraction steps. Adapted from Earth's Lab[32].

1.4. Fiber type and skeletal muscle function

Muscle fibers are classified according to the myosin heavy chain (MHC) isoforms expressed on them. The *MYH* genes encode the expression of four MHC isoforms: MHCI (*MYH7*), MHCIIa (*MYH2*), MHCIIb (*MYH4*), MHCIIx (*MYH1*). MHCIIb is not expressed in humans limb muscles[33], so only the other three MHC isoforms are present (I, IIa, and IIx).

Muscle fibers are also classified depending on the speed of contraction (fast or slow) and the metabolism used for energy uptake (oxidative or glycolytic). The combination of

these two factors catalogs the muscle fibers according to differences in contraction, metabolism, and resistance to fatigue. Type I fibers have slow contractile speed and an oxidative metabolism thanks to a high mitochondria content, making them resistant to fatigue. Type II fibers are split into type IIa: fast with oxidative metabolism, type IIb: fast with a glycolytic metabolism, and therefore less resistance to fatigue and type 2X with an intermediate phenotype (**Table. 1**).

	Slow twitch	**Fast twitch**		
Fiber type	**Type 1**	**Type 2A**	**Type 2X**	**Type 2B**
Speed of contraction	Slow <<	Fast <	Fast <	Fast
Metabolic type	Oxidative	Oxidative	Glycolytic	Glycolytic
Resistance of fatigue	High	> High	Low >	Low
MHC gene	MYH7	MYH2	MYH1	MYH4

Table 1. Fiber type classification in type 1 fibers (red) and type 2 (blue), according to the speed of contraction, metabolism, resistance to fatigue, and myosin isoform.

The skeletal muscle fiber type composition is subordinated to the muscle functions. Usually, muscles are composed of different fiber types in diverse proportions, according to the need for contraction speed or resistance. However, the fiber type composition is not constant over individuals nor during life. Indeed, regular training changes the fiber composition with a positive effect on the speed or endurance of the specific muscle, on contrary, a complete fiber reorganization with loss in muscle properties is observed in muscle disuse or muscle diseases[34].

1.5. Myopathies

Myopathies are clinical disorders of skeletal and cardiac muscles. Myopathies are classified as inherited, with a genetic component and an early age development, or acquired, with a sudden or subacute presentation at an older age. The manifestation of those diseases is usually in the form of weakness and muscle atrophy[35].

In this thesis within the inherited myopathies, we considered the hypertrophic cardiomyopathy (HCM) and protein aggregate myopathies (PAMs). HCM is a genetic condition that causes an increase in the wall thickness of the left ventricle, making it harder to pump the blood. It usually causes shortness of breath, chest pain, arrhythmias, or sudden death[36]. PAMs are a group of diseases of striated muscle with a very broad origin and cause misfolded proteins to aggregate, observed in pathologies like Alzheimer's and

Parkinson's disease, epidermolytic keratin disease, or heart and skeletal muscle-like myopathy[37].

Among the acquired myopathies, Ventilator Induced Diaphragmatic Dysfunction (VIDD) and limb muscle inactivity were the ones used as muscle diseases models in this study plan. VIDD is the loss of diaphragmatic force-generating capacity caused by the use of mechanical ventilation. The outcome will cause difficulties in weaning from the ventilator and potentially the death of patients in the Intensive Care Unit (ICU)[38]. Limb muscle inactivity, associated with patients forced to rest due to injury or illness, has an atrophic effect in the muscle tissue since the body will break it down to conserve energy.

Hyperglycemia is a severe condition associated with diabetes; a disease caused by the excess of glucose in the blood due to insulin-resistant problems. We adopted this pathological disorder to investigate how muscle regeneration is affected by high glucose conditions. Certainly, diabetes is usually associated with muscle mass loss due to the upregulation of factors like FOXO1 which trigger the skeletal muscle protein degradation pathways[39].

2. SMALL UBIQUITIN-LIKE MODIFIER PROTEIN (SUMO)

Protein post-translational modifications (PTM) are reactions catalyzed by enzymes where chemicals, lipids, sugars, or polypeptides are reversible or irreversible covalently attached to specific targets after their synthesis. These PTMs play an important role in dictating the target folding, function, localization, conformation, and activity. PTM are a fundamental part of the cellular and tissue signaling cascades that respond to intra and extracellular stimuli. A subgroup of PTMs showed the covalent linkage of small polypeptides, such as ubiquitin or ubiquitin-like molecules (UbLs, i.e., SUMO, NEDD8, ISG15, and ATG), providing a new structural reorganization of the target. This event possibly modulates the target-protein interactions, regulates the target activity, its subcellular localization, and stability or turnover.

The Small Ubiquitin-like Modifier (SUMO) is a protein of 101-amino acids (11 kDa). SUMO is covalently attached to the lysine residues on target proteins via the SUMOylation enzymatic cascade reaction. Five SUMO mammalian paralogs, SUMO1 to SUMO5, are transiently and reversibly conjugated to the substrate (**Figure 6**).

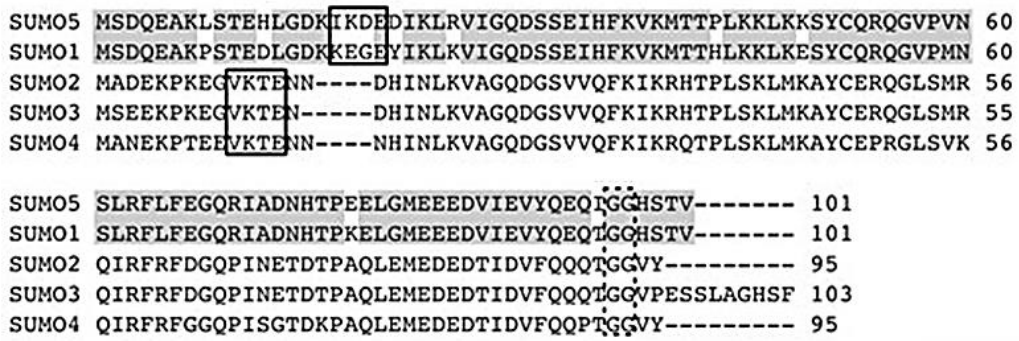

Figure 6: Alignment of amino acid sequences of SUMO5, SUMO1, SUMO2, SUMO3, and SUMO4. SUMO conjugation motifs are boxed by solid lines. The di-glycine motif for SUMO maturation is boxed by dashed lines. Yao Liang et al., Sci. Rep., 2016[40]

SUMO1, SUMO2, and SUMO 3 target a vast number of proteins for SUMOylation[41]. SUMO moieties are produced as precursors, and the isopeptidases, known as SUMO specific proteases, target them for its maturation[42]. Maturation generates a C-terminal carboxyl group, after a double glycine amino acid (SUMO-Gly-Gly-OH) that binds to the target protein lysine (K) residue in the consensus sequence, ΨKxD/E, via an isopeptide bond. In the consensus sequence, Ψ is a large hydrophobic residue, K is the target lysine and D/E are acidic residues[43]. SUMO2 and SUMO3 paralogs can polymerize, forming

polySUMO chains by covalent linkage of the C-terminal Gly-Gly-OH residue to a lysine available in their inner structure within a consensus sequence. SUMO1 lacks a consensus site and can't produce poly chains, usually working as a mono SUMOylation link or terminator in a polySUMO2/3 chain. The study of SUMO4 functions is difficult due to its specific tissue distribution, but the presence of a proline residue in its sequence prevents its maturation and conjugation to proteins[44]. SUMO5, the last addition to the family seems to play a role in the disruption of promyelocytic leukemia nuclear bodies (PML-NBs). SUMO5 helps to recruit components for its enlargement and enhance the conjugation of SUMO2/3 poly chains, inducing RNF4 activity for the PML-NBs disruption[40]. PML-NBs are interchromosomal accumulations of PML and other proteins that regulate diverse cellular processes like transcription, DNA repair, apoptosis, senescence, and tumor suppression.

2.1. The SUMO cycle and SUMO enzymes

SUMOylation is a dynamic reaction where only a small percentage of the targeted proteins is SUMOylated and their abundance is quickly affected as a response to internal or external cellular stimuli.

During SUMOylation, SUMO is attached to the target proteins through an enzymatic cascade mediated by the hetero-dimer E1 SUMO-activating enzyme, SAE1/2, the E2 SUMO-specific conjugating enzyme Ubc9, and in most cases, an E3 SUMO ligase, part of the PIAS enzyme family[45] (**Figure 7**).

The heterodimer of SAE1 and SAE2 triggers the activation of SUMO in an ATP-dependent way and transfers it in the conserved catalytic cysteine of the conjugation enzyme Ubc9. Then Ubc9, up to date the only E2 identified, delivers the SUMO moiety to the substrates. In some cases, Ubc9 alone is sufficient for the conjugation and ligation, however, the presence of the SUMO E3 ligases, facilitate the interaction of Ubc9 with the substrates, direct Ubc9 to specific targets, promote polySUMO chain formation, and introduce additional SUMO acceptor sites[46,47] (**Figure 8**).

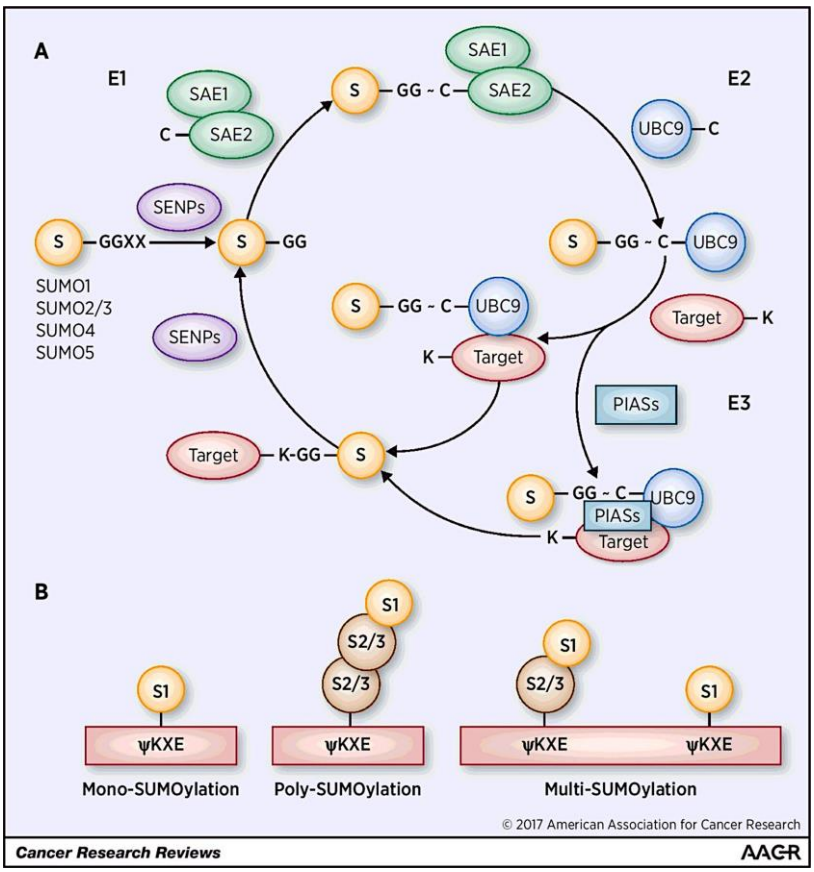

Fig. 7: Mechanism of SUMOylation. SUMO cycle with consecutive steps for the SUMO conjugation/deconjugation into the target (**A**). Different SUMO conjugation options with SUMO1 and SUMO2/3, from single units to poly chains (**B**). Andrea Rabellino et al., Cancer Res, 2017[48].

Fig. 8: PIAS proteins structure. SAP (DNA-binding motif), PINIT (nuclear translocation) RING (E3 SUMO protein ligase), ADSIM (acidic domain-containing SIM), S/T rich (Serine/threonine-rich C-terminal). Andrea Rabellino et al., Cancer Res, 2017[48].

In the case of SUMO, the covalent enzymatic reaction is reversible and mediated by specific proteases. In humans, three families are involved to assess the SUMO deconjugation and they are classified as SENPs[42], Desi[49], and USPL1[50]. These enzymes show specific activity among the different SUMO paralogues and different localization in

the cellular and nuclear compartments. SENPs contain a specific N-terminal domain in charge of the regulation of the cellular localization, proposing that each SENP may have a specific group of substrates[51] (**Figure 9**).

