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The role of SUMO pathway in pathophysiology of skeletal muscle

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The role of SUMO pathway in pathophysiology of skeletal muscle

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I dedicate this thesis to

My mother N.V. Ramana and my father N.Ch. Krishna Murthy

“Truth can be stated in a thousand different ways, yet each one can be true.”
— Swami Vivekananda

ABSTRACT

Skeletal muscles are highly evolved and essential organs comprising 40 % of the total human body weight. They are essential in maintaining posture, energy metabolism, secrete hormones and act as central reserves for amino acids. Despite many studies on muscle physiology, there is a lack of understanding in cellular and molecular mechanisms leading to muscle adaptation, regeneration and progression of muscular disorders.

Post-translational modifications (PTMs) markedly regulate the quality and the functionality of proteins in eukaryotic cells. One such PTM is the reversible conjugation of a 12 kDa moiety called Small Ubiquitin-like Modifier, SUMO, onto targeted proteins in a process termed SUMOylation. Alterations in expression or activity of SUMO conjugating/de-conjugating enzymes in association with genetic point mutations in the SUMO consensus sequence of specific targets have been implicated in conditions like cancer, diabetes, brain ischaemia, and cardiomyopathies. Given to the reversible and rapid dynamic response to detect alterations in physiological conditions, SUMO pathway is being extensively studied as a potential therapeutic target for some conditions of brain and cardiac muscle protection from diseases. Our interests are to translate the significance of the SUMO pathway to skeletal muscle health, investigate its modulation as consequence of adaptation to new muscle activities and study disturbances in the SUMO reaction that alter the SUMO conjugation on specific target proteins which are associated to skeletal muscle diseases.

Ventilator Induced Diaphragm Dysfunction (VIDD) is a condition characterized by muscle dysfunction that occurs as side effect of Mechanical Ventilation. In diaphragms isolated from rats exposed to Controlled Mechanical ventilated (CMV), we observed significant changes in the overall SUMO muscle proteins due to alteration in the abundance of SUMO enzymes transcripts resulting in determining a new subset of SUMO targets. We studied the beneficial use of the drug BGP-15 administrated during CMV treatment that recovered the muscle contractile function partially due to a reorganization of the SUMO reaction.

We further identified and characterized some specific skeletal muscle proteins targeted by the SUMO, which are associated with particular muscle functions. Mainly, we focused the attention on the E3 muscle ubiquitin ligase, MuRF1. We described the specific SUMO target site, enzymes involved in the SUMO reaction and the consequence of this PTM related to the properties of this protein. This discovery will open new avenues to understand the multiple functions of MuRF1 in muscle physiology and contribute to better understanding of muscular disorders that result from deregulation of MuRF1 activities mediated by SUMO conjugation.

Finally, we provided an important facet to the differences in abundance of SUMO enzyme transcripts that we found across the different skeletal muscles to control their specific role along the body position. In conclusion, we also provided strong evidence of how the SUMO cycle may also be used as a cellular pathway target for new treatments for various skeletal muscle diseases.

LIST OF SCIENTIFIC PAPERS FOR THESIS DEFENCE

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. The chaperone co-inducer BGP-15 alleviates ventilation-induced diaphragm dysfunction. Salah H, Li M, Cacciani N, Gastaldello S, Ogilvie H, Akkad H, **Namuduri AV**, Morbidoni V, Artemenko KA, Balogh G, Martinez-Redondo V, Jannig P, Hedström Y, Dworkin B, Bergquist J, Ruas J, Vigh L, Salviati L, Larsson L. Sci Transl Med. 2016 Aug 3.

III. 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)

IV. The abundance of SUMO enzymes is fiber type dependent among skeletal muscles and it is altered in early event of muscle disuse. **Namuduri AV**, Heras G, Lauschke MV, Vitadello M, Traini L, Gorza L, Gastaldello S. (Manuscript in preperation)

Additional publications (not included in thesis)

1. Sarcolemmal loss of active nNOS (Nos1) is an oxidative stress-dependent, early event driving disuse atrophy. Lechado I, Terradas A, Vitadello M, Traini L, **Namuduri AV**, Gastaldello S, Gorza L. J Pathol. 2018 Jul 31

CONTENTS

LIST OF ABBREVIATIONS

PTM(s)	Posttranslational modifications
SUMO	Small Ubiquitin-like Modifier
SAE1/2	SUMO activating enzyme subunit
Ubc9	Ubiquitin conjugating enzyme 9
SENP(s)	Sentrin-specific proteases
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
UPS	Ubiquitin proteasome system
MuRF1	Muscle RING-finger protein-1
PIAS	Protein inhibitor of activated STAT
HSP	Heat shock protein
VIDD	Ventilation induced diaphragm dysfunction
MyHc	Myosin heavy chain
Ubl	Ubiquitin like modifier
NEDD8	Neural-precursor-cell-expressed developmentally deep regulated 8
ISG	Interferon-Stimulated Gene
ATG	Autophagy-related protein
SIM	SUMO interacting motif
VIDD	Ventilator-induced diaphragm dysfunction
CMV	Controlled mechanical ventilation
Ub	Ubiquitin
FAT10	F locus adjacent transcript 10
RanBP2	RAN Binding Protein 2
TOPORS	TOP1 Binding Arginine/Serine Rich Protein
PC2	Polycomb protein
MAPL	Mitochondrial-anchored protein ligase
TRAF 7	TNF receptor associated factor 7
PAX6	Paired Box 6
MYH gene	Myosin heavy chain gene

BGP-15	3-Pyridinecarboximidamide, N-(2-hydroxy-3-(1-piperidinyl)propoxy)-, hydrochloride (1:2)

INTRODUCTION

1 Muscle

Muscle is a vital organ, which facilitates the movement of all kingdom *Animalia* and exhibits characteristics such as contractility, elasticity, and excitability. Muscle tissues enable the bodily functions that include conscious and unconscious actions, moving substances within the body, maintenance of posture, body temperature, digestion and many more. Mammalians consist of two different types of muscles smooth and striated, this type includes cardiac and skeletal tissues (Sink, 2006). Smooth muscle form inner hollow structures in blood vessels and digestive tract, and the cardiac muscle is only found in the heart. Skeletal muscle is attached to the skeleton with tendons and can be voluntarily controlled which is not achievable with cardiac and smooth muscles (Chal and Pourquié, 2017). Cardiac and skeletal muscles are characterized as striated muscles due to the structural arrangement of their muscle fibers (Shadrin et al., 2016).

1.1 Skeletal Muscle and Muscle Contraction

Skeletal muscle engage 30-50 % of total human body mass, constitutes of about 50-75 % of all human body proteins, very dynamic and plastic organ in nature. It is formed with multinucleated cells called fibers and coexist with blood vessels, tendons and bones. The main functions are include to generation of force and power for movement, maintaining posture, body temperature and contribute to the metabolic activities of the body by storing amino acids and carbohydrates. During starvation conditions, stored carbohydrates are released into blood circulation, which contributes to maintain glucose levels (Frontera and Ochala, 2015). Skeletal muscles also secrete cytokines named, myokines which regulate the metabolic process in muscle and other organs such as brain, liver, pancreas (Schnyder and Handschin, 2015) (Ojima et al., 2014). For example, some of the functions of myokines include, the brain-derived neurotrophic factor which regulates abilities such as learning and memory in brain, the interleukins (IL)-6 that regulates metabolism in muscles, IL-8 which regulates angiogenesis in muscles, IL-15 that promotes glucose uptake and fatty acid oxidation in skeletal muscles, (Schnyder and Handschin, 2015) (Duzova, 2012).

The skeletal muscle fiber is composed of fundamental modular structural units known as sarcomeres, which operate as functional units in the muscle. Sarcomere consists of a network of thick and thin myofilaments, which work as a contractile unit. While the thick filaments are composed of myosin and myosin associated proteins , the thin filaments are composed of actin, troponin and tropomyosin (Gao and Zhang, 2015). Myosin belongs to a superfamily of molecular motor proteins that can be identified by the presence of distinct MyHCs encoded by different MYH genes. Myosin is involved in a broad range of cellular processes like the movement of cells, cytokinesis, vesicle transport, Golgi organization and sensory transduction. Myosin interacts with another protein called actin to generate the force to power muscle contraction, movement and loads dependent anchoring cargoes on actin filaments along the eukaryotic cytoskeletal cells (Sellers, 2000; Sweeney and Houdusse, 2010). Myosin superfamily is divided into twenty-four classes based on the head domain similarities and domain organization. From this different classes, myosin II was first identified as a motor protein in skeletal muscle (Syamaladevi et al., 2012). This conventional form of myosin II in

muscle is composed of two heavy chains and four light chains (Weiss and Leinwand, 1996). Each myosin molecule is comprised of two globular head domains and a long tail often referred to as the rod. The globular head domains contain actin-binding site and exhibit ATP hydrolyze activity to generate force for muscle contraction. The C-terminal region of the two myosin heavy chains forms into coiled-coil rod region, which can interact with the other myosin molecules to form a bipolar filament (Holmes, 1987).

The binding of actin and myosin mainly depends on adenosine triphosphatase (ATPase) activity of globular head domain of myosin. Once the ATP is hydrolyzed to ADP, conformational changes occur in myosin and head domain strongly interacts with actin. On the contrary, when ATP reacts to the binding site of myosin, the affinity between the actin and myosin becomes weak. These conformational changes brought by the nucleotide binding, its hydrolysis and the product release are essential for the dynamic activity of myosin (Hartman and Spudich, 2012).

Actin is one of the most abundant and highly conserved proteins present in almost all eukaryotes; it is involved in more protein-protein interactions than any other known protein. Six different isoforms of actin were identified which include three α-actin isoforms (α-skeletal muscle, α-cardiac muscle, and α-vascular), one β-isoform (β-cytoplasmic) and two γ-isoforms (γ-cytoplasmic and γ-smooth muscle). The amino acid sequence of skeletal α-actin slightly differs from β and γ cytoplasmic actin isoforms mostly in the N-terminus region (Müller et al., 2013). While the cytoplasmic actin isoforms play an important role in cell motility, intracellular transport, and cell shape maintenance, skeletal muscle actin isoforms assist in contractility of muscle fibers (Müller et al., 2013)(Nowak et al., 2013).

The ability of actin to transform from monomeric globular (G-actin) to filamentous fibrous (F-actin) state due to the nucleotide hydrolysis, ions and large number of actin-binding proteins make it a crucial player in many cellular processes like cell motility, maintenance of cell shape and polarity, regulation of transcription (Roberto Dominguez and Kenneth C. Holmes, 2011). Typically, actin monomers are comprised of one small and one large domain, which are further divided into four subdomains. Two clefts are formed in actin monomer between the four domains due to its structural arrangement. One of the clefts between 2 and 4 domains binds to the nucleotide (ATP or ADP) and associated divalent magnesium cation (Mg^{2+}). The other cleft between 1 and 3 domains is made mostly by hydrophobic residues, it facilitates the primary binding site for actin-binding proteins, and thus it is called target binding site (Kabsch and Vandekerckhove, 1992).

A dynamic equilibrium is maintained between the monomeric G and polymerized F actin forms in all the actin isoforms. When the actin strongly binds to an adenosine nucleotide, the polymerization involved in the G to F-actin transition activates the ATPase activity. Actin dynamics are dependent on the ATPase activity, which drives actin filament treadmilling, where the polymerization at one end and depolymerization at the other occur at the same time. Treadmilling is regulated by actin regulatory proteins, troponin and tropomyosin which mediate the actin dynamics (Oda et al., 2009).

1.2 Skeletal Muscle: Fiber Type and Function

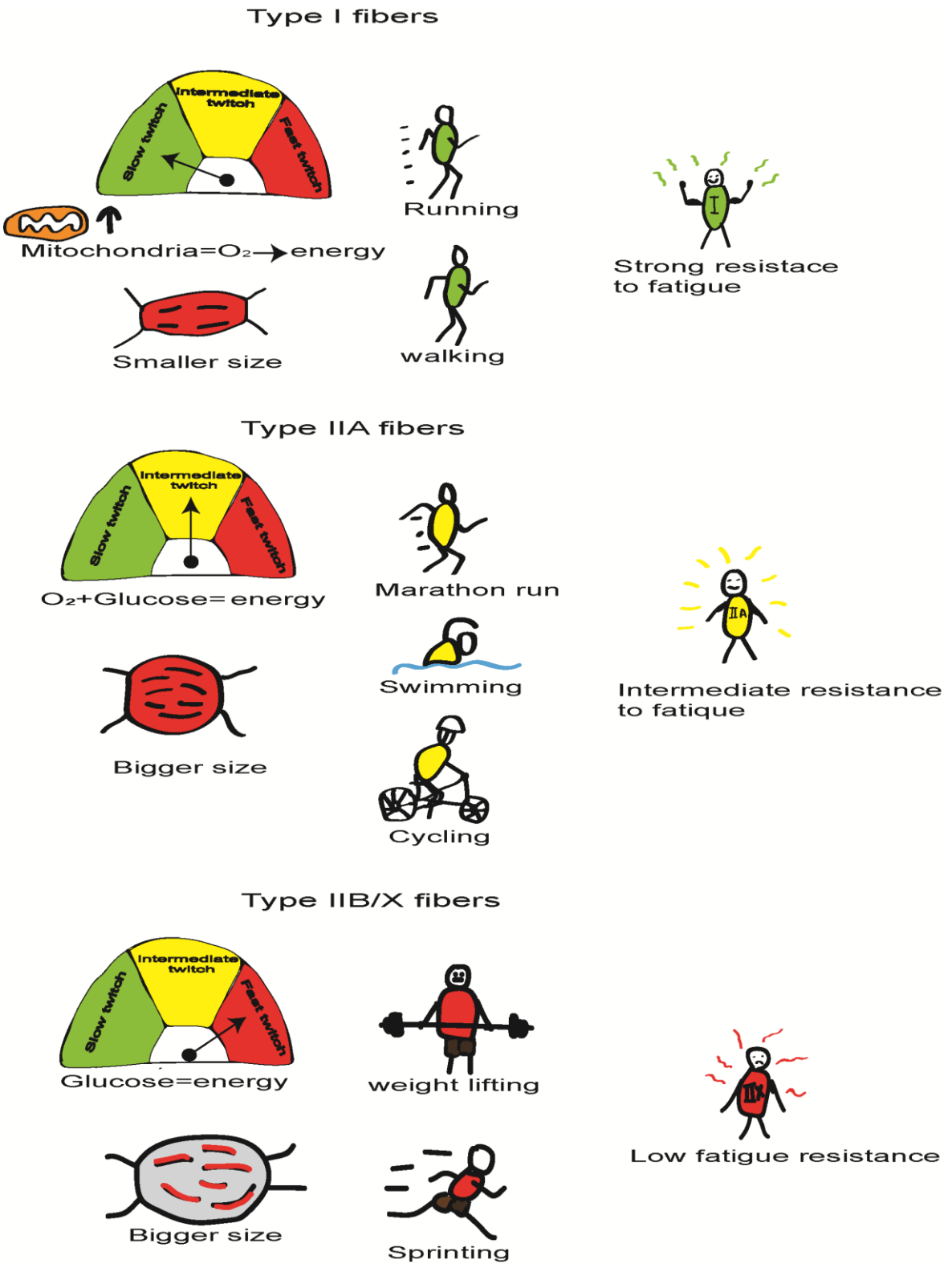

***Figure 1**. Schematic representation of different fibers types present in human skeletal muscles and their display of functional and metabolic characteristics in daily human needs.*

Skeletal muscles have an immense ability to get adapted to the enormous amount of changes that occur during the entire lifetime of a mammal. At the single fiber level, skeletal muscles are distinguished into four major fiber types: type I, IIA, IIX and IIB, based on the presence of specific myosin heavy chain (MyHC) isoforms. MyHC-1/slow, coded by the MYH7 gene,

MyHC-IIA, coded by MYH2, MyHC-IIX, encoded by MYH1, and MyHC-IIB, coded by MYH4 (Schiaffino and Reggiani, 2011). Muscle fibers are also classified into slow-twitch, oxidative and fast-twitch, glycolytic fibers. As the name suggests, fast and slow twitch fibers show significant differences in muscular functions like the speed of contraction, metabolism, and resistance to fatigue. Fast-twitch fibers are further categorized into three subtypes type IIA, IIB, IIX whereas there is only one type of slow twitch fibers, type I (Ciciliot et al., 2013). Type I fibers contain just slow myosin and have abundance in mitochondria. They use oxidative metabolism for ATP production and show strong resistance to fatigue. Type IIB contains only fast myosin, and less mitochondrial content thus have less oxidative metabolism relying on glycolytic metabolism and are susceptible to fatigue. Type IIA oxidative metabolism and contraction capacity lie between type I and type IIB fiber types (Deshmukh, 2016; Talbot and Genetics, 2017).

The fiber types in skeletal muscles are intermixed with varying proportions of type I and type II fibers in different groups. The composition of different fibers helps in accomplishing different functional muscle needs (Neunhäuserer et al., 2011). In a muscle section, the fiber types are typically recognized by their myosin heavy chain isoforms that can be differentiated with pH-sensitive ATPase assays. The myosin ATPase activity of fibers correlates with different MyHC and their contractile properties.

Muscle fibers have an extraordinary ability to adapt to different uses for example during high endurance exercise, where myosin heavy chain type I and type IIA fibers are recruited to suit the increased oxidative capacity and improved mitochondrial biogenesis (Seene and Kaasik, 2017). In healthy skeletal muscle, this may happen with intense exercise, which can promote an increase in the proportion of type I fibers making them resistant to fatigue (running) or type II resistant to endurance (bodybuilder). However, during pathological or paraphysiological conditions, the skeletal muscle fibers show a high vulnerability towards muscle disuse and bring in changes between the fiber types, force generation and moment. Fibers are also differently susceptible to muscle diseases: type I fibers are more prone to inactivity and denervation-induced atrophy, while type II fibers are vulnerable to secondary muscle involvement in case of cancer, diabetes, chronic heart failure, and aging (Miljkovic et al., 2015). In patients with type 2 diabetes, type I fibers tend to decrease with increase in type IIX fibers. Patients with Duchene muscular dystrophy have shown the onset of degeneration in type II fibers faster than type I fibers (Talbot and Genetics, 2017; Wang et al., 2004).

Many muscular disorders are caused as a consequence of mutations on muscular proteins and alterations in post-translational modifications (Davies and Nowak, 2006). Besides, abruption in the functioning of several signaling pathways, such as IGF1-Akt-FoxO signaling, inflammatory cytokines and NFκB signaling was also linked in the progression of several muscular disorders (Bonaldo and Sandri, 2013).

Skeletal muscle includes different classes of proteins related to muscle contraction, calcium binding and with enzymatic activities dedicated to maintaining the normal muscle physiology. Several skeletal muscle proteins are shown to be post-translationally modified to be functionally active. For example, actin has been shown to be heavily modified by phosphorylation, acetylation, glycosylation, SUMOylation and ubiquitination to regulate its stability and functionality (Terman and Kashina, 2014). The ryanodine receptor an important

protein which regulates calcium release in skeletal and cardiac muscle was shown to be S-palmitoylated to maintain its functional activity (Chaube et al., 2014). Calcium binding protein-like calsequestrin 1 has also been shown to be glycosylated to maintain its normal functionality (Sanchez et al., 2012). Tropomyosin, an important component of sarcomere, is shown to be phosphorylated to maintain its interaction with troponin during muscle contraction (Schulz et al., 2012). Since post-translational modifications have been shown to regulate different functions of proteins, it is imperative that knowledge on these modifications could unravel new unidentified functions of various proteins. Understanding the roles and modulations of PTMs on various muscular proteins could lead to new therapeutic approaches towards controlling or treating different muscular dystrophies (Walsh and Jefferis, 2006).

2 Post Translational Modifications (PTMs)

Proteins encoded by genes are transcribed from deoxyribonucleic acid (DNA) into messenger ribonucleic acid (mRNA), which are then translated into polypeptide chains by ribosomal machinery. These polypeptides or the folded mature proteins may undergo covalent modifications called PTMs. PTMs on target proteins mostly occur on amino acid side chains containing nucleophilic groups that can be easily targeted by electrophilic cofactors. Some examples of such amino side chains residues are Aspartic acid, Serine, Tyrosine and Lysine (Walsh et al., 2005). Depending on their enzyme machinery enzymatic activity, PTMs can be either reversible or non-reversible modifications on target proteins. PTMs regulate the key physical and chemical factors of proteins such as structure, stability, function, localization, activity and interaction with other proteins. For example, some of the well-studied PTMs are phosphorylation, which regulates signal transduction pathways, acetylation, which regulates of protein-DNA interactions, methylation, which regulates gene expression and ubiquitination, which maintains proteolysis (Santos and Lindner, 2017; Walsh et al., 2005).

PTMs play a significant role in the functional aspects of complex organs like skeletal muscle for the entire lifetime of a mammal. Among different PTMs, phosphorylation and ubiquitination are the most studied in relation with the pathophysiology of skeletal muscle. Phosphorylation is known to be a major contributor in enzymatic regulation of signal transduction pathways, muscle excitation-contraction coupling, sarcomere function, and inflammatory factors present in different compartments and pathways in skeletal muscle (Deshmukh, 2016). Ubiquitination, also known as Ubiquitin proteasome system (UPS) is one of the proteolytic pathways majorly involved in the maintenance of protein homeostasis in skeletal muscle (Bilodeau et al., 2016) .

2.1 The Ubiquitin pathway

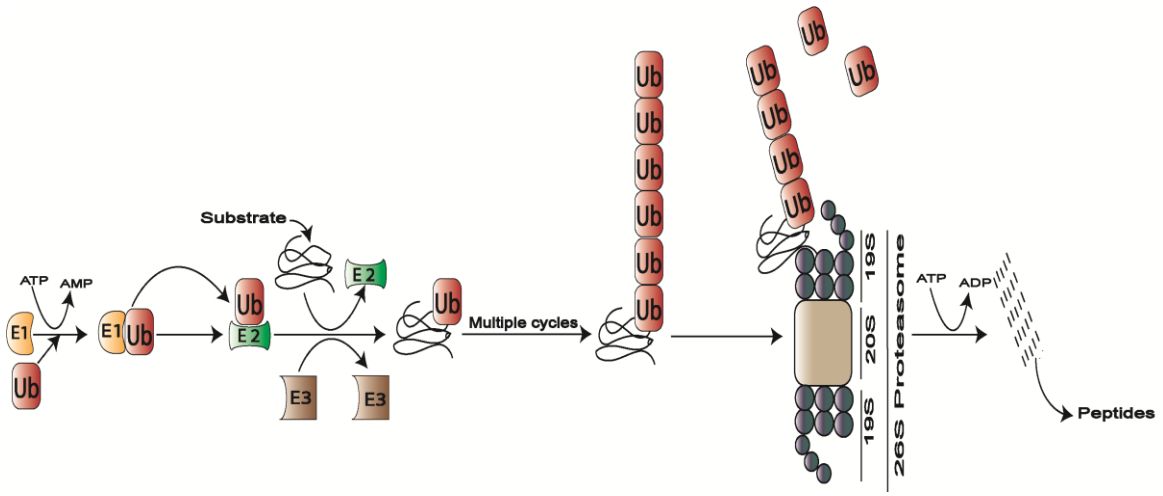

Figure 2. *Schematic representation of Ubiquitin pathway. Ubiquitin is activated by E1 activating enzyme. Activated ubiquitin is then transferred onto E2 conjugating enzyme. Then the E3 ligase forms a complex with E2 enzyme and ubiquitin. The substrate is specifically recognized by E3 ligase and catalyzes the transfer of ubiquitin onto the substrate from E2 enzyme. This process of conjugation continues until a chain of poly-ubiquitins are formed. The Poly-ubiquitin chains serve as degradation signal for 26S proteasome system. The substrates is then degraded by 26S proteasome system and reduced into small peptides*

Ubiquitin (Ub) is a small protein, which has a molecular weight of 8.5 kDa with 76 amino acid residues. The process of covalent attachment of Ub onto the target protein is defined as ubiquitination. Degradation of proteins by ubiquitination involves a vigorous action of three different classes of enzymes, mainly resulting in the formation of an Ubiquitin chain on a protein as a signal for degradation. Firstly, E1 activating enzymes with the help of ATP hydrolysis form a thioester bond with Ub. Then, the activated form of Ub is transferred onto an E2 conjugating enzyme, which forms a thioester bond at its cysteine residue with Ub. The E3 ubiquitin ligase then catalyzes the transfer of Ub on to a specific lysine on the substrate, which is either the target protein or the existing Ub molecule. ATP dependent process of ubiquitination continues until a chain of polyubiquitins are tagged to the target protein (Ciechanover, 1982) (Ciechanover, 2010). These polyubiquitin chains serve as a signal to 26S proteasome to degrade the target protein. The 26S proteasome structure contains a 20S core subunit and two 19S caps on either side of the 20S subunit. The ubiquitinated protein is recognized by one of the 19S caps, and before the entry into the barrel-like core subunit, the Ub chains are cleaved off from the target protein. Then with the help of ATPase activity, the proteins are unfolded from their native folded forms and enter into the 20S core subunit for degradation, where they are reduced into short peptides (Burger and Seth, 2004; Dohmen and Scheffner, 2012; Lecker, 2006; Metzger et al., 2014).