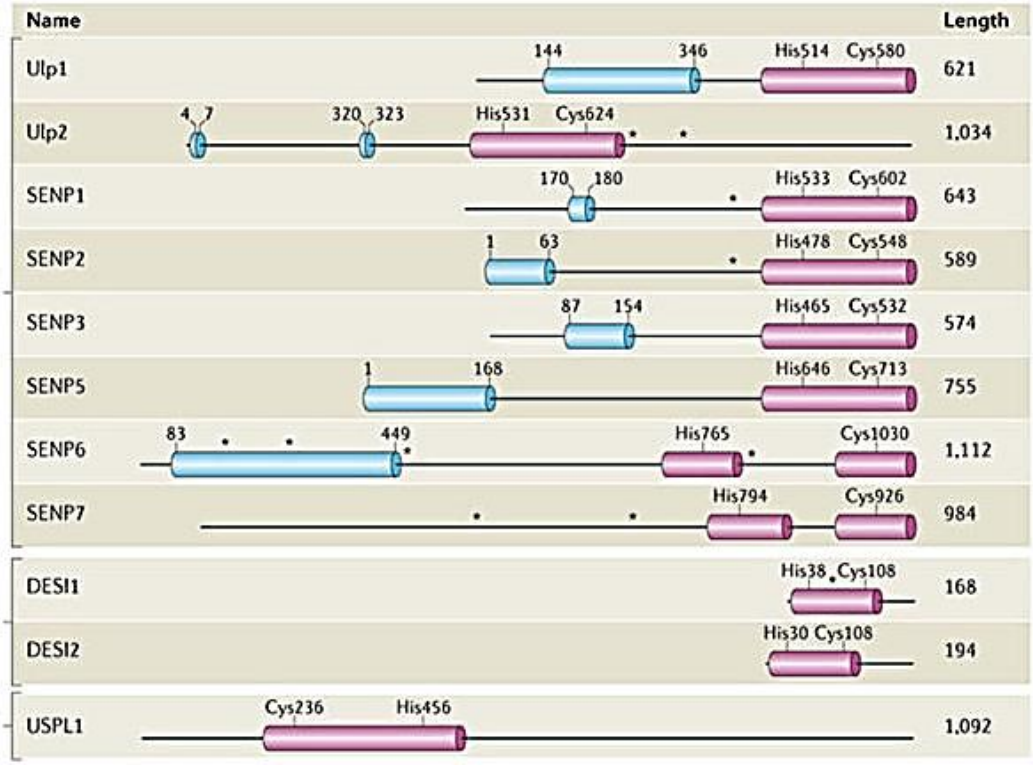

Figure 9: Structures of SUMO proteases. SUMO specific proteases with corresponding domains: catalytic domain in magenta, regions for intracellular localization in cyan, and SIM domains marked with asterisks. Christopher M. Hickey et al., Nature Reviews Molecular Cell Biology, 2012[52].

Besides SUMO conjugation into proteins, non-covalent protein interaction with SUMO is possible through the SUMO interaction motifs (SIMs). SIM domains contain a Val/Ile-X-Val/Ile-Val/Ile sequence that allows SUMO binding[53].

A connection between SUMO and Ubiquitin pathways was discovered with the SUMO-targeted ubiquitin ligases (STUbLs). STUbLs bind poly-SUMO2/3 chains via SUMO interaction motifs (SIMs) in tandem and target the SUMOylated protein with ubiquitin chains for degradation through the proteasome system. One example of this protein family is the mammalian RNF4, involved in the ubiquitin-dependent degradation of promyelocytic leukemia bodies (PML) after poly-SUMO2/3 conjugation, induced by different types of cellular stress[54]. The minimum amount of SIMs required to bind poly-SUMO chains is two, as it was demonstrated by the mutation of the different SIM domains in RNF4 sequence[55].

2.2. SUMOylation regulates diverse biological processes

SUMOylation is an important PTM involved in the regulation of crucial cellular functions including developmental and differentiation, and its alteration contributes to severe human diseases. However, most of the studies were performed on single eukaryotic cells from yeast to primary cells. The involvement of SUMOylation in regulating activity, functions, development, and disorders in complex systems like differentiated tissues (i.e., brain and muscles) is just emerging.

The SUMO network is vital for the survival of eukaryotic cells and changes in the expression levels of one enzymatic component may destabilize the entire cell behavior. For example, low levels of SAE1/2 will reduce the aggressivity of breast cancers with high expression of Myc[56], but in mammalian cells and mice embryos in development, the deletion of the SUMO E1 enzyme has a lethal efffect[56,57]. The high abundance of Ubc9 observed in the hippocampus and cerebral cortex suggests a potential role of SUMOylation in the synaptic and neuronal plasticity[58], but overexpression of Ubc9 is observed in ovarian, hepatocellular, prostate, and lung carcinomas[59].

The SUMO enzymes regulate the coupling and uncoupling of the SUMO moieties on the targeted protein in a balanced manner, as it has been observed in the cardiac gene regulation for the development and maintenance of the normal cardiovascular system[60]. Indeed, different abundances of SUMO enzymes were notified and associated with an altered SUMO reaction equilibrium, with a consequent variation in the total SUMOylated protein profiles observed, for example, in heart biopsies of patients who had severe developmental heart defects, including cleft lip and cardiac malformation[61].

The equilibrium of the SUMOylation reaction is also altered under different stress conditions including heat shock, high oxidative or osmotic environments, and ethanol poisoning. Stress situations are usually associated with a large increase in the global SUMOylation by SUMO2/3 in mammals, plants, and yeast[62]. Although this increase of SUMOylation is widespread in these situations, the affected substrates and regulatory circuits remain, however, largely unknown.

SUMO conjugation triggers the targeted protein in three different aspects: alters the activities, consents the localization or relocation in different cellular districts, or facilitates the interaction with other proteins. In fact, RIM1α, the Rab3-interacting molecule 1α, involved in the neuronal function, requires the conjugation with SUMO1 to increase the

synaptic vesicle presynaptic exocytosis activity[63] and the protein interaction of RanGAP1, (GTPase-activating protein for Ran that regulates the cytoplasm-nucleus transport), with the nuclear pore complex protein RanBP2 (RAN binding protein 2) and its nuclear translocation is allowed only when the first one is SUMOylated[64].

2.3. SUMO implication in skeletal muscle pathophysiology

The complexity of skeletal muscle physiology requires a crucial regulation and coordination of diverse muscle activities. As a new integrator pathway, SUMO orchestrates the correct functions of different cellular networks as they are summarized below.

SENP1 and SENP2 are important SUMO deconjugases operating in the myogenesis process[65,66]. As an example, the deSUMOylation of Sharp-1 mediated by SENP1, inhibits its activity, while SENP2 cleaves SUMO moieties from the transcription factor Mef2A, and promotes the skeletal muscle differentiation.

The sarcomeric organization is coordinated by the SUMOylation of different components like the Mef2 family of transcription factors[67], which enhances the transcription of Myomesin-1 for its incorporation into the sarcomeric structures[68]. Proteins involved in the mitochondrial adaptation to exercise like the myocyte enhancer factor-2 (Mef2), the peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC1α)[69], p38, and c-Jun[70], need the SUMO conjugation for its nuclear translocation.

The control of muscle energy metabolism is critical to avoid the development of metabolic syndromes or to correct obesity disorders. The inhibition of the Krüppel-like transcription factor 5 (KLF5) by SUMO attachment blocks its activity regulating the lipid metabolism[71]. Overexpression of Ubc9 protects the glucose transporter GLUT4 from degradation and increasing the response to insulin in adipocytes[72].

SERCA, the sarco/endoplasmic reticulum Ca^{2+}-ATPase has the role of transferring calcium from the sarcoplasm to the SR lume during muscle relaxation. SERCA2a is the isoform expressed in cardiac muscle and slow-twitch skeletal muscle. The conjugation of SUMO1 in SERCA2a has a protective effect during heart failure, with the increase of SUMO1 levels restoring SERCA2a levels[73].

There are some animal models developed to study the role of SUMO in different pathologies. SENP1 knockout or flox inducible for the study of apoptotic death during

transient brain ischemia/reperfusion[74] or His6-HA-SUMO1 KI mice for brain analysis of SUMOylated proteins[75].

2.4. SUMO as drug targetable pathway

A new line of research has emerged looking for compounds with effects in the modulation of SUMO PTM related to human diseases. New drugs are also designed to affect the main properties of the enzymes involved in the SUMOylation process to improve or reduce the interaction and/or conjugation of specific SUMO substrates. The massive changes in SUMO components associated with cancer, cardiac, and neurodegenerative diseases[76] give an insight into how important it is to keep a well-balanced SUMOylation profile. The peculiar reversible conjugation characteristic of SUMO makes it perfect for the development of specific pharmacological drugs to revert the effect of pathologies through its modification. In the last years, new drugs are starting to be established and to prove its positive effects in the treatment of heart failure, cancer, and other pathologies.

In vivo and in vitro studies provided new results showing the inhibitory effect of some drugs over the E1 activating enzymes SAE1 and SAE2. The gingkolic and anacardic acids impair the formation of the E1-SUMO intermediate[77] and show a positive effect in the treatment of non-promyelocytic leukemias[78]. A similar effect over the formation of the E1-SUMO intermediate was observed using kerriamycin B[79] and davidiin, having the last one a more potent effect even in lower doses[80]. The only drug under clinical trials right now is the inhibitor TAK-981, with an effect blocking the SUMO transference between the E1 and Ubc9[81].

On the contrary, the use of the small molecule N106 enhances the binding interaction of both E1 subunits, SAE1 and SAE2 increasing its conjugation activity and the SUMOylated fraction of SERCA2a, as a potential therapeutic strategy for heart failure treatment[82].

Ubc9 is also a drug target. Spectomycin B1 binds Ubc9 inhibiting its activity[83], and 2-Do8 blocks the SUMO transfer from the E2 to the substrate[84].

Topotecan decreases the total SUMOylation levels in glioblastoma cells by impairing SUMO1 conjugation of CDK6 and causing its degradation, but unfortunately, the mechanism involved in this activity is yet to be discover[85].

Finally, several new protein-based inhibitors and activity-based probes are useful for the inhibition of the SUMO deconjugases (SENPs) due to its implication in several cancer[86] (**Figure 10**).

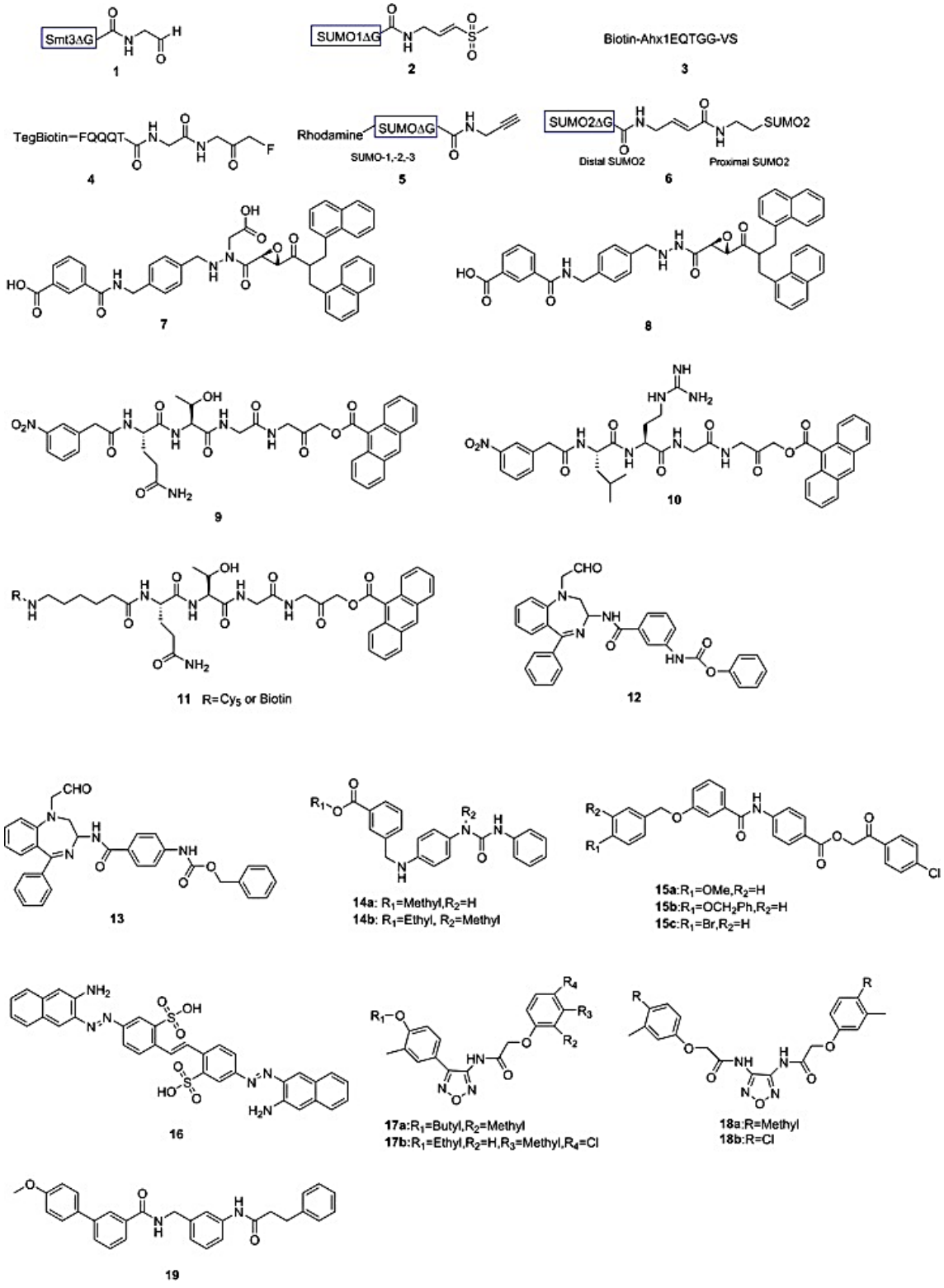

Figure 10: Overview of inhibitors and probes of SENPs. List of SENPs inhibitors and probes. Jia Yuqing, ACS Chemical Biology, 2019[86].