2.2 Muscle Specific Ring Finger protein 1 (MuRF1) - an E3 Ubiquitin Ligase and its role In Skeletal Muscle Pathophysiology

The selectivity and specificity of Ub conjugation on target proteins largely depends on E3 ubiquitin ligases, since they interact with both E2-Ub and the target protein. In mammalian cells, only one E1 Ub activating enzyme, around 40 E2 Ub conjugating enzymes and about 1000 E3 Ub ligases were identified to date (Santos and Lindner, 2017). Among the large pool of known E3 ligases, MuRF1 is one of the skeletal muscle-specific E3 ubiquitin ligases.

MuRF1 has been shown to interact with the giant protein titin and play a key role in maintaining sarcomeric M-line structure in cardiomyocytes. (McElhinny et al., 2002) (Mrosek et al., 2007). MuRF1 was also detected in nuclei of cardiomyocytes and shown to interact with a nuclear transcriptional regulator, the glucocorticoid modulatory element binding protein-1 (McElhinny et al., 2002). MuRF1 has been studied in regulating the production of reactive oxygen species in mitochondria of cardiac muscle in mouse models. Mattox and coworkers, suggested that SUMOylation or ubiquitination may facilitate the translocation process of MuRF1 into mitochondria (Mattox et al., 2014). However, it has not been clearly shown how and what promotes the actual process of MuRF1 translocation into this cellular compartment. Many studies on muscle pathophysiology show upregulation of MuRF1 at both mRNA and protein levels in muscle, during stress conditions like fasting, immobilization and denervation leading to muscle atrophy (Koyama et al., 2008; Lange et al., 2006). Upregulation of MuRF1 subsequently promotes ubiquitination on myofibrillar proteins like troponin I and myosin heavy chain during muscular atrophy (Mattox et al., 2014). On the contrary, in MuRF1 knockout (KO) mice, muscles were protected from developing contractile weakness (Hooijman et al., 2015) and have shown a decrease in the atrophy process in unloaded soleus muscle compared to unloaded soleus muscle in control mice (Labeit et al., 2010). It was also shown that skeletal muscle mass along with fiber cross sectional area significantly decreased with aging in control mice but was spared in MuRF1 KO mice (Hwee et al., 2014). Studies in skeletal muscles from various MuRF1 KO murine models suggest MuRF1 may have an extended role beyond maintaining protein turnover. For example, MuRF1 has been shown regulating carbohydrate metabolism and energy homeostasis in skeletal muscles (Baehr et al., 2011).

2.3 Ubiquitin Proteasome System in Skeletal Muscle

There are four main proteolytic pathways involved in protein degradation in muscle cells, the ubiquitin–proteasome pathway, the autophagy–lysosome system, the calpains and the caspase pathways. The Ubiquitin Proteasome System (UPS) is one of the major and studied networks commonly related to skeletal muscle pathophysiology. In normal physiological conditions, UPS maintains the balance of protein turnover in skeletal muscles to keep muscle mass stable. As regulator pathway, UPS plays a significant role in the elimination of unfolded proteins, which otherwise instigate degenerative disorders due to the accumulation of not functional or damaged proteins (Dohmen and Scheffner, 2012; Klaude et al., 2012; Sun et al., 2012).

2.4 Ubiquitin-like Protein modifiers

Ubiquitin-like protein modifiers (Ubls) are small proteins between 8 to 20 kDa in size and have a globular β-grasp domain and a flexible C-terminal tail resembling the ubiquitin molecule in structure. Functionally, all Ubls covalently conjugate to different substrates and regulate a wide range of their functionalities. Each Ubl mostly has a unique set of specific enzymes, which only recognize the specific moieties in each conjugation/deconjugation reaction (Taherbhoy et al., 2012). Some of the well-known Ubls are NEDD8, SUMOs, Atgs (Taherbhoy et al., 2012), ISG15 and FAT10 (Spinnenhirn et al., 2017). Among all these Ubls, SUMO pathway has received reputable attention with many substrates being identified as SUMOylated targets, and disturbances observed in the conjugation/deconjugation reactions

were implicated in the progression of fatal conditions like cancer, diabetes, heart failure and neurological diseases (Hochstrasser, 2000).

3 Small Ubiquitin-like MOdifier protein, SUMO

SUMO is a small 12 kDa protein and was discovered in 1996 as a substrates modifier in eukaryotic cells which was found to be covalently conjugated to GTPase activating protein RanGAP1 (Mahajan et al., 1997) (Hay, 2005) (Jürgen Dohmen, 2004). SUMOylation is a PTM reaction where the SUMO proteins are covalently conjugated and deconjugated to a large number of cellular targets involved in a variety of processes (Sarge and Park-Sarge, 2011). Since then, SUMOylation has emerged as a key regulatory mechanism for many cellular events in eukaryotes such as nuclear transport, transcription, replication, recombination and chromosome segregation. SUMOs are codified by SUMO-1 (sentrin, Pic1, GMP1, Ub11 and Smt3c), SUMO-2 (sentrin-3, Smt3a), SUMO-3 (sentrin-2, Smt3b) (Hay, 2005) (Johnson, 2004), SUMO-4 (IDMM5) (Li et al., 2017) and four isoforms designated SUMO-1, SUMO-2, SUMO-3 and SUMO-4 have been identified in mammals.

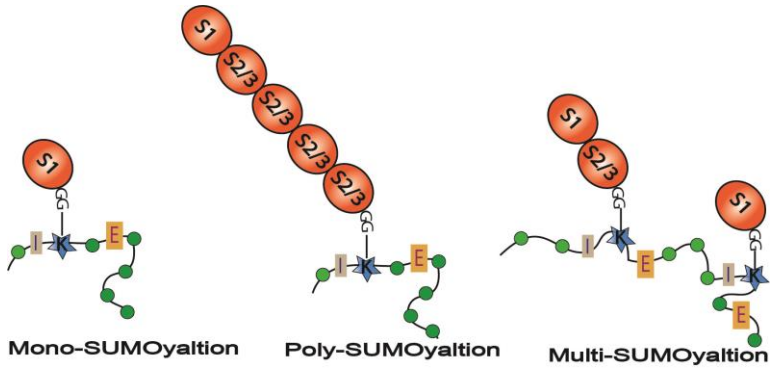

Figure 3. *Schematic representation of modification of substrates by SUMO-1 and SUMO-2/3 moeities. SUMO-2/3 can form polySUMO chains as they have a lysine residue present in their consensus sequence. SUMO-1 can only monoSUMOylate or act as a terminator for the polySUMO chains formed by SUMO-2/3.*

SUMO-1 is divergent from the other SUMO isoforms with only 47% sequence similarity with SUMO-2. SUMO-2 has 97% sequence similarity with SUMO-3 and 86% sequence similarity with SUMO-4. SUMO-2/3 can form polySUMO chains as they have a lysine residue present in their consensus sequence. In contrast, there is no lysine residue present in SUMO-1 consensus sequence, which makes it a terminator for the polySUMO chains formed by SUMO-2/3 isoforms. SUMO modification is a reversible and dynamic mechanism, which alters the localization and function of the conjugated protein substrates (Mukhopadhyay and Dasso, 2007; Sarge and Park-Sarge, 2011; Yeh, 2009). Specifically, SUMOylation of target proteins by SUMO-1 is vital for cellular processes like nuclear transport, cell cycle control, oncogenesis, and inflammation, while, SUMO-2 formation of poly chains regulate processes like chromosome segregation and DNA-damage response. SUMO-1 conjugation of target proteins regulates their interactions with other cellular components, whereas SUMO-2/3 conjugation is proposed to be a stress initiative reaction. It was suggested that SUMO-1 conjugation may be an antagonist to ubiquitin pathway, however poly-SUMO chains formed by SUMO-2 conjugation on a target protein serve as a signal for ubiquitination, where a ubiquitin E3 ligase, RNF4 recognizes the poly-SUMO chains formed by SUMO-2 via SIM domains and target them for ubiquitination (Kung et al., 2014; Sun and Hunter, 2012).

3.1 The SUMO pathway

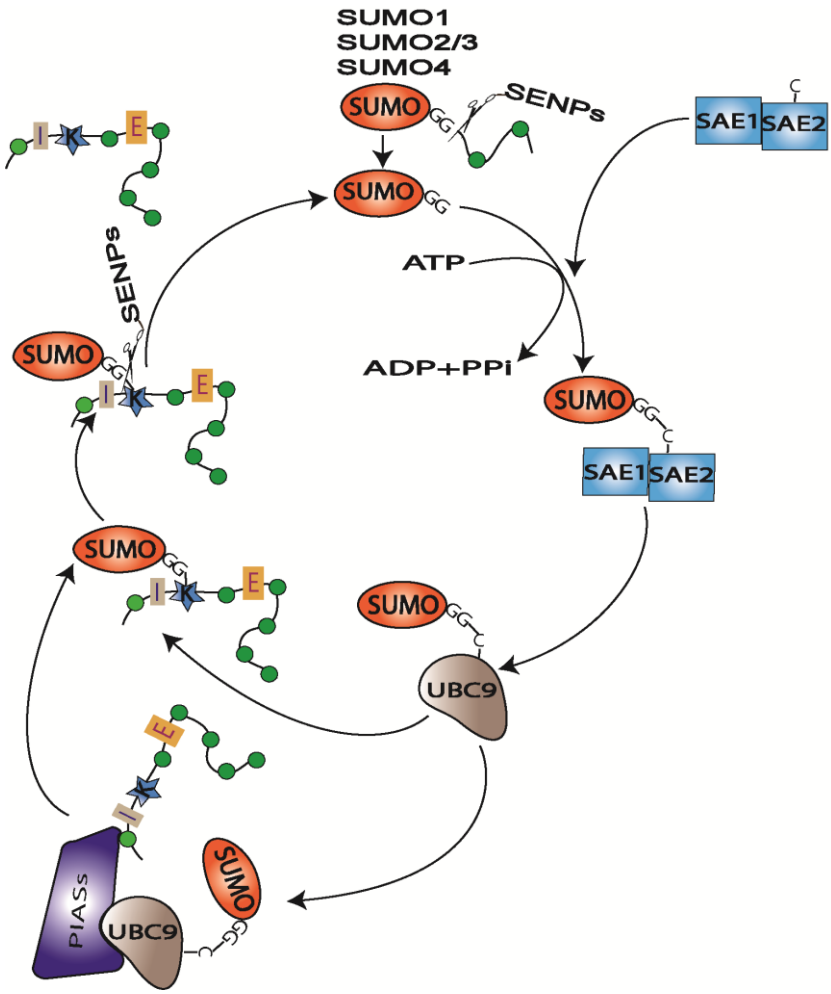

Figure. 4 *Schematic representation of SUMO pathway. SUMO is produced as a precursor protein, which is processed by sentrin-specific proteases (SENPs) and expose carboxy-terminal GG motif. SUMO is then activated by an ATP dependent reaction driven by SUMO activating enzyme1 (SAE1/2). The activated SUMO moiety is then transferred to the catalytic site of a cysteine on E2 conjugating enzyme, Ubiquitin like conjugating enzyme 9 (UBC9). Upon conjugation UBC9 recognizes the target protein and catalyzes an isopeptide bond between glycine residue on C-terminal of SUMO and the lysine residue present in sequence of target protein. UBC9 also catalyzes the SUMO conjugation to the target protein with E3 Ligases, protein inhibitor of activated STAT (PIAS). SUMOylated protein is then desumoyolated by SUMO de-conjugases of SENP family. The SUMO is then released from the target protein and is ready for another cycle of SUMOylation.*

Like other analogous Ubls, SUMO proteins are synthesized as inactive precursors that are cleaved to expose the C-terminal di-glycine motifs by SUMO-Sentrin specific proteases (SENPs), before conjugation onto lysine residues of target proteins. SUMO conjugation and deconjugation occur in a series of enzymatic cascades involving the sequential reactions of E1 SUMO-activating enzymes, E2 conjugating enzyme and E3 ligases. Apart from covalent attachments, proteins can also interact with SUMO molecules with their SUMO Interacting Motif (SIM) or SUMO interaction domains (Wilkinson and Henley, 2012). Alterations in the SUMO pathway bring dynamic global changes in SUMOylation of substrates and contribute to the development or onset of a disease (Gill, 2004). Further research can provide information on molecular mechanisms by which deregulation of SUMOylation and its substrates leads to different pathological conditions which in turn, can help in developing potential therapeutic strategies towards various diseases.