3. UBIQUITIN AND THE UBIQUITIN-PROTEASOME SYSTEM

Goldstein discovered the ubiquitin-protein in 1975[87]. Ubiquitin is a 76-amino acid peptide (9 kDa) and is covalently attached to the target through the ubiquitin cycle. The studies of the ubiquitin-mediated proteasomal degradation of regulatory proteins showed the essential role of the ubiquitin cycle in the cells. Ubiquitination controls many processes, including cell-cycle progression (cyclins and Cdk), signal transduction, transcriptional regulation (tumor suppressors, proto-oncogenes), receptor down-regulation, immune response, development, apoptosis, and endocytosis[88]. Pathological conditions, including malignant cell transformation, emerge when the ubiquitin pathway suffer alterations.

Ciechanover and Hershko found in 1978 that the ubiquitin-mediated proteolysis required the use of ATP as an energy source[89]. Ubiquitin is attached to the target protein through the sequential action of three enzymes. First, a specific activating enzyme (E1) activates the ubiquitin C-terminal Gly residue with ATP energy consumption. Through this step, an intermediate ubiquitin adenylate is created, PPi released and ubiquitin binds to a Cys residue of E1 in a thioester linkage, with AMP release. After that, the activated ubiquitin is transferred to a Cys active residue of a ubiquitin-carrier protein (E2) and finally a ubiquitin-protein ligase enzyme (E3) catalyzes the ubiquitin union in the substrate lysine residue by its C-terminus[90,91]. Once one ubiquitin molecule is attached to the protein, the process is repeated several times to form a polyubiquitin chain.

Ubiquitin has several lysines in its sequence helping to create different types of ubiquitin chains with specific functions. Lys48 ubiquitin poly chains mainly target substrates for the proteasome degradation, but new functions like the activity regulation of the transcription factor Met4 or the ubiquitin selective chaperone p97 activation are also performed[92]. Chains form through the Lys63 have non-proteolytic functions in different pathways including DNA damage repair, cellular signaling, intracellular trafficking, and ribosomal biogenesis, but new studies show its involvement in the degradation of some targets[93]. Unconventional ubiquitin poly chains of Lys6, Lys27, Lys29, and Lys33 have been found, but their function remains unknown.

3.1. Ubiquitinating enzymes

The E1 ubiquitin-activating enzymes catalyze the first step in the ubiquitination reaction[94]. The human genome encodes two E1 for the ubiquitin pathway:

UBA1 contains four different domains for the interaction with ATP[95] and Ub, the active cysteine, and the recruitment of specific E2s[96].

UBA6: Shares 40% of its sequence with UBE1 and some E2s, but it also has specific E2[97].

The E2 ubiquitin-conjugating enzymes participate in the protein substrate recognition, either alone or in combination with an E3 enzyme, being able of great specificity or overlapped functions. The E2-E3 interaction is very complex, involving different levels of specificity with a very precise effect over the target.

E3 enzymes are classified into three types of ubiquitin-protein ligases: HECT, U-box, and RING-finger. They have an important role in the specific bind of the E2 to the substrate and up to date, 500-1000 E3 ligases are described[98]. These enzymes localize in the different organs with tissue specificity and different localization in the cellular compartments, demonstrating how critical specificity is for the ubiquitin pathway.

3.2. Deubiquitinating enzymes

Before the ubiquitinated protein goes inside of the proteasome for degradation, the ubiquitin polychain is removed. Six families of enzymes are the ones performing the task: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), Machado-Josephin domain proteases (MJDs), ovarian tumor proteases (OTU), Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+) (JAMM), and the ZUFSP. USPs detach ubiquitin from the poly-ubiquitinated proteins, UCHs cleaves Ub/NEDD8, target ubiquitin precursors for maturation, and interact with Lys48 Ub chains to protect them from degradation. MJDs and OTU have high Ub chain specificity (K11, K48, K63) with specific roles[99]. JAMM are the only metalloproteases and use Zn^{+} in its active site for the deubiquitination[100]. ZUFSP has a single protein in the family, ZUP1 with an important role in genome stability.

3.3. E3 ubiquitin ligase MuRF1

MuRF1, MuRF2, MuRF3 compose the C-II family of TRIM proteins and are only expressed in cardiac, skeletal, and smooth muscles. All three proteins can homo- and hetero-dimerize via their coil domains. MuRF1 is a protein of 353 amino acids (40 kDa) encoded by the TRIM63 gene, MuRF2 (51 kDa) by TRIM55 and MuRF3 (40 kDa) by TRIM54 (**Figure 11**).

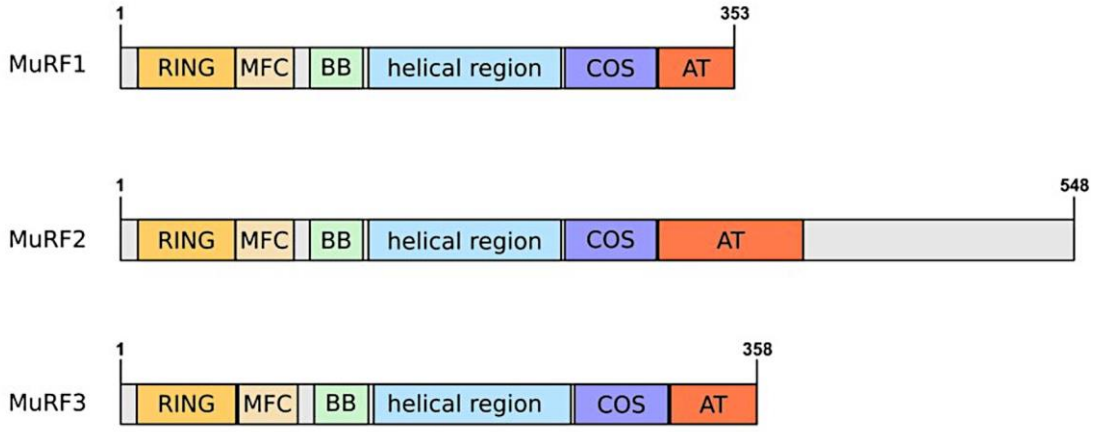

Figure 11: MuRF domains. MuRF protein family with corresponding domains. RING, MFC (MuRF family-specific domain), BB (B-box), helical region (coiled-coil domain), COS (C-terminal subgroup One Signature-box), and AT (acidic tail). Dr. Benjamin Suenkel, The Max Delbrück Center for Molecular Medicine[101].

Like the other TRIM proteins, MuRF1 follows the domain pattern of a RING-finger domain (E3 ubiquitin ligase function), followed by an MFC, a highly conserved region in the MuRF family for binding to PPAR (peroxisome proliferator-activated receptors). A B-box and two coiled-coil domains (helical region and COS) localize after them, for protein interactions and microtubule-binding, with an acidic tail at the end of the sequence that promotes binding with the microtubules with the help of the coiled-coil domains[102].

MuRF1 was first identified as a novel muscle-specific RING finger protein that bound to the kinase domain of the giant sarcomeric protein titin. MuRF1 localize at the M- and Z-line of the sarcomere[103] associated with MuRF2 and MuRF3. When there is inactivity, unloading, or muscle stress conditions MuRF1 is translocated into the myonucleus, where it may influence gene expression in the different atrophy models. The specific role could be direct, acting as a transcription factor or indirect, mediating polyubiquitin degradation of other transcription factors[104].

MuRF1 is the only family member related to muscle atrophy because its transcription is under the control of FOXO1 and FOXO3a that increases during certain forms of atrophy. The two major classes of proteins as putative substrates for MuRF1 are myofibrillar proteins and proteins occupied in ATP generation, in particular those involved in glycolysis, suggesting that MuRF1 could have a role in metabolic regulation since it was observed in cardiac mitochondria reducing the reactive oxygen species levels[105].

3.4. SUMO pathway and Ubiquitin Proteasome System

Ubiquitin and SUMO are part of the same family of ubiquitin-like proteins and common elements are shared in both pathways. They use similar enzymatic reactions involving the E1 activating, E2 conjugating, E3 ligase, and deconjugases enzymes to regulate the SUMO and Ub binding to the substrate's lysine. On the contrary, there are also some differences between the two pathways, SUMO enzymes cannot work as Ubiquitin-conjugating and deconjugating enzymes, and vice versa. More, predominantly, the main task described for the ubiquitin conjugation is the proteasomal degradation of proteins in the different cell compartments[106,107], while the SUMO moieties conjugation affects the enzymatic activity, protein-protein interactions, and cellular localization of the targeted proteins.

A large pool of proteins is a substrate for both SUMO and ubiquitin. Since both cycles usually compete for the same lysine, the expected result is an antagonistic effect[108]. Interestingly, a cooperative effect is observed in SUMO2/3 chains conjugated to specific substrates, which become a target of polyubiquitination reaction, mediated by the SUMO-targeted ubiquitin ligase (STUbL), RNF4, and addressed for proteasomal degradation[109].

Both ubiquitin and SUMO pathways can cross-regulate each other through the modification of their components, for example, the inhibition by SUMOylation of the ubiquitin E2-25k[110], the Gam1 ubiquitination of SAE1 for its degradation[111] or the complex relationship between the ubiquitin E3 enzyme Parkin and the SUMO E3 RanBP2. Parkin ubiquitinates RanBP2, promoting its degradation and RanBP2 binds non-covalently to Parkin enhancing its activity[112].

3.5. The ubiquitin and UbLs family

Besides Ub and SUMO previously mentioned, the ubiquitin-like protein family is formed by other proteins with similar structure but involved in the regulation of different cellular processes in the nuclear transport, proteolysis, translation, autophagy, and antiviral pathways[113] (**Figure 12**).

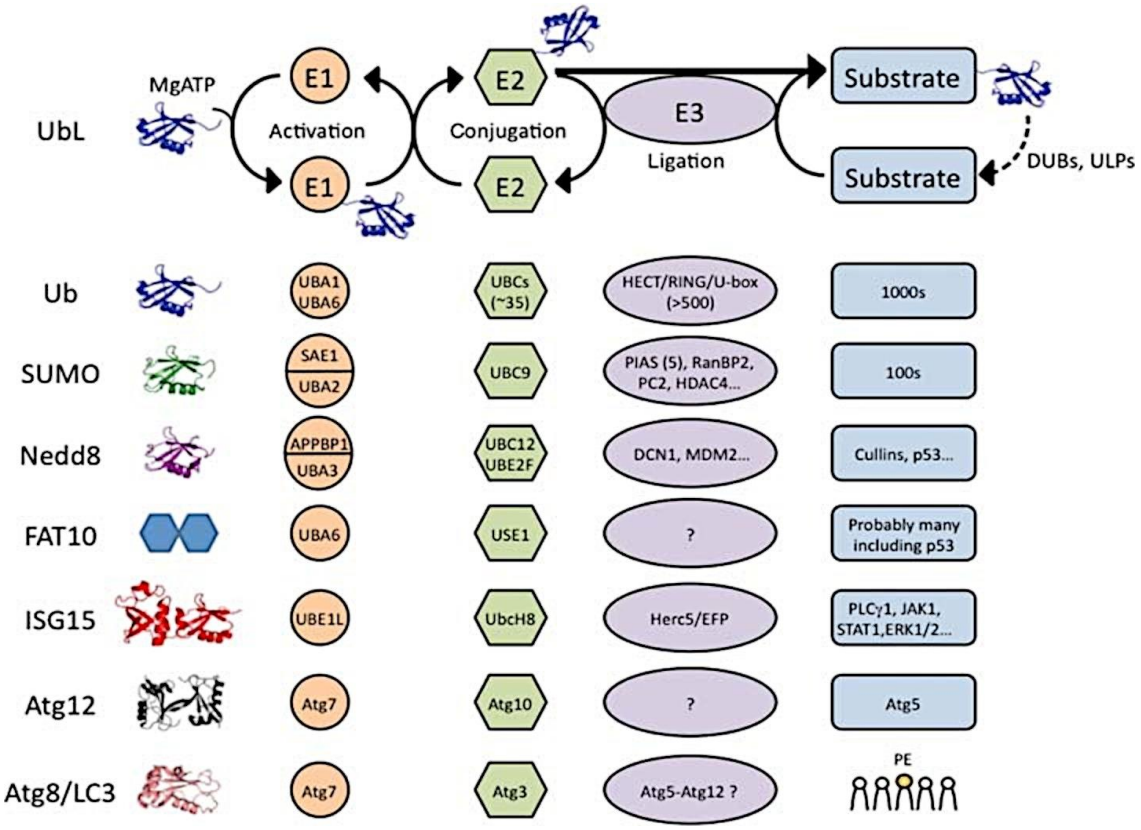

Figure 12: Schematic overview of the enzymatic cascades involved in the Ub and UbL conjugation pathways. Ubiquitin and ubiquitin-like family of proteins with 3D structure. Conjugation cycle with corresponding enzymes and known substrates for each UbL. Martine Biard-Piechaczyk et al. Biology of the Cell, 2012[114].

Nedd8 has a 58% similarity to Ub and was first found as one of the ”Neural precursor cell-Expressed, Developmentally Downregulated” genes expressed in embryonic mouse brain. Nedd8 regulates the Cul family members, needed for the assembly of multicomponent RING E3 ligases.

FAT10, the HLA-F adjacent transcript 10, is expressed in dendritic and B cells cytoplasm and is involved in the immune response, induction of caspase-dependent apoptosis, NF-κB activation, cell-cycle defects, and chromosomal instability.

ISG15, interferon-stimulated gene 15, was the first UbL identified and is involved in the interferon signaling after infection. Interferon type I mediates its expression and acts targeting viral and host proteins.

Atg8/LC3 and Atg12 regulate autophagosomal membrane growth and expansion: There is a correlation of Atg8 levels and the size of the autophagosomes, Atg12 forms a complex with Atg5 and Atg16L1 and works as an E3 ligase in the external side of the autophagosomal membrane.

4. AIMS OF THESIS

This thesis aims to understand the role played by the SUMO post-translational modification in the pathophysiology of skeletal muscle. We hypothesize that an invaluable role as a cellular sensor of environmental changes is attributed to the SUMO pathway for the correct muscle performance. Thus, by associating the alterations of the SUMO network with muscular diseases, we provide a new angle to understand the origins and offer potential innovative treatments for severe muscle pathologies.

4.1. Specific aims:

- To understand the functions of SUMOylation in the skeletal muscle physiology. Study the adaptation of the SUMO pathway under altered muscle conditions.

- To characterize the SUMO PTM on the E3 Ubiquitin ligase MuRF1. Understand how SUMOylation influences MuRF1 activity and cellular localization. Associate MuRF1-related human muscular disorders to its alterations in SUMO conjugation.

- To associate an unusual distribution in the SUMO enzymes and the SUMOylated proteins to the skeletal muscles localized in different body districts. Study the implications of the SUMO network modulation in muscle adaptation.

- To determine the variations of SUMO PTM in the myogenesis processes. Associate the impairment of muscle regeneration under hyperglycemic conditions with alterations in the SUMOylation reaction events during the myogenesis process.

5. METHODOLOGICAL CONSIDERATIONS

5.1. Eukaryotic cell lines

Immortalized murine myocytes, C2C12, and HeLa cells were cultured in DMEM-normal glucose (5.5 mM, 308-340 mOs/Kg) or high glucose (25 mM, 313-346 mOs/Kg) media, supplemented with 10% FBS and 100 IU/ml of penicillin-streptomycin antibiotics. Cells were grown in a humidified air atmosphere at 37 °C in presence of 5% CO_2.

5.2. Rodents and human samples

Rats: Female Sprague-Dawley rats were deeply sedated by isoflurane inhalation, paralyzed with α-cobratoxin, and maintained in constant protein, and fluid balanced from the beginning to the end of all mechanical ventilation processes. Animals were monitored continuously to detect any pain reactions (EEG activity, heart rate, and intra-arterial blood pressure). For the pharmacological assay, rats were treated with BGP-15 drug (40 mg/kg dose) during the desired period of mechanical ventilation. Control rats, not subjected to mechanical ventilation, were anesthetized with isoflurane for two hours and then euthanized. The experimental procedure was ended at different time points, from few hours to 2 weeks of mechanical ventilation, and animals were sacrificed by thoracotomy, hearts were removed, and all tissues were harvested. Surgery was performed under sterile conditions. All necessary steps were taken to minimize animal suffering described in the ethical permit N263/14.

Wistar-Han rats were caged individually and randomly assigned to experimental groups: freely ambulating as controls, 1-, 2-, and 4-days hind limb-unloaded animals. Hind limb muscles were unloaded using the tail suspension rat model, by wrapping the tail root with tape under general anesthesia, induced with intraperitoneal administration of 20 mg/kg of zolazepam chlorhydrate and tiletamine chlorhydrate. All animals were euthanized in the presence of anesthesia with isoflurane according to the ethical permit 502/2015-PR. Limb muscles were excised and frozen in liquid nitrogen.

Mice: BKS Cg-Dock7^{m}+/+ Lepr^{db}/J male mice, were used as a model of chronic hyperglycemia, this strain is used as a model in phases I - III of diabetes type II and obesity. 8- to 10-week-old mice were sacrificed by cervical dislocation and the respiratory muscles were immediately isolated and deeply frozen in liquid nitrogen and storage at −140°C.

Human: Muscle biopsies were isolated from the vastus lateralis muscle of volunteers participating in a campaign of muscle inactivity performance at 0, 8, and 35 days after a bed rest period. Procedures were described in the ethical permit 502/2015-PR.

5.3. ORFs cloning in expression vectors

To clone the ORFs of the analyzed proteins in prokaryotic and in eukaryotic plasmids, cDNAs generated from rodents or human skeletal muscle RNAs were used as templates and PCRs were performed together with designed primers containing specific restriction sites for the cloning. The PCR reactions were performed with 40 ng template, 20 μM forward and reverse primers, 20 mM dNTP's mix, High Fidelity Buffer, and 2 U Pfu enzyme in a final volume of 50 μl. The PCR cycling program was: denaturation 95°C for 5 min, followed by 25 cycles of denaturation 95°C for 30 sec, annealing (temperature according to the primers TM) for 30-60 sec, polymerization 72°C for 1 min, and a final step of 72°C for 10 min elongation. Amplicons were purified in agarose gel and extracted by using a gel extraction kit following the manufacture instructions. Ligase reactions were performed combining the digested plasmids and amplicons (molar ratio 1:3) in presence of ligase enzyme with the appropriate buffer, for 16 h at room temperature. Ligase reactions were used to transform competent DH5α and plated on bacteria LB agar medium, supplied with the appropriate antibiotic selection. Colonies were visible after 24 h of incubation at 37°C.

5.4. Side direct mutagenesis

The site direct mutagenesis was performed on the ORFs nucleotide sequences to generate proteins with different amino acid sequences. For this purpose, the QuikChange II Site-Directed Mutagenesis kit was adopted and complementary primers including the desiderated mutations were designed for each case. The mutagenesis reaction contained 100 ng of plasmid template, 20 μM of primers, 20 mM of dNTP's mix, High Fidelity Buffer, 2 U of Pfu Taq DNA polymerase, in a total volume of 50 μl. When required, 8% DMSO was added to the final reaction. The cycling program was: denaturation 95°C for 3 min, followed by 16 cycles of 95°C for 30 sec, 65°C for 1 min, 72°C for 5 min with a final step of 72°C for 10 min. After PCR, samples were treated with the DpnI enzyme at 37°C for 1 h in the presence of the appropriated reaction buffer. 20 μl of the digested sample was used to transform 50 μl of DH5α competent bacteria and plated on bacteria LB agar medium, supplied with the appropriate antibiotic selection. Colonies were visible

after 24 h of incubation at 37°C. Colonies were grown in the appropriated media overnight and the plasmid DNAs were extracted using Mini-Prep commercial kit, quantify and send to the sequence to confirm the nucleotide mutations.

5.5. Eukaryotic cells transfection

Exponential C2C12 and HeLa cells were transfected with the desired plasmids using lipofectamine probes according to the manufacturer's protocol.

5.6. Cells and muscle lysates

Cells were lysed with RIPA buffer (25 mM Tris-Cl pH 7.5, 50 mM NaCl, 0.5% NP40, 1 mM EDTA pH 8.5, 1 mM DTT, 20 mM NEM, protease inhibitors) and passed 2–3 times through a syringe provided with a G28 needle to disrupt genomic DNA. Crude lysates were clarified with centrifugation for 15 min at 10000 g, 4°C, and protein concentration was measured with BIORAD protein assay kit using BSA standard curve.

Frozen muscle biopsies were incubated with lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% SDS, 0.5% DOC, 0.5% NP40, 1 mM DTT, 10 mM NEM, 20 mM Iodoacetamide, fresh protease inhibitors cocktail) for 15 min on ice, then homogenized in a 1.5-ml tube with pestle. Muscle homogenizes were centrifuged for 20 min at 10000 g, 4°C. Clear supernatants were collected and protein concentrations were measured with the BIORAD protein assay kit using BSA standard curve.

5.7. Immunoblotting

Desired amounts of sample lysates were resuspended in loading buffer and denatured for 10 minutes at 95°C, loaded and fractionated in acrylamide Bis-Tris 4%-12% gradient gels. Proteins were transferred onto PVDF membrane for 60 minutes at 0.34 A, and blocked in TBS (50 mM Tris-Cl, 150 mM NaCl, pH 7.6) containing 0.1% Tween-20% and 5% skimmed milk. Membranes were incubated with the specific primary antibodies overnight at 4°C, followed by a 1-hour incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies. The immunocomplexes were visualized by chemiluminescence and detected by ChemiDoc MP Imaging System. Band signals were acquired and analyzed with the correspondent imaging analysis software, version 5.0.

5.8. Quantitative real-time PCR

Gene transcripts were assayed by qPCR using specific primers designed with PRIMER3 software. RNAs extraction was performed following the manual instructions included in the GeneJET RNA purification kit. Extracted RNAs were purified from genomic DNA contamination with DNase I/RNAse-free treatment. The correspondent cDNAs were produced using oligo (dT)18 and random primers by following the instruction of RevertAID H Minus First-strand cDNA synthesis kit. qPCR reactions were performed with 100 ng of cDNA template, in presence of the specific primers and SYBR Green Master Mix, in a 20 μL of the final volume. The analysis was performed with QuantStudio 3 Real-Time PCR Systems instrument, with the following cycling program: initial at 50°C for 2 minutes, denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and at 60°C for 1 minute. A final step of "melting curve" cycle between 65°C and 90°C, 1°C/s temperature speed was incorporated. Fold change relative to a housekeeping control gene (gapdh) was calculated as $2^{-\Delta Ct}$ where: $\Delta C_t = C_t(\text{target}) - C_t(\text{gapdh})$, according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline. To calculate relative gene expression levels between differently treated samples, $2^{-\Delta\Delta CT}$ was used to determine fold transcript changes, where $\Delta\Delta C_t = \Delta C_t(\text{target gene after treatment}) - \Delta C_t(\text{target gene before treatment})$. All samples were analyzed in triplicate and mean and ± SD were obtained.

5.9. Protein purification

Prokaryotic cells: E. coli BL21 bacteria were transformed with the desired plasmids and colonies were selected within the appropriate LB agar medium. After exponential growth, IPTG-protein induction was performed. Bacteria were collected and lysed. The proteins of interest were purified using the different methods according to the specific conjugated tag. Purified proteins were either separated by SDS-PAGE gel and stained with Coomassie blue or directly used for enzymatic assays.

Eukaryotic cells: Cells were harvested using cold PBS, when necessary in presence of 0.2 M iodoacetamide, and lysed with specific lysis buffers. Lysates were centrifuged at for 10 min, 10000 g, at 4°C. Native or overexpressed-tagged proteins were purified using the specific antibodies conjugated to sepharose beads or with commercial tag-trap columns, following the manufacture´s purification protocols.