3.2 SUMO E1 Activating Enzymes (SAE1/SAE2)

After the cleavage of additional amino acid residues near the di-glycine motif at C-terminus of SUMO proteins, they need to be activated by SUMO E1 Activating Enzymes (SAE1/SAE2). SUMO E1 activating enzyme is a heterodimer, where SAE1 and SAE2 encoded by Aos1 and Uba2 genes respectively (Johnson, 2004). The SUMO E1 activating enzymes form a complex with SUMO proteins using ATP hydrolysis. The E1 enzyme forms a thioester bond between its conserved Cys residue and the C-terminal of SUMO proteins, releasing AMP. Following a transesterification reaction, SUMO proteins are then transferred onto a conserved Cys residue on E2 conjugating enzyme (Mukhopadhyay and Dasso, 2007) (Colby, 2006)

3.3 SUMO E2 conjugating enzyme, Ubiquitin-like Conjugating Enzyme 9 (Ubc9)

In contrast with ubiquitin conjugation machinery, SUMO conjugation involves a single E2 conjugating enzyme known as UBC9 encoded by UBE2I gene, which reacts with all four SUMO paralogues in mammals (Ihara et al., 2008). After the transfer of SUMO onto UBC9 by E1 enzyme, a thioester bond is formed between the C-terminus region of SUMO and conserved Cys residue of UBC9. (Borja-Cacho and Matthews, 2008; Mo and Moschos, 2005; Rytinki et al., 2009).

3.4 SUMO E3 Ligases

Although UBC9 can solely recognize the acceptor lysine on the substrates, SUMO E3 ligases further enhance the substrate specificity and catalyze the transfer of SUMO onto the substrate from UBC9 (Flotho and Melchior, 2013). RanBP2 (Kirsh et al., 2002), TOPORS (Weger et al., 2005), Pc2 (Kagey et al., 2003), MAPL (Rabellino et al., 2017), TRAF7 (Yutaka Morita et al., 2016), ZNF451 (Pichler et al., 2017) and PIAS family proteins are some known SUMO E3 ligases (Rabellino et al., 2017). Among these SUMO E3 ligases, PIAS family proteins are well studied in mammals. Four PIAS genes are identified including, PIAS1, PIAS2 (with two splice variants PIASxα and PIASxβ), PIAS3 (with the splice variant PIAS3γ), and PIAS4 (also known as PIASγ, with splice variant PIASγE6) (Rabellino et al., 2017). All five isoforms, PIASxα, PIASxβ, PIAS1, PIAS3, and PIASγ show different selective specificity of substrates (Wang, 2011). PIAS family proteins also act as E3 ligase of SUMO on various transcription factors like p53, STAT, c-jun. (Duval et al., 2003; Rytinki et al., 2009)

3.5 SUMO Sentrin Specific-Proteases (SENPs)

SUMO proteins are translated as precursors and must be matured to be functionally active; specific proteases known as SENPs catalyze this maturation process. SENPs are responsible for cleaving the additional amino acid residues present at the C-terminus of SUMO proteins, and thus expose the di-glycine residue required for the conjugation process (Yeh, 2009). SENPs are also involved in deconjugation of SUMO from target protein after the process of modification (Hay, 2005). So far, six genes of SENPs have been discovered in mammals designated as SENP 1, 2, 3, 5, 6, 7, among them SENP1 and SENP2 act as deconjugating enzymes of all SUMO isoforms, whereas SENP 3, 5, 6, 7 specifically deconjugate SUMO2/3 (Drag and Salvesen, 2008) (Wang, 2011). SENPs are differently localized within the

mammalian cell, and this different localization of SENPs also illustrate that SUMOylation is essential at different subcellular components (Mukhopadhyay and Dasso, 2007).

3.6 SUMO consensus sequence

The SUMO conjugation reaction generates a covalent bond between the C-terminal carboxyl group of the SUMO moiety and an acceptor lysine residue on the substrate. In several identified SUMO substrates the lysine residue was surrounded by short specific conserved consensus sequence ψKXE/D, where (K) is the lysine on which the modification occurs (Gareau and Lima, 2010). In this consensus motif, ψ is a bulky aliphatic amino acid, X is any amino acid, and E/D is an acidic residue. This short amino acid sequence mostly serves as a recognition site for all the SUMO proteins to SUMOylate the target protein. The E2 conjugating enzyme UBC9 directly interact with the lysine residues within this consensus sequence on substrates and conjugates SUMO onto it (Hickey et al., 2012). However, not in all the SUMO substrates, the SUMO acceptor lysine was situated in the SUMO consensus motif, ψKXE/D. In some substrates, the SUMO consensus sequence was adjoined by longer domains including phosphorylation-dependent SUMO motifs (PDMs) and negatively charged amino acid-dependent SUMO motifs (NDMs) (Gareau and Lima, 2010). For example, a mass spectrometry analysis of SUMO substrates Nucleolar protein 58, RNA Binding Motif Protein 25, Anaphase-promoting complex subunit 4 and DNA topoisomerase 2 revealed the presence of SUMOylated lysines and the phosphorylated serine with a preferred spacer of four amino acids between them (Matic et al., 2010).

3.7 SUMO Interaction Motif (SIM)

SIM domain promotes the non-covalent interactions between SUMO moieties and SIM domain-containing proteins. The SIM domain consists of a short stretch of hydrophobic amino acids, (v/I) X (v/I) (v/I), which are bordered by acidic residues. SUMOs interact non-covalently with their binding partners via SIM mediated interactions through these short sequences, When SUMO-SIM complex is formed, a parallel or antiparallel β-strand conformation extends the SUMO β-sheet to allow the hydrophobic side chains of the SIM to interact with a hydrophobic pocket on the SUMO surface. The acidic residues present on SIM domain following the hydrophobic region on proteins containing SIM contribute the SIM-SUMO interaction. The SIM domain binds to all SUMO isoforms commonly. SIMs are reported to be present in some SUMO E3 ligases PIASX and Ran binding-protein 2 (RanBP2/Nup358), and Promyelocytic leukemia protein (Da Silva-Ferrada et al., 2012; Song et al., 2004)

3.8 Outcome of SUMOylated Proteins in eukaryotic cells

Initially, SUMOylated proteins were identified predominantly in nucleus, but in recent times, more SUMO substrates are being discovered in other cellular compartments. Although the molecular consequences of SUMOylation are diverse and challenging to predict, they can be summarized in these three categories, and SUMOylation can influence the (i) stability, (ii) localization and (iii) functional activity of the target proteins (Wilkinson et al., 2012). When a substrate is SUMOylated, the modified SUMO moiety extend the substrate surface and influence or promote interactions with other macromolecules, for example, only the

SUMOylated Ran GTPase-activating protein 1 (RanGap1) interacts with RanBP2 and relocates to nuclear pore from cytosol, alternatively, RanGap1 doesn't do (Mahajan et al., 1997).

SUMOylation can bring conformational changes in the substrate that can alter their enzymatic functions. For example, SUMOylation triggered conformational changes on thymine–DNA glycosylase enhances its enzymatic activity in base excision repair of G:U/T mismatches and in transcription (Steinacher and Schär, 2005)(Smet-Nocca et al., 2011). SUMOylation can promote protein-protein interactions by SIM domain that can bring changes in substrate localization, function, and stability; for example, SUMOylated transcription factor ElK2 can interact with histone deacetylase (HDAC) 2 via SIM domain, which in turn decreases its transcriptional activity. In all these processes SUMOylation brings stable changes in target proteins, and deSUMOylation plays an opposite role. (Geiss-Friedlander and Melchior, 2007; Wang and Dasso, 2009; Wilkinson and Henley, 2012; Zhao, 2007)

SUMO reactions have emerged as an important process that controls fundamental mechanisms for regular activity in eukaryotic cells. SUMOylation was also assigned to be a fine sensor that can detect and respond immediately to normal cellular alterations as a consequence of external or internal perturbations. For example, during non-physiological conditions, oxidative, osmotic and heat shock stress, might lead to alterations in expression levels of SUMO enzymes and consequently change the status of SUMOylated proteins (Anderson et al., 2012).

SUMO modifies and regulates key biological properties of a large number of proteins that are involved in many cellular pathways. For example, SUMO modifies proteins like TOP-II, RanGAP 2 in cell cycle regulation, proliferating cell nuclear antigen in DNA replication and DNA damage repair, the dynamin-related protein in mitochondrial fission and transcriptional factors like HDAC1 and HDAC4. SUMOylation is also involved in the regulation of transcription factors by multiple mechanisms and enhances the functions of transcription activators, or it inhibits and affects the interaction with co-regulators. SUMOylation mediated inhibition has often been correlated with translocation of the transcriptional activators to nuclear bodies and transcriptional repressors into nuclear reservoirs. For example, SUMO-modified transcriptional activator Elk-1 recruits HDAC2 to promoters, which results in transcriptional repression. SUMOylation of HDAC1 and HDAC4 was shown to enhance their ability to repress transcription (Jürgen Dohmen, 2004).

3.9 SUMO in brain

Deregulation of SUMOylation was also shown as a vital factor in the progression of neurological disorders, including neurodegenerative diseases, such as Alzheimer and Parkinson's diseases, spinocerebellar ataxias, cerebral ischaemia and epilepsy (Coelho-Silva et al., 2017).

Tyrosyl DNA phosphodiesterase-1 is a protein that is important in resealing single-strand DNA breaks. It is shown to be SUMOylated to maintain its functionality. A disruption in SUMOylation of tyrosyl DNA phosphodiesterase-1 interrupts its function and has been linked to causing ataxia in humans (Coelho-Silva et al., 2017). DJ-1 a multifunctional protein

that is implicated in the progression of Parkinson disease was shown to be SUMOylated. DJ-1 is SUMOylated on lysine 130, and mutation on its SUMO site led to blockade in its functionality such as repression of p53 transcriptional activity and impairment of translocation into the nucleus to be a transcriptional regulator (Droescher et al., 2013). Several SUMO substrates were identified in relation with signaling pathways that contribute in the progression of Alzheimers disease. For example deregulation in SUMOylation of substrates such as amyloid precursor protein, amyloid β, and tau proteins is implicated in the development of Alzheimers disease (Coelho-Silva et al., 2017).

3.10 SUMO in muscle

With the growing evidence day-by-day, SUMO pathway and SUMOylated proteins are linked to many cardiomyopathies and muscular dystrophies. In several human cancers, up-regulation of SUMO E1 activating enzyme, UBC9 the E2 conjugating enzyme and E3 enzymes of PIAS family were reported (Sarge and Park-Sarge, 2009). It has been discovered that SUMOylation plays an essential role in muscle differentiation. The knockout of SUMO E2 conjugating enzyme UBC9 in C2C12 cells has shown depletion in the SUMOylation protein levels which had consequently compromised the myoblast differentiation (Riquelme et al., 2006). Important contractile proteins α and β-actin were shown to be a SUMO-1 and a SUMO-2 moieties targets, suggesting that this PTM may have a regulatory function on muscle contraction in skeletal muscles (Uda et al., 2015). Some important muscle related proteins like SERCA2a (Kho et al., 2015), PAX7 (Wazen et al., 2014), snoN (Wrighton et al., 2007) were discovered to be SUMO substrates and are being investigated for their role in muscle pathophysiology.

3.11 SUMO in cardiac muscle

SUMO pathway has been shown to be playing a significant role in controlling cardiac gene activity and heart development. For example, the Serum responsible factor, GATA4, a zinc finger-containing transcription factor, Myocyte Enhancer Factor-2, Prospero-related homeobox are shown to be SUMOylated, which is essential for the morphogenesis and normal function of the heart (Wang and Schwartz, 2010).

Lamin A, a protein that plays an important role in maintaining structure and function of the nucleus was shown to be SUMOylated to retain its standard functionality. Mutations in lamin A instigate decrease in its SUMOylation, which led to diseases like muscular dystrophies, and cardiomyopathy in humans. Depletion of SUMO-1 levels was observed in heart failures in human, mouse and porcine models. Contrastingly, SUMO-1 overexpression notably improved the heart condition in mouse. SERCA2a an endoplasmic reticulum–associated Ca^{2+} transporting ATPase in cardiac muscle was shown to be SUMOylated by SUMO-1 to maintain its functionality and stability. Although it is yet to be discovered why SUMO-1 levels are reduced in heart failures, it remains that SUMOylation plays a crucial role in both the developing and adult heart (Flotho and Melchior, 2013).

3.12 Drugs targeting SUMO pathway

SUMO proteins modulate crucial functions of tumor suppressor proteins like p53, pRB, p63, p73, and mdm2, and deregulation in SUMOylation of these proteins may lead to different cancers (Pichler et al., 2017)(Sireesh et al., 2014). Considering the importance, involvement and regulation of SUMO pathway in different processes of eukaryotic cells and several disease pathways, one can speculate that SUMO pathway can be a possible target towards treating various diseases. The SUMO pathway could be modulated pharmacologically by manipulation of any of the enzymatic activities associated with the SUMO reactions, or by preventing the binding of specific SUMO substrates to members of the SUMOylation/deSUMOylation machinery.