Muscles: Approximately 200 mg of muscles tissue were lysed in an optimized lysis buffer (Tris-Cl 150 mM, NaCl 150 mM, SDS 0.5%, NP-40 1%, DOC 0.5%, EDTA 5 mM, DTT 1 mM, fresh NEM 20 mM, and protease inhibitors, pH 7.6). Muscle lysates were precleared with protein G-agarose beads coupled to anti-normal mouse IgG for 3 h and gently rotated at 4 °C before the incubation with protein G-agarose beads coupled to specific antibodies. Beads were collected by centrifugation and washed twice with PBS containing 0.01% NP40. Immunocomplexes were eluted by competition with specific peptides. Elutes were used for mass spectrometry analysis or protein-protein interaction assays.

5.10. Immunocytochemistry

C2C12 cells were seeded in 13-mm diameter coverslips precoated with poly-D-lysine in 12-well plates. When required, exponential cells were transfected with plasmids for protein expression assays or differentiated into myotubes with an appropriate medium. Coverslips were washed three times with cold PBS and fixed with filtered 4% paraformaldehyde dissolved in PBS, for 20 min at RT. Fixed cells were permeabilized with 0.5% Triton-X100 for 1 h at RT. 3% BSA in PBS was used as a blocking solution and slides were incubated for 30 min at RT. After that, 1 h incubation with primary antibodies, three PBS-washed, and 1 h incubation with the secondary antibody were performed. Coverslips were transferred on an objective glass with a mounting solution containing DAPI probe to stain nuclei. Confocal or fluorescent microscopes were used to acquire the images.

5.11. Immunohistochemistry

Frozen muscle biopsies were fixed with Compound for Cryostat Sectioning (OCT) and cryosectioned into 10 μm slices at −20°C. Cryosections were incubated at room temperature (RT) for 5 min, rehydrated in PBS for 15 min at RT, fixed in cold acetone at −20°C for 15 minutes, dried for 1 minute at RT, and blocked with 3% BSA in PBS for 40 minutes at RT. Incubation with primary antibodies was performed for 90 minutes at RT, then washed 3 times for 5 minutes with PBS, followed by a 1-hour incubation with secondary antibodies and washed again with PBS. The slides were mounted with Fluoroshield medium containing DAPI. Pictures were acquired using a ZEISS laser scanning confocal microscope using a fixed protocol for all sections.

5.12. ATPase pH 10.4 staining

Cryosections were incubated with alkaline solution 1 (0.1 M Glycine, 5.4 mM $CaCl_2$, 0.1 M NaCl, pH 10.4 adjusted with 0.1 M NaOH) for 9 min at 37°C. Then, washed with water, and incubated with solution 2 (17 mg Na_2ATP in 10 ml of Solution 1, pH 9.4 adjusted with HCl) for 30 min at 37°C. Samples were washed and incubated consecutively with 1% $CaCl_2$ for 3 min at RT, 2% $CoCl_2$ for 3 min, 1% $(NH_4)_2S$ for 1 min at RT, with washes performed between the different incubations. Finally, slides were mounted in glycerine gelatin. Pictures of staining were acquired using Nikon Phase Contrast 0.90 dry microscope.

5.13. NADH-TR staining

Cryosections were incubated in 30 ml of NADH-TR solution (28 mg Nitroblue Tetrazolium, 6.25 ml 0.1 M MOPS solution pH 7.4, 10 mg Nicotinamide adenine dinucleotide in 30 ml H_2O) for 20 min at 37°C. Then the sections were washed with running distilled water to remove any excess NADH-TR solution on glass slides. Sections were kept for drying at RT for 15 min and cover glass was mounted with glycerine gelatin. Pictures of staining were acquired using Nikon Phase Contrast 0.90 dry microscope.

5.14. Images processing software

ImageJ software was used as a processing program to quantify band intensity from western blots, and fluorescences from immunofluorescence muscle section pictures.

5.15. ROS detection

Exponential C2C12 cells and derived myotubes cultured in medium containing different amounts of glucose with and without ROS inhibitors, or overexpressing specific proteins, were seeded in 24/96-well plates. Intracellular and mitochondrial ROS were measured using commercial kits following the manufacturer's protocol.

5.16. Myogenesis

Confluent C2C12 cells were differentiated in myotubes using differentiation medium (DMEM + 2% horse serum). Fresh medium was supplied to the culture every two days during differentiation to provide a constant amount of glucose. When required, 10 μM anacardic acid or 2 μM topotecan were added as indicated. These drug concentrations were selected based on C2C12 and myotubes toxicity tests performed previously. Stocks

were prepared in DMSO and the final working concentration did not exceed 0.01% v/v, resulting in no DMSO-induced cellular cytotoxicity or alteration of the myogenesis process. All four myogenesis processes (NG, HG, HG+anacardic acid, and HG+topotecan) were performed in parallel.

5.17. Cellular senescence assay

C2C12 cells were cultured and myogenesis was induced in media. β-galactosidase assays were performed with the senescence cells histochemical staining kit according to the manufacturer's protocol.

6. RESULTS AND CONCLUSIONS

6.1. Paper I

A well-maintained muscle is very important for the activities of daily life. The good status of the muscle is conserved by intricated and interconnected pathways able to quickly adapt to different situations. Our hypothesis was to understand how SUMOylation collaborated in the correct skeletal muscle functions under normal activity, and how it responded in cases of skeletal muscle diseases.

To achieve the results, we analyzed diaphragm muscles from ambulatory rats as control, and under controlled mechanical ventilation (CMV) as an acquired muscle disease model. Indeed, prolonged exposure to CMV treatment results in the development of Ventilator-Induced Diaphragm Dysfunction (VIDD). VIDD is a common disease in ICU patients that causes a delay in the weaning from intubation due to diaphragm weakness and affects the quality life of the patients[115].

6.1.1. SUMOylated proteins are unevenly distributed in the muscle fibers

The distribution of SUMO conjugated proteins in the different muscle fibers is unknown. To investigate this, we performed consecutive diaphragm cryosectioning from control rats and immunostained the sections either with SUMO1 or SUMO2/3 antibodies or with fiber type identifier markers NADH-TR (to identify oxidative or glycolytic fibers) or myosin ATPase (to identify myosin type I or II fibers) (**Figure 13**).

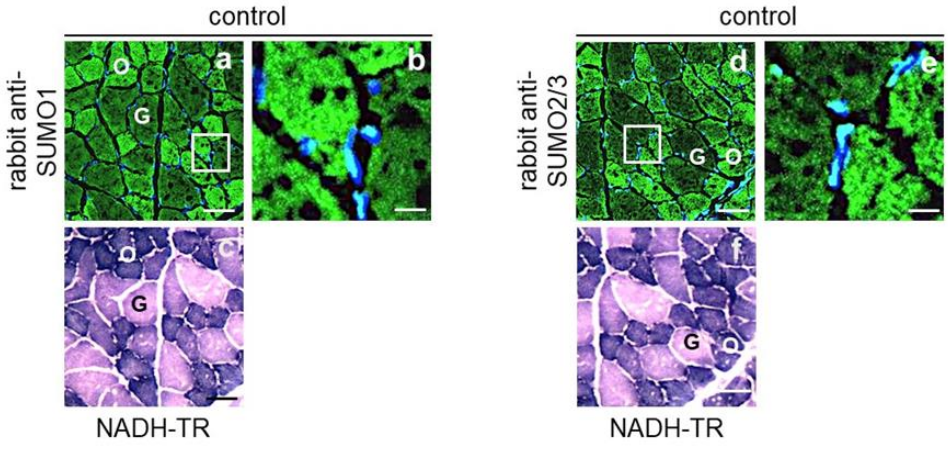

Figure 13: Immunofluorescence analysis of SUMO conjugates in the control diaphragm. Muscle fibers stained with antibodies to SUMO1 (**a**) and SUMO2/3 (**d**). White boxes are enlarged, showing the nuclear localization of both SUMO1 and SUMO2/3 (**b** and **e**). Consecutive serial muscle sections were subjected to NADH-TR staining to recognize the oxidative (O, dark purple) and glycolytic (G, light purple) fibers (**c** and **f**). Scale bars, 50 µm (panels **a**–**d** and **c**–**f**), and 20 µm (panels **b**–**e**).

The results obtained surprisingly showed a mosaic-like distribution of SUMOylated proteins between fibers types, showing oxidative fibers higher accumulation of SUMO conjugated proteins compared to the glycolytic fibers.

6.1.2. SUMO reaction is perturbed in the diaphragm during CMV treatment

The levels of total SUMO conjugated proteins are altered in stress situations due to changes in the expression of the SUMO enzymes in cells and organs[116]. We hypothesized that the SUMOylation will change as a consequence of acquired muscle pathology, using VIDD as a model.

We compared the total levels of SUMOylated proteins for both SUMO1 and SUMO2/3 in the diaphragm of control and CMV treated rats. The results showed a progressive elevation in the total amount of SUMOylation for both SUMO1 and SUMO2/3 target proteins, with short times of CMV (**Figure 14**).

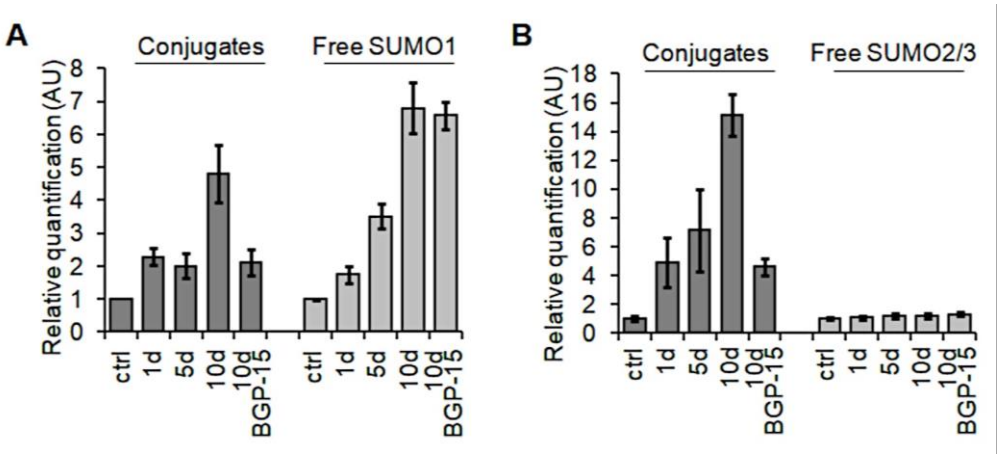

Figure 14: Quantification of free and conjugated SUMO1 (**A**), and SUMO2/3 (**B**). The intensities of free SUMO and SUMO conjugates were obtained from western blot expressed in AU after normalization by the correspondent GAPDH loading.

6.1.3. SUMOylated proteins increase in all fiber types as a response to CMV

Since CMV increased the total amount of SUMOylated proteins, we hypothesized that an increase in the SUMO conjugation in the different fiber types will be observed. We compared the previous cryosections from control rats with the ones obtained at different time points of CMV.

We observed a progressive increase of the SUMO1 and SUMO2/3 signals with the treatment, being the glycolytic fibers highly affected, and reaching the same intensity levels as the oxidative fibers at the end of the measurements (**Figure 15**).

Furthermore, we found the presence of both SUMOs in the myonuclei in the different conditions, validating the nuclear localization of SUMOs previously described in the literature[117].

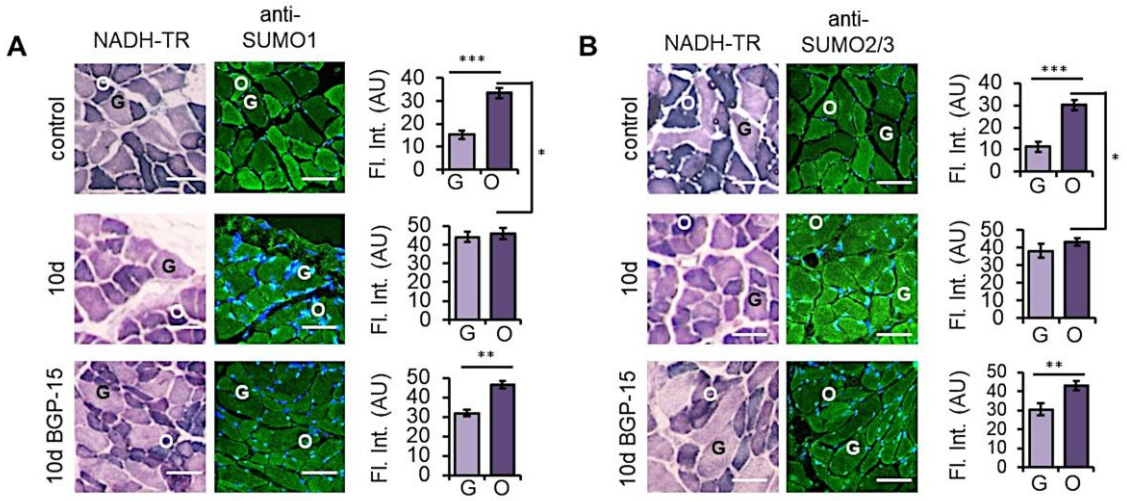

Figure 15: Localization of SUMO1 (**A**) and SUMO2/3 (**B**) proteins on diaphragm muscle cross-sections from control and mechanically ventilated rats. Cryo-sections stained for NADH-TR, and SUMO1-2/3 antibodies in diaphragms from control and mechanically ventilated rats 10 days, and with BGP-15 for 10 days (10d BGP-15). Scale bars, 50 µm.

6.1.4. SUMO substrates in skeletal muscle

The specific SUMOylation levels in the muscle fibers and how they increased during CMV opened the question about which proteins were SUMOylated in the diaphragm during normal conditions and what changes undergo with the CMV treatment.

With a combined approach of immunoprecipitation, mass spectrometry, and bioinformatics analysis on rat diaphragms lysates, we identified a vast amount of new SUMO interacting proteins involved in a wide spectrum of cellular activities with a broad distribution in the cell compartments. In normal circumstances, the majority of those proteins are involved in diverse pathways for energy production, muscle remodeling, and contraction.

During the different points of mechanical ventilation, we found the recruitment of new SUMO1 and SUMO2/3 substrates from different processes including transcription factor, translation regulator activity, response to an external stimulus, and response to stress. These results validated our hypothesis about SUMO playing an important role in muscle physiology and the adaptation to pathology.

6.1.5. SUMO substrates validation

By immunoprecipitation with SUMO1 and SUMO2/3, we obtained all the SUMO interacting proteins, including SUMOylated proteins, proteins interacting with SUMOylated proteins, and proteins with SIM domains that interact with SUMO moieties. To confirm which of them were SUMOylated, we selected ten highly abundant SUMO-protein candidates with different roles and localizations with a positive score in potential SUMO lysines, and we performed prokaryotic and eukaryotic assays. We obtained positive SUMOylation results for the E3 ubiquitin ligases MuRF1 and MuRF3, the motor protein myosin, the mitochondrial proteins aspartate aminotransferase (AATM), Ornithine aminotransferase (OAT), ATP synthase subunit epsilon (ATP5E), and ATP synthase subunit alpha (ATP5A) and for the ryanodine receptor-related proteins calsequestrin 1 (CASQ1), calsequestrin 2 (CASQ2) and triadin (TRDN).

In the case of myosin, calsequestrin 1, triadin, and ATP synthase subunit alpha, a western blot analysis performed using the enriched SUMO1 and SUMO2/3 peptide eluted immunocomplexes confirmed their SUMOylation in the skeletal muscle.

6.1.6. SUMO cycle components adapt to pathological conditions

Previous studies suggest that stress situations induce alterations in the expression of the SUMO machinery components[118]. These alterations could be the explanation for the different levels in SUMOylation observed along with the CMV treatment. We performed a transcriptomic analysis from rat diaphragm muscle from control and CMV. The results showed changes in the mRNA expression levels of the SUMO cycle components in control and during 1, 5, and 10 days of CMV (**Figure 16**).

The increased expression levels of SUMO conjugating enzymes like the E1 dimers, (Sae1/Uba1), the E2 (Ubc9), and some E3 ligases (Pias1-3-4, RanBP2, TOPORS), and the reduced levels of the E3 ubiquitin ligase RFN4 could explain the increase in the global SUMOylation profile. The transcriptomic values were validated for some candidates at the protein level via western blot or immunofluorescence in cryosections.

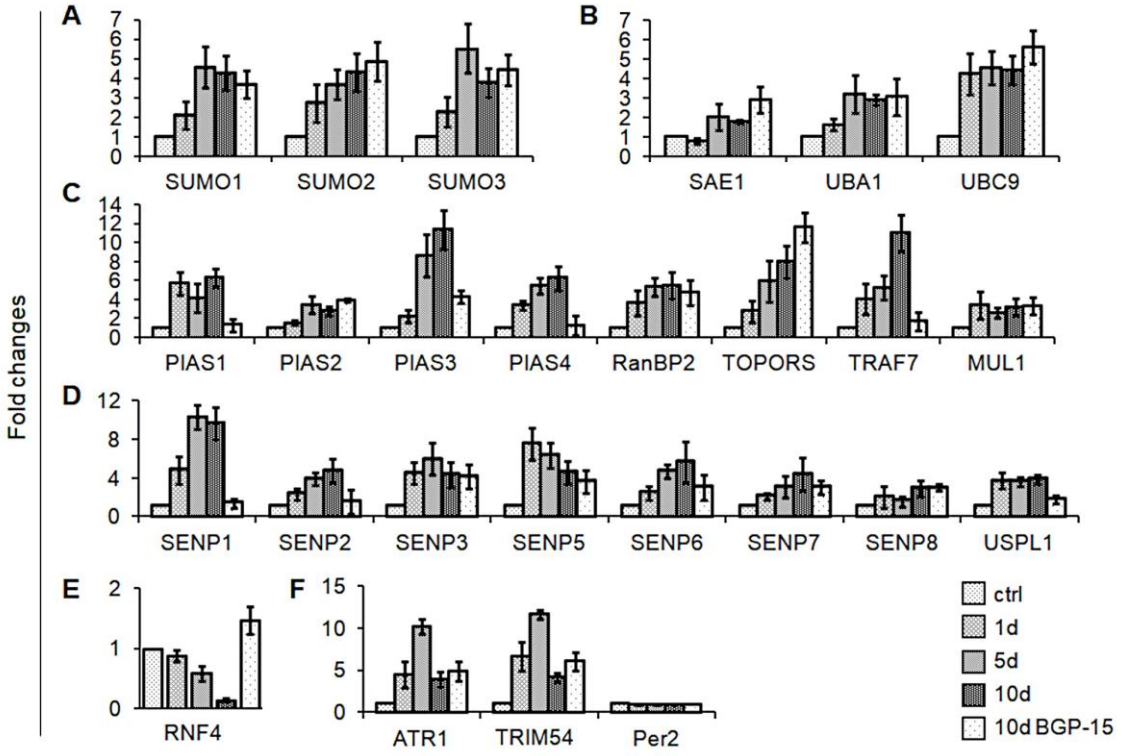

Figure 16: Transcriptome analysis of SUMO-related enzymes in control and mechanically ventilated rats without and with drug treatment. Quantitative PCRs performed on SUMOs (**A**), E1- and E2 SUMO enzymes (**B** and **C**), SENPs (**D**), STUbL RNF4 (**E**), and ATR1, TRIM63, and the circadian Per2 (**F**).

6.1.7. BGP-15 reverts the SUMOylation levels altered by CMV

BGP-15 is a co-inducer of HSP72 expression and was useful for the cardiac function improvement[119] and the recovery of the mitochondrial function during CMV in rat diaphragm[120]. Heat shock proteins (HSP) are overexpressed in response to stressful situations with a chaperone activity and a protective role against ROS[121]. ROS increase during prolonged exposure to CMV[122] and high ROS levels increase SUMOylation by enhancing the activity of PIAS1 and PIAS4 and blocking SENPs[123].

We hypothesized that treating rats with BGP-15 while they were under CMV conditions could revert SUMOylation levels. We used diaphragms extracted from rats after 10 days of CMV and treated with BGP-15 to measure the general SUMO levels and compared them with non-treated rats. We observed a clear effect of BGP-15 reducing the total SUMOylation levels after 10 days of CMV (**Figure 14**), the immunofluorescence intensity was similar to the control slides (**Figure 15**), and both SUMO pathway components proteome and transcriptome were protected (**Figure 16**).

6.1.8. Conclusion of Paper I

For the first time, we showed a heterogeneous distribution of SUMO conjugated proteins in the muscle fibers according to the fiber type. We observed an adaptation of the SUMO pathway in response to the progression of VIDD. Finally, the positive effect of BGP-15 impairing the pathological effect in the SUMO pathway opens the door for the SUMO cycle as a target for the development of a pharmacological approach in the treatment of pathologies.

6.2. Paper II

MuRF1 plays an important role in the muscle pathophysiology and we found it was SUMOylated by SUMO1 in paper I. MuRF1 is a muscle E3 ubiquitin ligase known to be involved in the muscle protein turnover[124] for muscle maintenance and has a protective role during cardiac hypertrophy[125]. MuRF1 is also related to the development of protein aggregate myopathies (PAMs)[126] and hypertrophic cardiomyopathy (HCM)[127,128] when its protein sequence is mutated.

We wanted to characterize the SUMO conjugation of the E3 ubiquitin ligase MuRF1 and its implication in MuRF1 activity and localization. We also wanted to correlate the alterations in MuRF1 SUMOylation and its association with muscle pathologies.

6.2.1. SUMO1 conjugates MuRF1 on lysine 238

Knowing that MuRF1 is SUMOylated, we tried to find the exact lysine in the MuRF1 sequence involved in the SUMO conjugation. With a combination of bacteria SUMOylation assay, immunoprecipitation, and mass spectrometry followed by bioinformatics analysis we isolated ten potential lysines involved in the SUMOylation of MuRF1.

To define which lysine could be involved in the SUMO conjugation, we generated single mutations for all the ten identified lysines (K) into arginine along the GFP-MuRF1 amino acid sequence and performed eukaryotic assays. Exclusively, the GFP-MuRF1 containing the mutation in the 238 position showed a complete absence of the slow migrating bands above the native GFP-MuRF1 protein, and this result was confirmed by the total absence of PTM bands above GFP-MuRF1 corresponding to SUMO1 after immunoprecipitation.

This result suggests that K238 is the unique lysine in the MuRF1 protein that could become a target of SUMO1 conjugation (**Figure 17**).

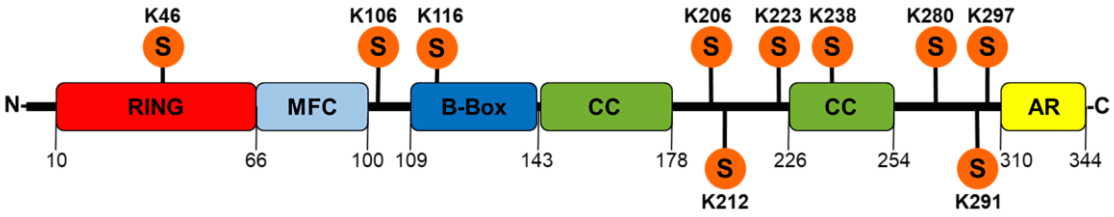

Figure 17: MuRF1 protein structural domains indicated in different colors, RING, MFC (MuRF family conserved domain), B-box, CC (coiled coin, and COS domains), AR (acidic tail). Potential SUMOylation binding sites for single mutants generation marked in orange.

6.2.2. Ubc9 and PIAS 4 promote the SUMOylation on MuRF1

The correct SUMO conjugation into the target proteins is mediated by an enzymatic pathway as it was earlier mentioned. Such conjugation requires the use of the only known E2 conjugation enzyme Ubc9 and maybe the use of an E3 SUMO ligase. To identify the enzymes involved in MuRF1 SUMOylation, we performed eukaryotic assays with the co-transfection of GFP-MuRF1 with Ubc9 or several E3 SUMO ligases. An increase in the SUMOylated fraction of MuRF1 when combined with Ubc9 but not with its catalytic mutant (C93S) and with the specific use of the E3 SUMO ligase PIAS4 validated both enzymes.

6.2.3. MuRF1 mutation impairs its enzymatic activity for troponin degradation

Mutations in the polypeptide chain sequence can affect the protein 3D structure and inactivate it. We hypothesized that alterations in the MuRF1 sequence influence its activity. To address this question, we compared the MuRF1 wild type (wt) with the K238R in the enzymatic degradation of a well-known target, troponin.

We observed that the degradation of troponin was significantly reduced in MuRF1 K238R compared to the wt. This result suggested that the mutation affects the substrate ubiquitination, probably due to a conformational protein change. This change in conformation could generate a non-functional E3 ubiquitin ligase or cause an impairment between enzyme-substrate interaction.