Many drugs are now found to inhibit different steps involved in the processing of SUMO by targeting SUMOylation. Pharmacological intervention towards only SUMO E1/E2 enzymes SAE1/SAE2 and UBC9 in SUMOylation pathway, would cause a global modulation of total cellular SUMOylation (Anderson et al., 2012). For example, synthetic compounds like ginkgolic acid and davidiin are found as SUMO E1 inhibitors (Takemoto et al., 2014). N106 a small compound was shown to enhance SUMO E1 enzyme activity and improve cardiac function in mice with induced heart failure (Kho et al., 2015). Spectomycin B1 and GSK145A are found to be UBC9 inhibitor (Yang et al., 2018). Similarly, the 2', 3', 4'-trihydroxyflavone (2-D08), an oxygenated flavonoid derivative, is a synthetic flavone that obstructs the SUMOylation by inhibiting the binding of SUMO in the E2-SUMO intermediate to the substrate. Several synthetic compounds like peptide vinyl sulfones, benzodiazepine analogs, 1, 2, 5-oxadiazole, peptide aza-epoxides were discovered modulating the enzyme activity of SENPs which process the maturation and deSUMOylation of SUMO proteins (Sireesh et al., 2014). Downregulation of SUMOylation by Topotecan is shown to be beneficial in treating glioblastoma and other cancers (Yang et al., 2018).

A thorough investigation is necessary to design potent inhibitor molecules that can be used to block the SUMOylation of respective target proteins specifically. Thus, SUMOylation must be investigated for a better understanding of its significance in various pathological conditions, and this may serve as a potential therapeutic target for future drug development of various diseases including muscular atrophies, cardiovascular, neurodegenerative disorders and cancers (Sireesh et al., 2014).

4 Diaphragm

In mammals, the diaphragm is one of the most essential and unique skeletal muscle necessary for the inspiratory phase during the physiological ventilation. The diaphragm is located between the thoracic and abdominal cavities and serves as a barrier between them (Merrell and Kardon, 2013). The diaphragm is one of the essential skeletal muscle that is composed of mixed fiber type. Depending on the parameters of ventilation demands across different species, the relative proportions of fatigue-resistant motor units in the diaphragm vary (Sieck et al., 2012). In humans, the diaphragm is composed of type I and type IIA fibers whereas in murine it is composed of type I, type IIA and type IIX and IIB fibers (Meznaric and Cvetko, 2016; Sieck et al., 2012). Diseases of the diaphragm can be developed from birth, for example, in humans, congenital diaphragmatic hernias are prevalent congenital disabilities,

that are a result of an underdeveloped diaphragm, leading to high morbidity and mortality in infants (Merrell and Kardon, 2013). Dysfunction of the diaphragm can also result from metabolic or inflammatory disorders, trauma or surgery, mechanical ventilation, during myopathies or neuropathies (Kharma, 2013). Patients from these conditions develop weakness or blockade in contractile function of diaphragm muscle, which leads to severe respiration-related problems.

4.1 Mechanical Ventilation (MV) and Ventilator Induced Diaphragm Dysfunction (VIDD)

MV is a lifesaving intervention, commonly used in ICUs. It is used in patients with respiratory failure, cardiovascular instabilities, brain traumas, neuromuscular diseases, drug overdosage, different types of organ failures, during surgery and post-surgical recovery (Gayan-Ramirez and Decramer, 2002). Despite being a lifesaving intervention, it also has some negative effects on both short and long-term treated critically ill patients in ICUs. Generally, patients in ICUs spend 40% of their time in weaning from the mechanical ventilator. This delay in weaning procedure not only affects the patient's mortality but also affects morbidity and increases the overall costs of stay in ICUs for patients. VIDD is characterized as diaphragm weakness that occurs following short or longer durations of (Controlled Mechanical Ventilation) CMV. Presence of VIDD has negative effects on the recovery of ICU patients. So far the molecular mechanisms leading to the pathophysiology of VIDD have been poorly understood (Dasta et al., 2005; Ntoumenopoulos, 2015). Studies done on understanding the molecular mechanisms underlying the pathogenesis of VIDD, suggests that there is a transient increase in the oxidative stress, post-translational modifications, altered regulation of muscle contraction and atrophy in response to short to long-term CMV in rat diaphragm muscle fibers exposed to modelled ICU conditions (Corpeno et al., 2014).

4.2 BGP-15

Heat shock proteins (HSPs), are essential for restoring normal cellular functions during stress conditions in the body. BGP-15 a co-inducer of HSP72 when administered has shown tremendous improvement in mitochondrial efficiency and decreased reactive oxygen species production in CMV rat's diaphragm. As the mitochondrial dysfunction and reactive oxygen species production are important factors in the pathogenesis of VIDD, BGP-15 can be viewed as a potential therapeutic agent (Dasta et al., 2005; Ntoumenopoulos, 2015; Salah et al., 2016). BGP-15 has been shown to improve muscular strength and contractile function in mice with Duchenne muscular dystrophy (Gehrig et al., 2012).

5 AIM

The major aim of this thesis is to investigate and obtain better knowledge on the role of post-translational modifications mediated by SUMO moieties on proteins during skeletal muscles pathophysiology. The novelty of my thesis was to identify and characterize new muscle substrates which the SUMO conjugation and deconjugation could modulate functions in normal and in altered pathological conditions. This work affirmed utterly new approaches in both muscle and SUMO post-translational modification fields with the future perspectives to build bridges between basic research and clinical trials by providing information, which could be translated into pharmaceutical treatments to human patients with severe muscular disorders.

Specific aims:

- To show SUMOylation as an indispensable reaction that regulates the muscle activity and it is altered during diaphragm dysfunctions. To identify muscle proteins that are involved in the SUMO PTM in normal and muscle disease conditions.

- To investigate the positive effects of the HSP72 co-inducer, (BGP-15) drug, on diaphragm muscle in rats exposed to ICU condition, mainly focused on the alteration in SUMOylation to myosin protein.

- To explore the effects of SUMO-1 conjugation and deconjugation as a possible mechanism to regulate enzymatic activity and myocytes localization of the E3 Ubiquitin ligase MuRF1. Associate a specific protein role when it is localized in different cellular districts in high glucose conditions.

- To investigate, the different abundance of SUMO enzymes asserted in diverse groups of skeletal muscles in ambulatory rats. To demonstrate alterations in the SUMO machinery components followed to muscle inactivity.

6 METHODOLOGICAL CONSIDERATIONS

Animals

Female adult Sprague Dawley rats were used in the first and second papers. The rats were deeply sedated, post synaptic neuromuscular blockade (α-cobra toxin), mechanically ventilated extensively monitored and supplied parenteral nutrition during the entire duration of experiment (2 hours until 10 days). Control rats used in papers I, II and IV, were sham operated which were not paralyzed by α-cobra toxin but anaesthetized with isoflurane by a precision mass-flow controller and euthanized within 2 hours after initial anesthesia and surgery. In papers I and II, a separate group of rats were treated with BGP-15, a daily dosage of 40 mg/kg is given through intravenous fusion along shorter to 10 days of mechanical ventilation.

In paper IV, Wistar Han IGS rats were caged individually and were randomly assigned to either of all experimental groups, control until 4-day hind limb-unloaded animals. Hind limb muscles were unloaded using the tail-suspension model, by wrapping the tail root with tape under general anesthesia induced with intraperitoneal administration of 20mg/kg of Zolazepam chlorhydrate and tiletamine chlorhydrate. Animals were euthanized after inducing anesthesia with isofluorane.

Eukaryotic cell lines

C2C12 (a mouse skeletal muscle cell line) and HeLa (a female human breast cancer cell line) were used in paper I, III. All cultures were done in DMEM with 5.5 mM or 25 mM glucose concentration supplemented with 10 % heat activated FBS and 1 x 100 IU/ ml penicillin. All the cell incubations were done in 37° C incubators with 5 % CO_2 in air.

Transfection of Eukaryotic cells

For transfection, C2C12 cell lines or HeLa cells lines were cultured in 6 well or 12 well plates until they reach 40%–50% confluence then were transfected with the desired plasmids using lipofectamine. Cells for SDS PAGE were harvested after 24 h by centrifugation (3000 RPM for 10 min at 4°C) and washed with cold phosphate buffered saline (PBS) containing 0.2 M iodoacetamide. After 24 hours, cells for immunocytochemistry were washed with PBS and were preceded by fixation with 4% paraformaldehyde in PBS.

Transformation and induction in prokaryotic cells

6x HIS-pSUMO 1, 2 and 3 plasmids with conjugating enzymes (E1 dimers (SAE1-SAE2) and the SUMO E2 conjugating enzyme UBC9) along with GST-MURF1 plasmids were co-transformed into BL21 competent cells through heat shock method. Transformed samples were plated on agar plates with 100mg/ml ampicillin/50mg/ml streptomycin. Single colonies were picked and grown over night in 2 ml of 2X LB medium containing 100 mg/ml ampicillin and 50 mg/ml streptomycin. Overnight cultures were then diluted in fresh 300ml medium with appropriate antibiotic and grown at 37º C until the OD reaches 0.6. Then the samples were induced using isopropyl-b-D-thiogalactose (IPTG) with the final concentration of 250µM for 5 hours at 30ºC on a shaker and then transferred into 37°C for 30 min. These cells were then lysed with lysate buffer (50 mM Tris-Cl pH 7.5, 300 mM NaCl, 0.1% NP40,

0.05% SDS, 1 mM DTT, 20 mM NEM, protease inhibitors) and centrifuged at 13K rpm at 4º C for 15 min.

Lysate preparation

Cell lysates were prepared and soluble protein fractions were extracted 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 through a syringe 2–3 times. Crude lysates were clarified by centrifugation (13000 rpm for 15 min at 4°C) and protein concentration were measured and separated using SDS-PAGE after denaturing lysates at 95°C in presence of loading buffer.

Muscle lysates for protein expression analysis were prepared from frozen muscle biopsies and incubated with lyses 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, 20 mM NEM, protease inhibitors) for 15 min on ice, then homogenized in a 1.5 ml tube with pestle. Muscle homogenizes were centrifuged for 20 min at 13000 rpm at 4°C. Clear supernatants were collected and protein concentration was measured with a Protein Assay kit.

Muscle Lysates preparation for enrichment of SUMOylated Proteins for Mass Spectrometry analysis

For each control or mechanically ventilated group, a total of 200 mg of diaphragm muscle tissues were collected from at least five different rats and pooled into a group. We adopted protocols described in Becker et. al. and Barysch et. al., to perform the muscle lysates and solutions required for the SUMO immunoprecipitation, peptide elution and recycling of the affinity matrices as well as the procedure of protein G-agarose beads coupling with monoclonal anti-SUMO1 and SUMO2 antibodies produced in hybridoma cell lines. Diaphragm tissues were homogenized in lyses buffer (Tris-Cl 150 mM, NaCl 150 mM, SDS 0.5%, Nonidet P-40 1%, deoxycholate 0.5%, EDTA 5 mM, DTT 1 mM, fresh NEM 20 mM, and protease inhibitors, pH 7.6).

A pre-clearing step was performed using muscle lysates with protein G-agarose beads that were coupled to anti-mouse IgG for 3 hours and gently rotated at 4º C on a rotor. Precleared muscle lysates were divided into two portions and incubated one portion with anti-SUMO1 antibodies (50% 21C7 and 50% 76–86) and the other with protein G-agarose beads coupled with anti-SUMO2 antibodies (8A2) for the SUMO1 or SUMO2/3 complex enrichment. Then to further concentrate and remove contaminants from the SUMO immune complex, the samples were subjected to TCA precipitation. Then 150 µg of each TCA-precipitated elutes were separated on SDS-PAGE (4–12% gradient gel) and stained with Coomassie Blue. The gel pieces were cut according to the molecular weight and sent for mass spectrometry analysis.

Immunoblotting

Desired amount of lysates were denatured for 10 min at 95°C in loading buffer (NuPage 4X, Reducing Agent 10X) and fractionated in acrylamide Bis-TrisCl 4-12% gradient gel. After transferring onto PVDF membranes for 45 min at 0.34 A, the filters were blocked in TBS (50 mM Tris-Cl, 150 mM NaCl, pH 7,6) containing 0.1% Tween-20 and 5% non-fat milk and

incubated on the shaker with the specific primary antibodies for overnight at 4°C followed by incubation for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibodies. The complexes were visualized by chemioluminescence, detected by ChemiDoc MP imaging system, and analyzed with the correspondent imaging analysis software, version 5.0.