6.2.4. SUMOylation is essential for MuRF1 translocation into the nucleus

Since SUMOylation promotes cellular translocation we focused our next goal in the analysis of the cellular distribution of MuRF1 in mouse myoblasts. We compared the cellular distribution of transfected GFP tagged MuRF1 wt and K238R in C2C12 cells using cell fractionation and immunofluorescence imaging. The results showed that SUMO1 conjugation to MuRF1 was required to translocate into the nucleus even though it was cleft after and that the K238R mutation induced a high proliferation of MuRF1 aggregates in the cytoplasm (**Figure 18**).

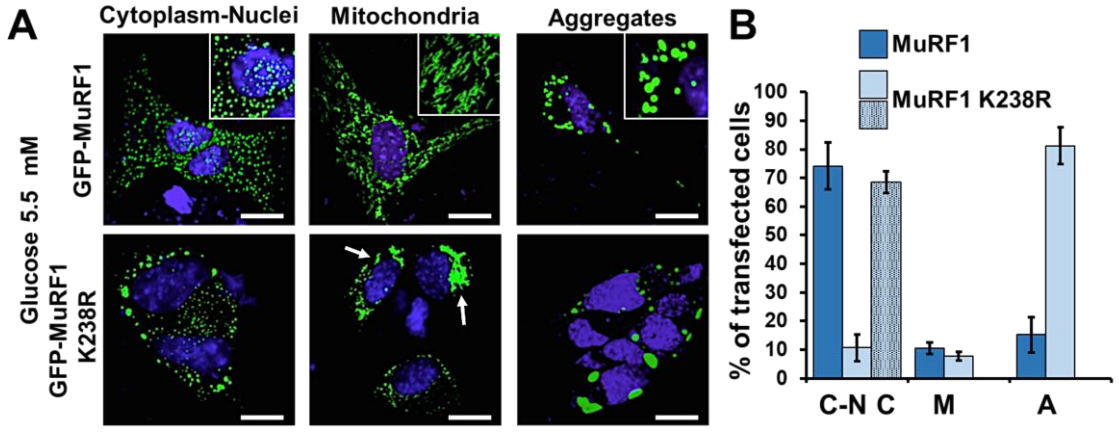

Figure 18: Confocal microscopy analysis of GFP-MuRF1 and GFP-MuRF1-K238R cellular localization. Protein distributed in cytoplasm and nuclei, in mitochondria, or forming aggregates in the cytoplasm (**A**). Statistical distribution of protein localization in transfected cells (**B**). White squares show enhanced details, arrows show alteration in mitochondria morphology due to increased ROS levels. Scale bar 10 µm.

6.2.5. MuRF1 protects from SUMO deconjugation under high glucose conditions

To investigate the potential role and the biological significance of MuRF1 localization in mitochondria, we analyzed the level of total SUMOylated proteins in murine myoblasts. We compared no transfected cells with ones transfected with MuRF1 wt and K238R placed in normal (5,5 mM glucose) and high glucose medium (25 mM glucose). A significant decrease in the SUMO2/3 conjugated proteins signal was detected in no transfected cells or K238R ones in high glucose compared to normal glucose, but no difference was noticed in cells overexpressing MuRF1 wt. This result was connected with a reduction of approximately 50% of cellular ROS in the cells transfected with wt MuRF1, demonstrating that MuRF1 had a positive effect preserving the SUMOylation of cellular proteins by reducing the level of cellular ROS.

6.2.6. Conclusion

The new insights provided on MuRF1 SUMOylation highlight the critical role played by SUMO1 in the modulation of both MuRF1 activity and localization. Since the specific lysine involved in the SUMO conjugation is localized in the coiled-coil region of MuRF1, with the role of microtubule association, the effect caused by SUMOylation impairment could affect the correct localization of MuRF1.

6.3. Paper III

The knowledge provided by our previous work done on paper I showed that SUMOylated proteins accumulate in different degrees according to the muscle fiber type. We also observed that VIDD causes alterations in the expression levels of the SUMO machinery components and at the end alterations in the global SUMO conjugation.

In this paper, we wanted to investigate if the correlation between SUMOylation levels and fiber type found in the diaphragm was also observed with other muscles along the body. Also, if a new stress situation caused by soleus unloading had a similar effect on the SUMOylation levels as CMV.

6.3.1. The SUMO system is specific to each skeletal muscle

To understand if there were variations among the different skeletal muscles in their SUMOs profile, we compared the SUMO1 and SUMO2/3 western blots obtained from the lysates of nine different rat skeletal muscles (tibialis anterior, EDL, soleus, diaphragm, plantaris, gastrocnemius superficial, gastrocnemius deep, gastrocnemius proximal and masseter). We found variations in the intensity levels of the SUMO substrate conjugation associated with the different skeletal muscles. These results confirmed our hypothesis that the SUMO pathway adapts to the needs of the skeletal muscles according to the specific role played by each one of them.

6.3.2. SUMO conjugation levels are specific according to skeletal muscle fiber

In the paper I, we observed a mosaic distribution of SUMOylated proteins in the diaphragm connected with the metabolism of the muscle fibers. We hypothesized that the other skeletal muscles would have a similar distribution with a muscle fiber type correlation and the abundance of SUMO conjugates.

Immunostaining analysis with SUMO1 or SUMO2/3 antibodies, and ATPase pH 10.4, and NADH-TR stainings, performed in consecutive cryosections from ambulatory rats, provided a mosaic of fluorescence intensities with higher levels of SUMOylation localized in the skeletal muscle fibers with an oxidative metabolism (**Figure 19**).

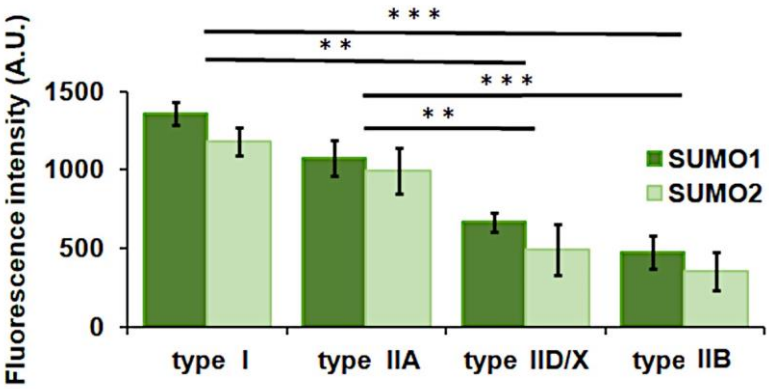

Figure 19: Fluorescence intensity measurement for each fiber type. 100 fibers per type from the nine different skeletal muscles were used in the analysis. Results were normalized to the corresponding myofiber area and values were expressed in arbitrary units (A.U.).

These results validated our previous findings in the paper I where we showed the same distribution of SUMOylated proteins in the diaphragm.

6.3.3. Transcriptome analysis of the SUMO machinery components

For a better understanding of the heterogeneity in the SUMOylation expression in the different skeletal muscles, we analyzed the transcriptomic levels of the SUMO moieties and enzymes from the same nine rat skeletal muscles. The RNA measurements showed differences in their expression levels within the skeletal muscles. The proteomics analysis confirmed the results from the transcriptomics, validating the correlation of the SUMO enzymes expression and the specific function of the skeletal muscles.

The principal component analysis provided with interesting information. The different expressions of the SUMO components allowed us to classify skeletal muscles according to muscle embryogenesis, anatomical position (vertical), and fiber type composition (horizontal) (**Figure 20**).

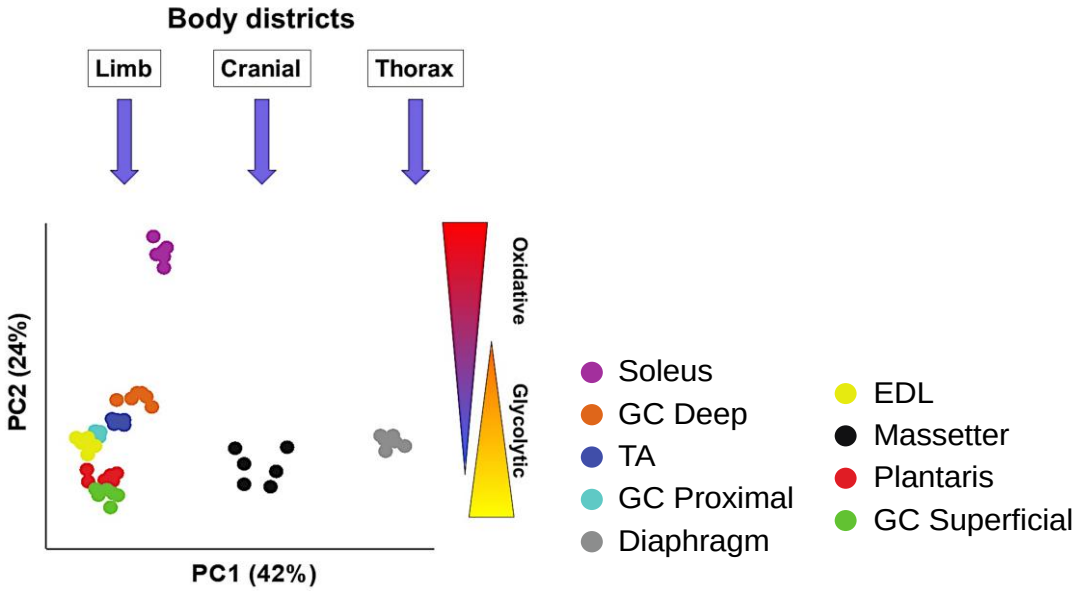

Figure 20: Principle component analysis of transcriptome reveals the formation of distinct clusters, indicating differences in the SUMO expression signature.

6.3.4. Muscle activity impairment affects SUMOylation levels

The previous experiments were performed over healthy, ambulatory rats and we wanted to observe the effect of a different pathology over the SUMO pathway. In the paper I, we observed changes in the SUMO pathway due to CMV in rat diaphragm, so we hypothesized that other alterations in the muscle activity could induce modifications in the expression of the SUMO network components and therefore changes in the general SUMOylation levels.

Using soleus muscle extracted from ambulatory and tailed-suspended Wistar-Han rats during 1, 2, and 4 days of unloading, we performed a western blot analysis of SUMO1 and SUMO2/3 followed by the transcriptomic analysis of the SUMO machinery. To complete the study, an immunohistochemistry study of cryosections was performed. Results showed an increase of SUMOylation levels combined with alterations in the enzymes right after unloading before any signs of protein degradation mediated by the UPS. This could be explained by a protective effect of the SUMO pathway in the proteins by delaying its ubiquitination and further protein degradation (**Figure 21**).

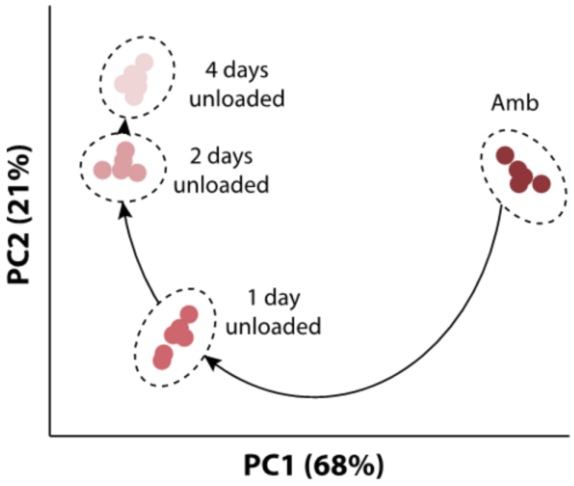

Figure 21: Principle component analyses depicting the effect of unloading the SUMO machinery. Note that expression of SUMO related factors changes rapidly within 1 day after unloading.

6.3.5. PAX6 increase Ubc9 expression in early events of muscle inactivity

In our papers studying the effect of muscle stress in the SUMO levels (papers I and III), we observed an increase in the SUMO conjugated proteins in parallel with the treatment at the same time as Ubc9 is overexpressed.

Deeper bioinformatics inquires of the Ubc9 promoter region gave us a list with potential transcription factors including HTF, ARP1, and ATF. We recognized PAX6 as a candidate among them and investigated its expression levels and localization by western blot and immunohistochemistry.

Interestingly the overexpression of Ubc9 was not connected to PAX6 overexpression but to its accumulation in the nucleus during short times of unloading. Besides we found that PAX6 could be SUMOylated as it is mentioned in the bibliography[129], which might explain its translocation to the nucleus.

6.3.6. Conclusion

We have validated the role of the SUMO network and the expression levels of each component linked to the functional requirements of each muscle according to its fiber type composition.

Again, we confirmed the importance of the SUMO network and its alteration during muscle pathologies and the possibility of becoming a therapeutic target to alleviate the effects of atrophy. For that effect, we showed how PAX6 is involved in the control of

Ubc9 expression and its potential use as a target for the regulation of the SUMO conjugation.

6.4. Paper IV

In this manuscript, we wanted to understand the involvement of the SUMO pathway in the regulation of muscle regeneration. Muscle regeneration is one of the outcomes of myogenesis along with embryonic muscle development and postnatal growth. In all the situations myoblasts follow a very tightly controlled program that leads to the formation of myofibers. Finally, we developed an in vitro model using high glucose concentrations to study muscle regeneration under hyperglycemic condition, similar to the ones observed in diabetic patients. Our hypothesis was that this condition will cause changes in the normal behavior of the SUMO pathway.

6.4.1. SUMO conjugation changes with the progression of myogenesis

To answer our question about the effect of the myogenesis progress in the SUMO conjugation we performed an analysis of SUMOylated proteins during the different time points studied (exponential, confluent, and 1, 3, and 5 days of myogenesis). We observed a peak in the proteins SUMOylated by SUMO1 and SUMO2/3 in the confluence stage followed by a progressive decrease during myogenesis.

6.4.2. The effect of hyperglycemia during myogenesis

To understand the effect of hyperglycemia in global SUMOylation levels during myoblast differentiation, mouse myoblasts were grown and differentiated in normal (NG) and high glucose (HG) medium and monitored at the different time points mentioned. Myotubes developed faster in the HG, they were bigger, and with a higher nuclei count compared with the NG ones. They also started contracting and reached the senescence status earlier.

SUMOylation levels analyzed by western blot also showed a tendency in the decrease of SUMOylated proteins along with the development of myogenesis but with maintained higher levels for both SUMO1 and SUMO2/3 in the case of HG.

The alteration in the SUMO pattern matched the results obtained by the transcriptome analysis. We found that the gene expression of the SUMO network was rearranged, and transcripts levels were easily clustered into NG or HG conditions.

6.4.3. ROS role during myogenesis

ROS have a crucial function as secondary messengers for a multitude of pathways[130], and even the SUMO components are regulated by them[131]. We pursued the investigation of the ROS levels during the different stages of myogenesis with and without the influence of a high glucose concentration.

The results obtained from the measurement of both cytoplasmic and mitochondrial ROS during the different time points of myogenesis showed alteration of ROS levels. In normal conditions, both cytoplasmic and mitochondrial ROS had a higher peak on day 1 and then decrease. However, in the case of high glucose conditions, the ROS peak was reached in the exponential phase and was maintained until the end of the experiment.

This effect was also associated with the early senescence of the myotubes grown in high glucose combined with a disorganized pattern of the striation in the sarcomeric structure (**Figure 22**).

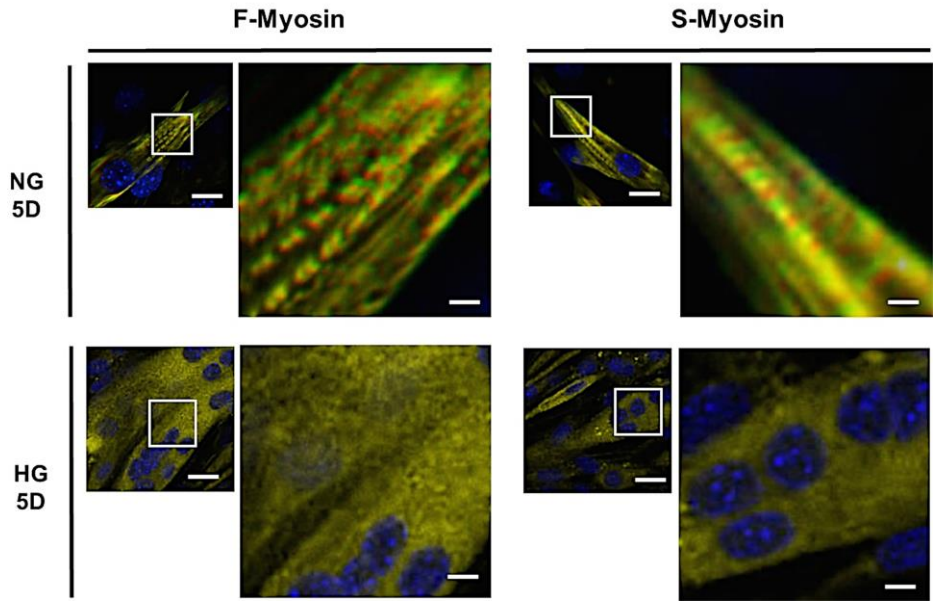

Figure 22: Confocal images of myotubes derived from C2C12 cells after 5 days from the differentiation, in NG and HG. Samples were double-stained for fast myosin (F-Myosin, red) and slow myosin (S-Myosin, red) together with alpha-actinin (green); the sarcomere structure showed partial co-localization with myosin and alpha-actinin (yellow). The selected areas (white square) were magnified on the right side. Scale bars = 100 μm, scale bars = 10 μm for the magnified images.

6.4.4. Anacardic acid protects against hyperglycemia

We have described that SUMOylation plays an important role in the myogenesis process and a pharmacological approach for the reversion of the SUMOylation mediated

by SUMO2/3 levels could help the myogenesis in hyperglycemic conditions. This approach could be mediated by the use of SUMOylation inhibitors like anacardic acid or topotecan.

The combination of the myogenesis development in NG and HG with the addition of the drugs demonstrated our hypothesis but only in the case of the anacardic acid. The use of topotecan had not the expected positive effect over the myogenesis since it interfered with myoblast fusion. The SUMOylation pattern of the myoblast grown in HG with anacardic acid had a significant reduction in the intensity and it was similar to the ones grown in NG. We also observed a similar myogenesis development, with no early senescence and a normal sarcomeric distribution. Finally, the proteomic and transcriptomic expression levels of the SUMO components were similar to the ones of cells growing in normal conditions (**Figure 23**).

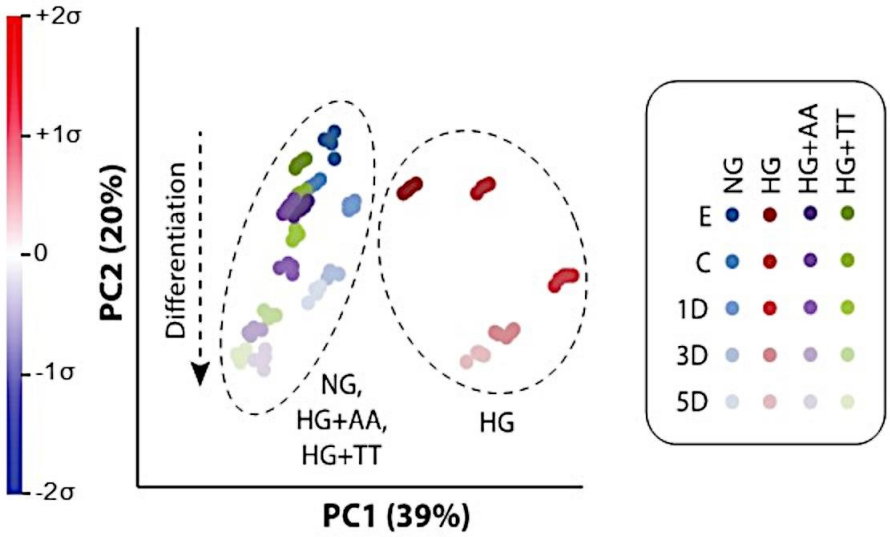

Figure 23: Principle component analysis of transcriptome revealed the formation of two distinct clusters, indicating differences in SUMO expression signature during myogenesis.

6.4.5. SET7/9 regulation by SUMO2/3

The regulation mediated by SUMO2/3 of the histone-lysine N-methyltransferase SET7/9 was previously described for the sarcomeric organization[132]. We use this property to our benefit and performed the immunoprecipitation of SET7/9 in the different conditions (NG, HG with and without anacardic acid) and our findings confirmed that only in HG conditions SET7/9 was SUMOylated and therefore inactivated. The treatment with anacardic acid reduced the SUMO conjugation of SET7/9.

6.4.6. Conclusion

These results seem to link the correct SUMOylation levels with a viable myogenesis process. We also confirmed that during hyperglycemic conditions myogenesis was accelerated and senescence was reached earlier than in normal conditions due to a starting point with higher ROS levels.

The transcriptomic and proteomic expression levels of the SUMO machinery components were altered due to the excessive ROS production by the HG conditions, but this effect could be ameliorated using anacardic acid thanks to its properties as SUMO inhibitor and antioxidant.

Finally, we linked the increase of SUMOylation produced by high glucose with the SUMOylation of SET7/9, an important player in myogenesis regulation. The inhibition mediated by SUMO conjugation of SET7/9 activity as a transcriptor factor caused the disorganization of the sarcomeric structures.

7. DISCUSSION

One of the major problems with muscle pathologies is the lack of early detection events to provide immediate treatments. Diseases like muscular dystrophies and inflammatory myopathies begin to develop years before their diagnosis, and by the time patients suffer the symptoms, only palliative treatments are available. Traditional methods of diagnosis include medical history, blood, and genetic tests, biopsies, and imaging. The development of new ways for the early detection of pathologies could help with the prognosis.

This thesis provides new potential results for an early diagnosis approach for skeletal muscle pathologies, considering the protein regulation mediated by SUMO posttranslational modification. Our discovery shows that SUMO enzymes and the global SUMOylation process are quickly altered before the activation of protein degradation, in the starting phases of the three muscle disorders described (VIDD, muscle inactivity, and muscle regeneration in hyperglycemia). Indeed, the expression of some SUMO enzymes can be used as biomarkers for the premature detection of muscle pathologies as we confirmed in the rat diaphragm and soleus, and in the vastus lateralis from humans.

Until now, we only had access to few animal models of acquired pathologies, however extra studies in animals with inherited myopathies or in human muscle biopsies are needed to confirm the potential use of the SUMO network components as biomarkers.

Regulate the SUMO conjugation in our favor can be beneficial for treating patients. An extended analysis of PAX6 role regulating the expression of Ubc9 during muscle diseases is necessary to confirm its effect. The development of a pharmacological approach to increase o decrease the presence of PAX6 in the myonucleus could interfere with the SUMO conjugation levels through the control of Ubc9 expression and reduce or block harmful effects of muscle inactivity.

We discovered a vast amount of new classes of protein related to SUMO. This result emphasizes how crucial is the SUMO pathway role in the regulation of muscle functions. In-depth analysis performed with some candidates showed the potential protective effect against degradation of myosin mediated by SUMO conjugation. More, we confirmed the role of SUMOylation of the E3 ubiquitin ligase MuRF1 in lysine 238 and compared the negative effect of the SUMOylation impairment in terms of activity and cell localization

which can be associate to hypertrophic cardiomyopathy and protein aggregate myopathies. Also, we described one potential molecular mechanism describing the alterations in the myogenesis process in hyperglycemia; the transcriptor factor SET7/9 becomes inactivated through SUMOylation and impairs muscle regeneration in high glucose conditions. Finally, other SUMO related candidates associated to the calcium regulation events (ryanodine receptor, calsequestrin, and triadin), and mitochondrial proteins (aspartate aminotransferase, ornithine aminotransferase, ATP synthase subunits-α and -ε) were not yet studied, but their involvement in the muscle contraction makes them valuable new SUMO targets for potential implication in physiology and myopathies studies.

The positive effect of some drugs, as BGP-15 observed in diaphragm from mechanically ventilated rats, and anacardic acid for the treatment of myogenesis in hyperglycemic conditions validates our hypothesis that targeting the SUMO network will ameliorate the effects of some SUMO-related muscle pathologies. The screening of new drugs to interfere with the SUMOpathway will provide new pharmacological approaches in the treatment of muscle diseases.

Future perspectives involve a deeper analysis of the previous findings:

Develop a CRISP-CAS9 methodology in murine or human myoblasts and generate transgenic mouse models with mutations in the SUMO protein consensus domain will allow us to understand the physiological function of SUMO PTM to the investigated skeletal muscle proteins. To complete the SUMO PTM picture, a unique mouse model with inducible expression of UBC9 will give us a better insight into the implications of SUMOylation in the regulation of different skeletal muscle activities.

A specific study of MuRF1 mutations associated with human pathologies found close to the SUMO binding site could explain the implication of this E3 ubiquitin ligase in the development of human diseases like hypertrophic cardiomyopathy and protein aggregate myopathies.

Cancer is one of the pathologies that beneficiates more for the early diagnosis, increasing the chances for successful treatment. Our future project combines the study of the alterations in SUMO conjugation and the myogenesis dysregulation in rhabdomyosarcoma and the test of new drugs targeting the SUMO pathway.

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