Quantitative real time PCR (qPCR)

The SUMOylation machinery components transcripts were analyzed by qPCR using concerned primers and designed with PRIMER3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). RNAs extraction was performed following the manual instructions included in GeneJET RNA purification Kit (K0731, ThermoFisher Scientific, Lithuania). Extracted RNA was purified from DNA contamination with DNase I treatment (EN0521, ThermoFisher Scientific, Lithuania). The correspondent cDNAs were produced using both oligo (dT) 18 and random primers by following the instruction of RevertAID H Minus First strand cDNA synthesis Kit (K1632, ThermoFisher Scientific, Lithuania). qPCR reactions were performed with 100 ng of cDNA template using PowerUp SYBR Green Master Mix (A25742, Thermo Fisher Scientific, Lithuania) in a 20 µL of final volume. The analysis was performed with QuantStudio 3 and 5 Real-Time PCR Systems with the following cycling program: initial 50°C 2 min, denaturation 95°C 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A final step of melting curve between 65°C to 90°C, 1°C/sec temperature speed was incorporated. Relative fold change relative to housekeeping control gene (GAPDH) was calculated as $2^{-\Delta Ct}$ (x1000) where: ΔCt = Ct (target) – Ct (GAPDH), according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline 54. All single samples were analyzed in triplicate and mean and ±SD were obtained with the samples included in each group of CMV, tail-suspended and Control rats.

Histological staining

Immunocytochemistry: A 30,000 C2C12 cells were cultured per well in a 12-well plate over coverslips pre-coated with poly-D-lysine. After 24 h, cells were transfected with 2 µg of desired plasmids. After additional 24 h, coverslips were fixed with 4% paraformaldehyde in PBS solution for 20 minutes. Then cells were fixed with 0.5% triton x100 in PBS for 90 min at RT, washed 3 times with PBS. Incubation with primary antibodies was performed for 90 min at RT, then washed 3 times with PBS followed by 1h incubation with secondary antibodies and washed again 3 times with PBS and mounted with Fluoroshield mounting medium with DAPI. Confocal microscope was used to acquire the images.

Immunoflurescence, NADH-TR staining and ATPase staining: Frozen muscle biopsies from rats were fixed with Compound for Cryostat Sectioning (OCT) and cryosectioned into 10 µm slices at -20˚ C.

Immunofluorescence: Skeletal muscle cryosections were incubated at room temperature (RT) for 5 min, rehydrated in PBS for 15 minutes at RT, fixated in cold acetone at -20º C for 15 min, dried for 1 min at RT, and incubated with blocking solution (3% BSA in PBS) for 40 min at RT. Incubation with primary antibodies was performed for 90 min at RT, then washed 3 times for 5 min with PBS followed by 1h incubation with secondary antibodies and washed

again with PBS. The slides were mounted with fluoroshield mounting medium with DAPI and pictures were acquired using ZEISS laser scanning confocal microscope.

NADH-TR staining: Skeletal muscle cryosections sections 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 H2O) 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 2 hours and cover glass was mounted with glycerine gelatin.

ATPase staining: Consecutive cryosections were preincubated with solution1, pH 10.3 (Glycine-100 mM, CaCl2-72 mM, NaCl-107 mM, and NaOH-100 mM) for 9 min at 37˚ C water bath and were rinsed with running distilled water. After preincubation cryosections were incubated with solution 5, pH 9.4 (ATP-3.35 mM prepared in solution 1) for 30 min at 37˚C water bath and rinsed in running distilled water. The sections were then incubated in Solution 2 (1% CaCl2) for 3 minutes and rinsed, further immersed in Solution 3 (2% CoCl2) for 3 min and rinsed in 3-4 changes of distilled water. Then they were incubated with Solution 4 (1 % (NH4)2S) for 1 minute and were rinsed with running distilled water. After rinsing, cover glass was mounted with glycerine gelatine and pictures of staining were acquired using Nikon Phase Contrast 0.90 dry microscope.

Images processing software: ImageJ software was used to measure band intensity from western blots, fluorescence intensity and area from immunofluorescence pictures of C2C12 cells and muscle cryosections.

Statistical analysis

One-way analyses of variance (Anova) and student t-test were used to compare multiple groups. $P < 0.05$ was considered statistically significant. Data are presented as average ± standard deviations. One-way analyses of variance and Tukey's test were used to compare multiple groups, and p 0.05 was considered statistically significant, data are presented as average S.D.

7 RESULTS AND CONCLUSIONS

7.1 Rationale for paper I

SUMO pathway is established as a critical regulator of various cellular processes in eukaryotic cells (Zhao, 2007). Deregulation of SUMO conjugation/deconjugation reaction was associated with several pathologies in humans such as neurodegenerative disease, cardiomyopathies and cancers (Yang et al., 2017). Although SUMOylation was extensively studied in single eukaryotic cells, brain tissue and cardiac muscle, it is not so well described its contribution in skeletal muscles. We, therefore, set out to investigate the significance of the SUMO pathway in skeletal muscle pathophysiology.

A previous study has shown a significant decrease of contractile force in rat diaphragm muscle fibers with a parallel increase of oxidative stress generated during early hours of CMV (Corpeno et al., 2014). SUMO conjugation and deconjugation reactions were reported to act in a very dynamic and equilibrate process. Consecutively, their kinetics properties were affected by alterations of the normal equilibrium leading sensitive deregulation of the SUMOylated proteins which contributed to the development of some human pathologies, as described in oxidative stress conditions (Feligioni and Nisticò, 2013). We hypothesized that the SUMO network as a fine sensor to detect non-physiological situations, it may respond differently to the altered condition in diaphragm activation pattern during short and long periods of CMV by bringing changes in the total muscle protein SUMOylation set, which can contribute to the observed decrease in contractility. We further postulated that alteration in SUMO enzymes during CMV treatment could assist in the progression of the severe muscle disease.

For this study, diaphragm biopsies was available from controls and rats exposed to ICU condition. Animals were deeply sedated, paralyzed by neuromuscular blockers and subjected to short and long term CMV (2 hours to 10 days), (Lars Larsson's group, Karolinska Institutet, Sweden), (Corpeno et al., 2014).

7.2 Variation of SUMOylated proteins in rat diaphragms with the effect of CMV (paper I)

Following with our hypothesis, Western blot analysis on diaphragm lysates from control and rats exposed to CMV has revealed that the conjugation of substrates by both SUMO1 and SUMO2/3 proteins have progressively increased with CMV treatment. For the first time through this study, we demonstrate the effect of CMV on global modulation of SUMOylation patterns in rat diaphragms.

7.3 Alterations in the localization of SUMOylated proteins between fiber types in rat diaphragms with the impact of CMV (paper I)

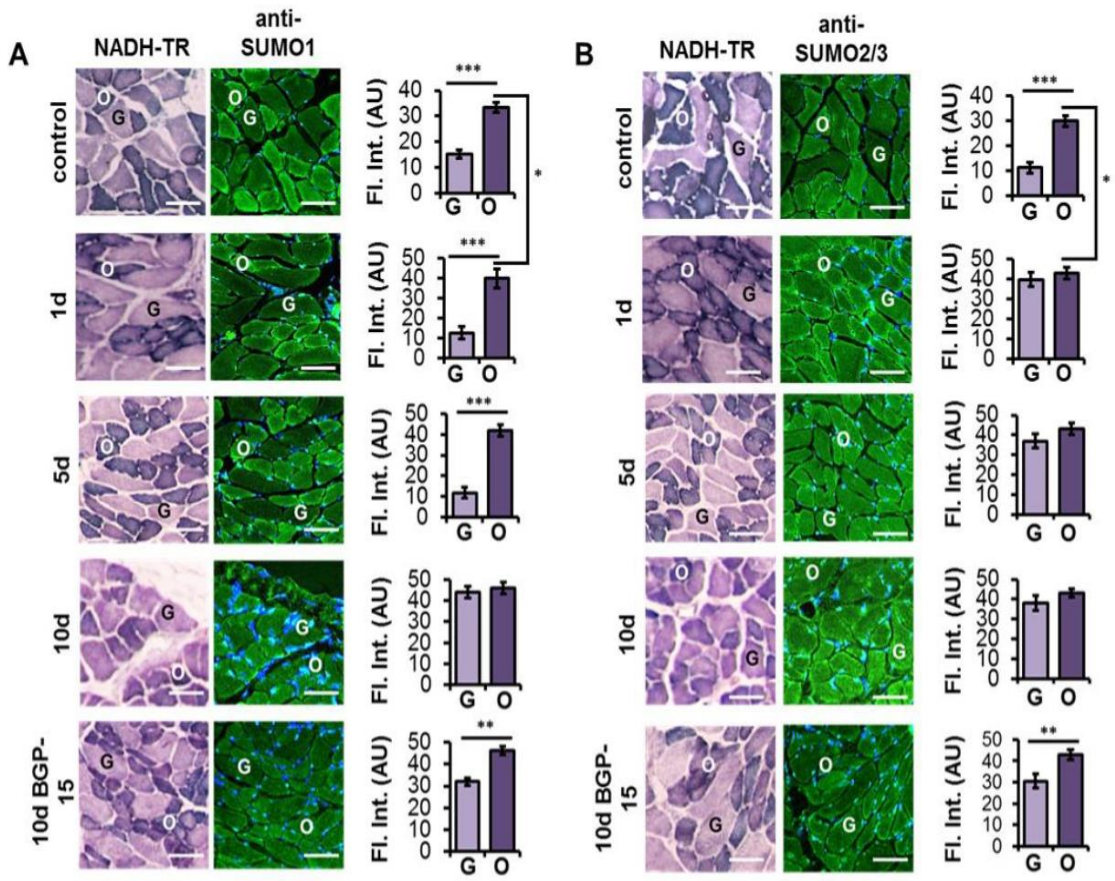

Figure 5. *(A) and (B) Images of rat diaphragm muscle sections from control and different stages of CMV ranging from 1 day, until 5 days and 10 days with and without BGP15. Left column shows NADH-TR staining, middle column shows IF staining with anti SUMO-1 (A), anti SUMO-2/3 (B) antibodies. Right column shows the florescence intensity of SUMO-1 (A) and SUMO-2/3 (B) in O-oxidative fibers and G-glycolytic fibers. IF pictures were taken using confocal microscope and NADH-TR pictures were taken from bright field microscope. (AU-arbitrary units).*

The immunoblot analysis results have raised several questions like, in which fiber types SUMOylated proteins are mainly localized? Which are the most affected fibers type in rat diaphragm during CMV treatment based on the SUMO protein content?

Immunofluorescence assay was performed on described diaphragm muscle cryosections using anti-SUMO-1 and SUMO-2/3 antibodies. The consecutive sections were also stained with NADH-TR reaction to differentiate between oxidative and glycolytic fiber types, according to their metabolic activity. The fluorescence signal intensities in control diaphragm suggested that the localization of SUMO-1 and SUMO-2/3 conjugates were higher in oxidative fibers in comparison to glycolytic fibers. We discovered that the distribution of SUMOylated proteins was altered between different types of fibers along CMV intervention. Furthermore, the immunofluorescence of SUMO-1 and SUMO-2/3 substrates were increased in oxidative fibers during the early phase of CMV. The abundance of SUMO-1 conjugates was maintained constantly in oxidative fibers until 5 days of CMV following a gradual increase after 10 days of the treatment. Whereas, the SUMO-2/3 conjugates were increased in both fiber types just after 1 day and remained constant along the duration of CMV in rat

diaphragms. Altogether, the accumulation of SUMO conjugates was increased in oxidative fibers during early phase, while the glycolytic fibers were affected at longer durations of CMV in rat diaphragms. Interestingly, BGP15 treatment significantly decreased the conjugation of both SUMO-1 and SUMO-2/3 conjugates in glycolytic fibers at 10 days of CMV. Following the literature that described the presence of SUMO in the cellular nucleus (Andreou and Tavernarakis, 2009), we observed the localization of SUMO in myonuclei in all diaphragm fibers.

7.4 Recruitment of new endogenous SUMO substrates under CMV treatment in rat´s diaphragm (paper I)

The results from the Western blot analyses and immunofluorescence assays suggested an increase in the SUMOylation of proteins during CMV treatment in rat diaphragms compared to the control.

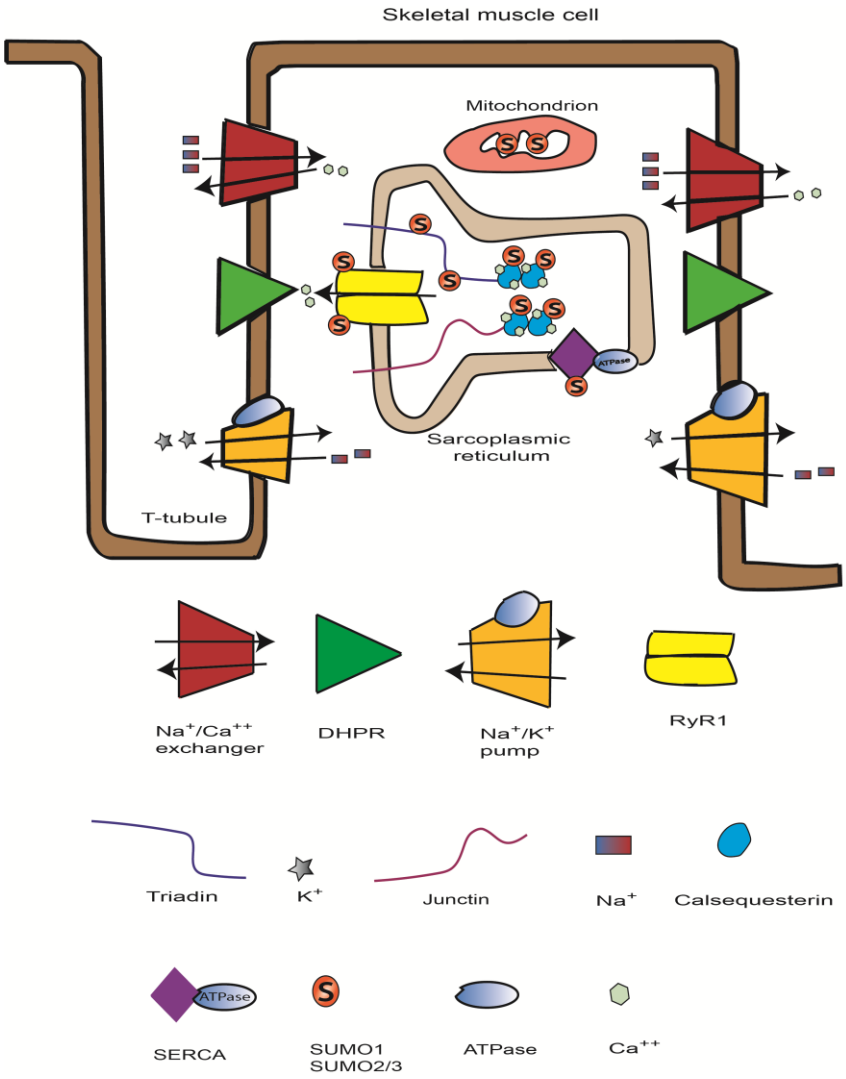

***Figure 6**. Schematic representation of endogenous SUMO substrates identified in rat diaphragms from control and rats exposed to CMV. The substrates included, the mitochondrial proteins-aspartate aminotransferase (AATM), the ornithine aminotransferase (OAT), the ATP synthase subunit-epsilon (ATP5E), the ATP synthase subunit-alpha (ATP5A), the calcium regulator proteins-calsequestrin 1 (CASQ1), calsequestrin 2 (CASQ2) and triadin (TRDN). We validated the potential SUMOylation reaction of the above mentioned substrates in prokaryotic and eukaryotic systems. A previously known SUMO substrate sarcoendoplasmic reticulum calcium*

transport ATPase (SERCA) was discovered in our mass spectrometry data, validating the novelty of our experimental approach.

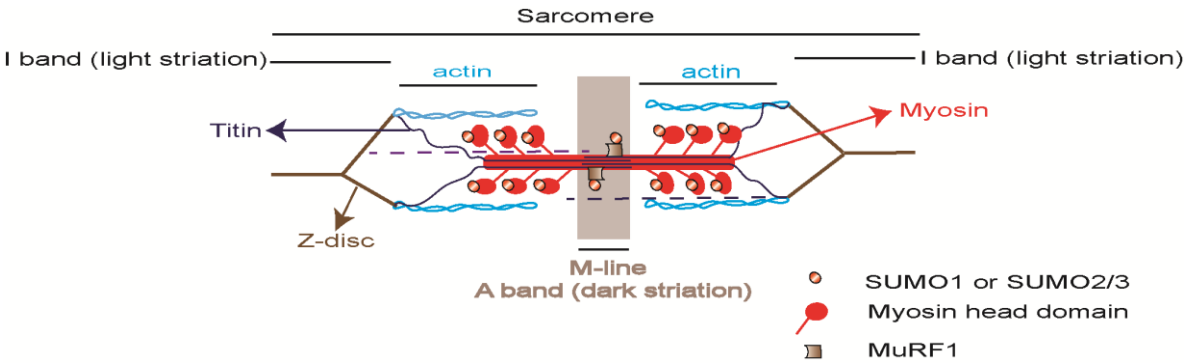

Figure 7*. Schematic representation of a single sarcomere of a muscle fiber with identified endogenous SUMO substrates from control rats. The substrates include the ubiquitin ligases-TRIM63 (MuRF1), the motor protein myosin. We validated the potential SUMOylation reaction of MuRF1 and myosin ATPase domain as substrates of SUMO conjugation in prokaryotic and eukaryotic systems.*

Based on this result, we were intrigued to identify these new SUMOylated proteins in control and during different stages of CMV treatment in rat´s diaphragms.

For this purpose, we combined immunoprecipitation, mass spectrometry, and bioinformatics approaches, and discovered a large pool of endogenous SUMO substrates in rat diaphragms involved in essential muscle functions like muscle remodeling, contraction, calcium regulation and mitochondrial proteins. Interestingly, there was a time-dependent recruitment of new SUMO substrates related to significant cellular and molecular pathways during CMV, which were not modified by SUMOs in normal physiological conditions. Our mass spectrometry data included previously known SUMO substrates like sarcoplasmic reticulum calcium ATPase (Chen et al., 2016), and α-actin (Terman and Kashina, 2014) proving the novelty of our immunoprecipitation approach. This data further supports our hypothesis that modulations in SUMOylation of proteins during CMV may contribute to the altered contraction in diaphragm fibers.

From the large pool of endogenous SUMO substrates, which were identified by mass spectrometry, we selected four groups of SUMO substrates with divergent roles and localizations for the muscle activity and physiology. The selected substrates include ubiquitin ligases-TRIM63 (MuRF1), TRIM54 (MuRF2), the motor protein myosin, the mitochondrial proteins-aspartate aminotransferase (AATM), the ornithine aminotransferase (OAT), the ATP synthase subunit-epsilon (ATP5E), the ATP synthase subunit-alpha (ATP5A), the calcium regulator proteins-calsequestrin 1 (CASQ1), calsequestrin 2 (CASQ2) and triadin (TRDN). We further validated the potential SUMOylation reaction of these substrates in both prokaryotic and eukaryotic systems.

7.5 Modulations in transcripts of SUMO components machinery in rat diaphragms with the effect of CMV (paper I)

It is well described in the literature that modulations in SUMO components machinery induced by non-physiological conditions, stress, infections, or congenic diseases, alter the expression levels of SUMO enzymes and, consequently change the status of conjugated

proteins at both cellular and organ levels (Pichler et al., 2017). We next wanted to investigate whether the observed modulations in SUMOylated proteins along the CMV are associated with any changes transcript levels of the SUMO pathway components machinery.

To address this, we performed transcriptome analysis on total mRNA isolated from rat diaphragm biopsies collected from control and different time points of CMV. The results indicated that there are significant alterations in mRNA expression levels of SUMO moieties and SUMO related enzymes during various stages of CMV in the analyzed samples. SUMO conjugases UBC9, PIAS1, PIAS3, TRAF7, and SUMO deconjugases SENP1, 5 and 6 exhibited considerable changes in their expression levels at both transcript and protein levels at different time points of CMV. These modulations of the transcripts were further validated at protein levels by Western blots using specific antibodies. This result more also verified our hypothesis that imbalances in SUMO conjugated and deconjugated enzymes during CMV treatment could contribute in the progression of VIDD.

7.6 Concluding remarks of paper I

From this study, for the first time, we show that changes in the functional state of rat diaphragm muscle induced by CMV brought significant modulations in global SUMOylation patterns. We show that SUMOylated proteins are majorly localized in oxidative than in glycolytic fibers of diaphragm muscles in ambulatory rats. We demonstrate that localization of SUMOylated proteins were significantly altered in different fibre types alongside the effect of CMV in rat diaphragms. There was a time dependent recruitment of new endogenous substrates by SUMO proteins with the effect of CMV treatment. We identified and validated the potential SUMOylation reaction of four classes of muscle proteins, which are involved in different skeletal muscle functions. The alterations in SUMO components machinery at both transcriptional and translational levels during CMV indicated a possible mechanism, which promoted the accumulation of SUMOylated proteins during the treatment.

We demonstrate that alterations in SUMOylation of muscle proteins can influence other molecular mechanisms and contribute in the progression of muscular pathologies including VIDD. Our study provided novel information on the dynamics of the SUMO pathway in rat diaphragm during a not physiological contraction activity, which also can be considered a new potential target for new therapeutic interventions.

7.7 Rationale of paper II

Many studies have implicated progression of VIDD with increases in oxidative stress processes, disruption of myofibrillar proteins and various remodeling responses across the diaphragm muscle fibers (Petrof et al., 2010). BGP-15 an antioxidant drug was shown to have protective effects against oxidative stress-induced diseases in mammalians (Sumegi et al., 2017). Previous studies have demonstrated an increase in irreversible PTMs, like oxidation, nitrosylation, on myofibrillar proteins triggered by oxidative stress in early hours of CMV in rat diaphragms (Corpeno et al., 2014). It is well known that the SUMO pathway is an excellent sensor of redox species and was shown to be altered significantly by the exposure to oxidative species (Feligioni and Nisticò, 2013). Considering all these mentioned factors, we hypothesized that BGP-15 administration along with CMV would regulate the production of reactive oxygen species resulting in the decrease of oxidative stress and improve diaphragm

health. We hypothesized that BGP-15 could recover the abnormal reversible PTMs on myosin produced by SUMO which could be beneficial in preserving its functionality during CMV treatment, and delay or slowdown the progression of VIDD, thus improving the weaning process.

7.8 Administration of BGP-15 reduced abnormal SUMO PTMs on myosin during CMV in rat diaphragms (paper II)

In this study, we show that CMV induced loss in diaphragm contractile functions, which have correlated with the increase of PTMs on the motor protein myosin. The administration of chaperone co-inducer BGP-15 along with ten days of CMV had improved the mitochondrial structure and functionality, which resulted in the decrease of reactive oxygen species production in rat diaphragm muscle fibers.

7.9 Administration of BGP-15 reduced poly-SUMOylation on Myosin (paper II)

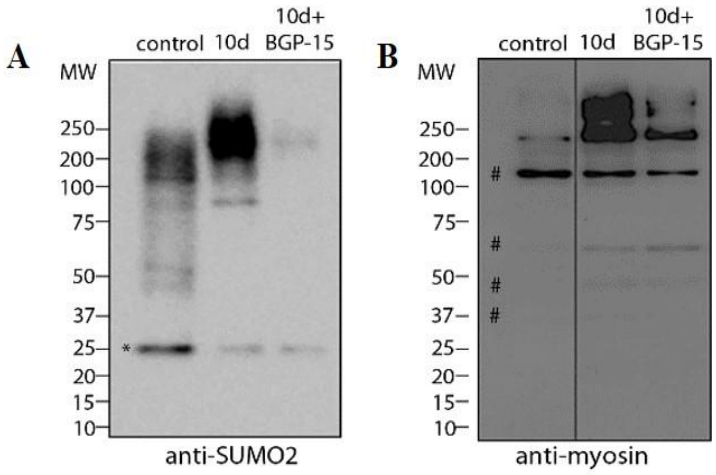

Figure 8. *BGP-15 modulates the endogenous SUMO-2/3 muscle proteins. (A) SUMO-2/3-enriched myofibrillar proteins isolated from diaphragm samples were identified by incubating with anti-SUMO-2/3 antibodies. The asterisk indicates low-chain immunoglobulins, which are a residue of the immunoprecipitation. (B) A enriched fraction of SUMO-2/3 muscle proteins was incubated with anti-myosin antibodies to identify the myosin PTMs by SUMO-2/3.*

The CMV treatment induced poly-SUMOylation on myosin during ten days of CMV in rat diaphragm, so we wanted to investigate the effect of antioxidant BGP-15 treatment on PTMs produced by SUMO on myosin during CMV. Western blot analysis on diaphragm lysates enriched with SUMO-2/3 substrates has revealed myosin as a target of SUMO2 in control conditions, but it became a poly-SUMOylated target during ten days of CMV. Ten days treatment with BGP-15 during CMV has significantly decreased the poly-SUMOylation of myosin protein, which was comparable to the levels in control samples. These results suggested that abnormal PTMs produced on myosin by SUMO2 during CMV might contribute in disruption of its functional abilities as a contractile protein.

7.10 Myosin is a substrate of SUMO conjugation in control rat diaphragms (paper II)

We demonstrated myosin as a target of SUMOylation by performing an in vitro SUMO conjugation assay with E1, E2 conjugating enzymes together with and without ATP molecule by using myosin extracted from rat diaphragm muscle fibers.

7.11 BGP-15 regulation of SUMO enzymes during CMV (paper II)

Then we wanted to investigate which SUMO enzymes are modulated during the CMV treatment in rat diaphragms. Transcription analysis revealed that ten days of CMV treatment caused alterations in the transcription of SUMO conjugases PIAS 1, 2, 3, 4, and deconjugases like SENP 1, 2, 5, and 6. On the contrary, the administration of BGP-15, along with ten days of CMV, returned the mRNA levels of the E3 ligases PIAS1, and 4 and deconjugases SENP1, and 2 to normal levels comparable to controls samples. We further validated modulations in transcripts at protein levels with western blots using specific antibodies against few SUMO enzymes, PIAS1, PIAS3, SENP1, SENP2, SENP5, and SENP6. These results suggest that the alterations induced by CMV in SUMO conjugases/deconjugases may contribute in an accumulation of SUMOylated proteins and contribute in the progression of VIDD.

7.12 Concluding remarks of paper II

Our study provides novel information on how BGP-15 could help to preserving contractile properties of the diaphragm muscle during CMV. BGP-15 could be a potential preventive/therapeutic intervention in reducing the damaging effects of CMV on mitochondrial, myosin functionality and be protective for patients exposed to CMV in ICUs. We demonstrate that BGP-15 could reverse the abnormal PTMs on myosin brought down by SUMO during CMV and contribute partially in alleviating the progression and the severity of VIDD. These results suggest that SUMOylation may also have a role in regulating the functional properties of myosin. Further research signifying the role of SUMO regulation on myosin functions may provide links to understand muscular diseases, which are associated with myosin dysfunction.

7.13 Rationale of paper III

In my first paper, we discovered many endogenous SUMO substrates in normal and in pathological diaphragms rat (i.e., during CMV conditions). From this pool of several SUMO substrates one particular protein MuRF1 caught our attention due to its presence also in control rat diaphragms. MuRF1, one of the skeletal muscle-specific E3 ubiquitin ligases, has been shown to be upregulated during atrophic conditions (Murton et al., 2008) and shown to have a protective effect against cardiac hypertrophy (Patterson et al., 2011). Mutations in MuRF1 gene have been associated with progression of hypertrophic cardiomyopathy and other congenital muscle diseases. In addition to this, mutations in MuRF1 gene are shown to be causing abnormal expression of the protein resulting in cellular mislocalization and promoting impaired ubiquitination in adult cardiomyocytes leading to cardiomyopathy (Watanabe and Hatakeyama, 2017). It has been well established that SUMO attachment modulates the functional features and localization of the various cellular substrates (Andreou and Tavernarakis, 2009). Based on the above mentioned factors, we hypothesized that SUMOylation might have a specific role in MuRF1 localization and regulation of its functions in physiological conditions. We also assume that mutations on SUMO site may lead to mislocalization of MuRF1 and alter its Ubiquitin E3 ligase activity and transcriptional regulator.

7.14 MuRF1 is mono-SUMOylated by SUMO-1 on lysine 238 (paper III)

We first validated MuRF1 as SUMO-1 target using a SUMO conjugation assay in bacteria with recombinant MuRF1. Then, using mass spectrometry combined with bioinformatics analyses on purified recombinant MuRF1, we identified ten putative lysine residues which could be resposible for SUMO conjugation. To locate the specific lysine residues, we generated the correspondent variants of GFP-MuRF1 mutants, where each of all the ten putative lysine amino acids were converted to arginine using site-directed mutagenesis protocol. Transfection and expression of these ten mutants into C2C12 cells revealed the lysine residue 238 (K238) as the unique amino acid involved in SUMOylation. Results from immunoprecipitation reconfirmed that MuRF1 was a target by single moiety of SUMO-1 on the K238 residue. We also discovered that the SUMO conjugating enzymes UBC9 and PIAS 4 are essential in the SUMOylation of MuRF1.

7.15 SUMOylation is required to regulate MuRF1 enzymatic activity as an E3 ubiquitin ligase (paper III)

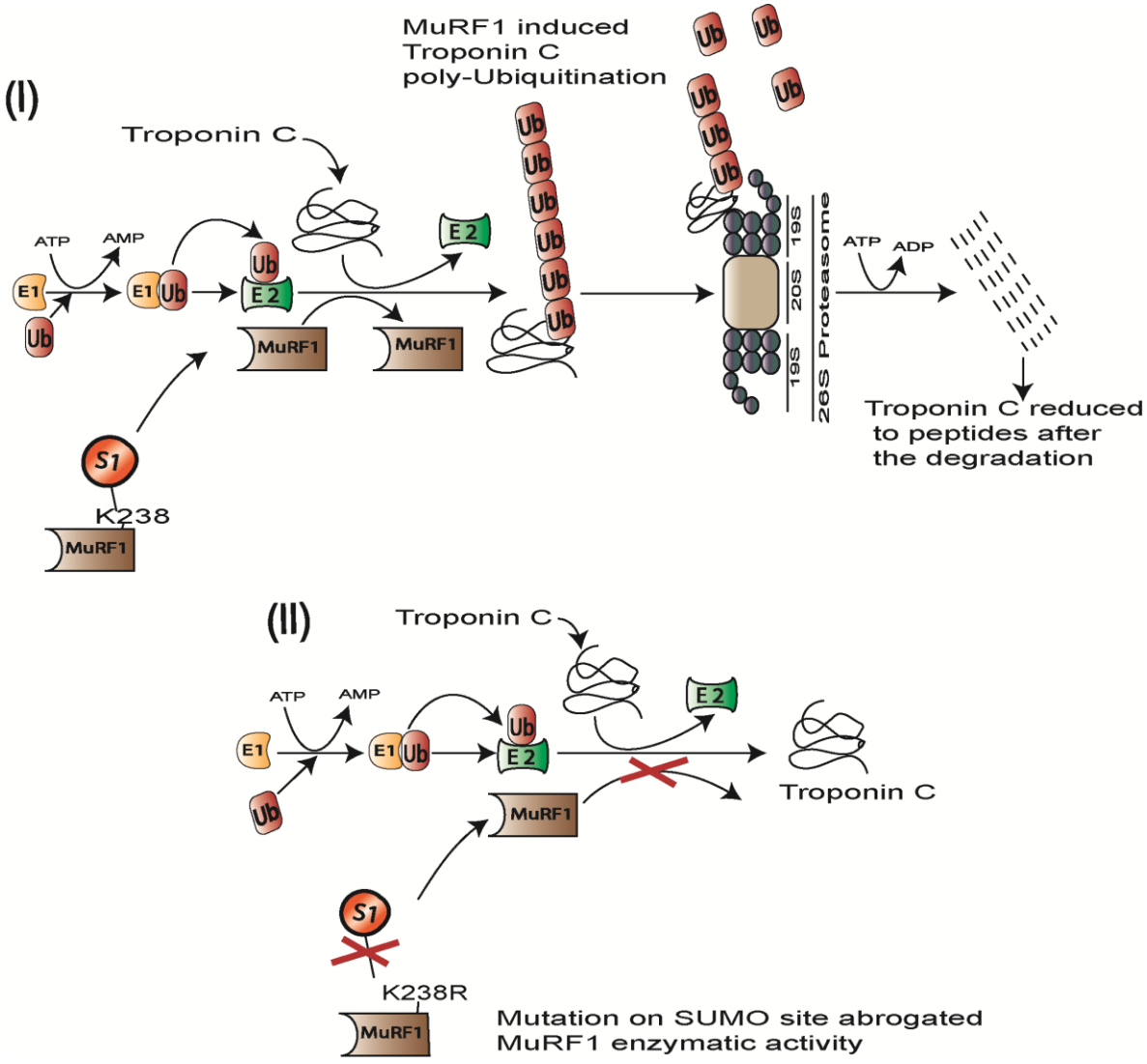

Figure 9. *(I) and (II) Schematic representation showing importance of PTM of MuRF1 by SUMO-1. SUMOyation of MuRF1 was required for its E3 ubiquitin ligase activity to promote degradation of troponin C in C2C12 cells. Mutation on lysine 238 on MuRF1 sequence altered its function as E3 ubiquitin ligase signifying the importance of SUMO modification on MuRF1.*

We were then curious to find if the mutation on K238 affected the enzymatic activity of MuRF1 as an ubiquitin ligase. Readouts from co-transfections of MuRF1 or K238R mutant MuRF1 with its well-known substrate, troponin C, revealed that the mutation K238R indeed had impaired the enzymatic activity of MuRF1 in the poly-Ubiquitination conjugation function. This result could be explained due to a conformational change caused by the mutation, which leads to a no SUMOylated product or an impairment in recognition of the substrate

7.16 SUMOylation is essential for translocation of MuRF1 from the cytoplasm to nucleus (paper III)

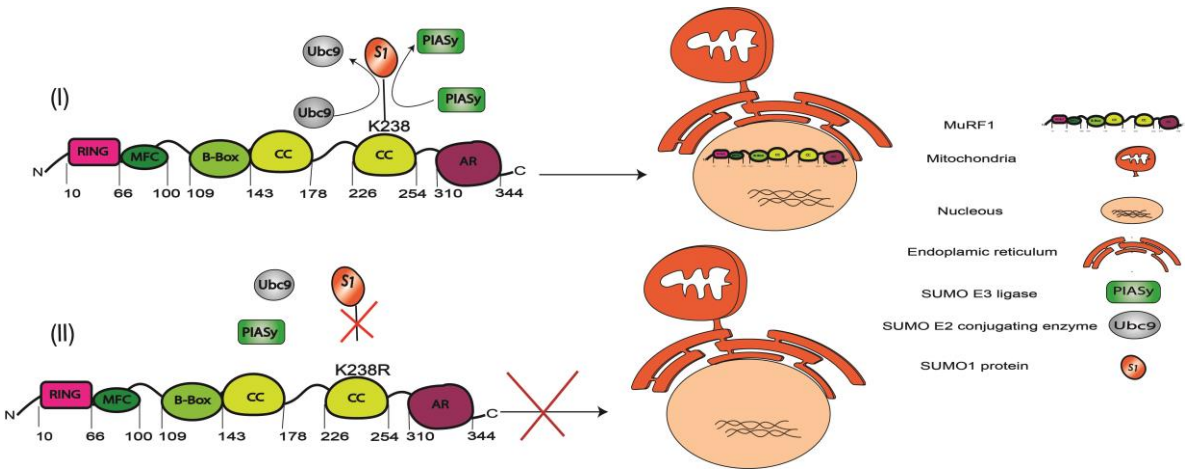

Figure 10. *(I) Schematic representation of PTM of MuRF1 on lysine 238 by SUMO-1, which promoted its translocation into nucleus in C2C12 cells. (II) Mutation on lysine 238 on MuRF1 abrogated its translocation into nucleus in C2C12 cells.*

It has been well established that SUMO-1 modification primarily facilitates the translocation of the target proteins into different cell compartments (Saitoh and Hinchey, 2000). To assess how SUMOylation affected the localization of MuRF1, we studied its localization comparing the wild-type and K238R SUMO mutant. Immunofluorescence assays using GFP recombinant MuRF1 showed that this protein was localized in different cellular compartments at varying proportions: about 78% equally distributed both in the cytoplasm and the nuclei, around 10% in mitochondria, and about 18% in the cytoplasm as aggregates. In a quite contrast, 80% of the MuRF1 K238R mutant was distributed predominantly in the cytoplasm as aggregates with a significant decrease in mitochondrial distribution and with almost no distribution in the nuclei. This result strongly indicates that the lack of SUMO conjugation of MuRF1 not only impairs its enzymatic property but also blocks its translocation from cytoplasm to the nucleus.

7.17 Concluding remarks of paper III

Overall, this study sheds new light on the significance of SUMOylation for hampering the muscular atrophies related to MuRF1 deregulation.

7.18 Rationale for paper IV

In my papers I and II, we showed that SUMOylation might play an important role in regulating the physiology in rat diaphragms. We also demonstrated that SUMO network was considerably altered to adapt to the non-physiological conditions, which were caused in rat diaphragm muscle during the CMV. Considering these results, we hypothesized that there could be a strong correlation between the functional diversity of skeletal muscles and maintenance of stoichiometry of SUMOylation reaction and abundance and ration among SUMO components machinery.

7.19 Quantification of SUMOylated proteins in ambulatory rat skeletal muscle groups (paper IV)

To evaluate the profiles of the SUMOylated proteins, we subjected skeletal muscles tibialis anterior, extensor digitorum longus, soleus, diaphragm, plantaris, gastrocnemius superficial, gastrocnemius deep, gastrocnemius proximal and masseter from ambulatory Sprague Dawley rats to Western blot analysis. The correspondent SUMO immunoblots revealed that the conjugation profile of SUMOylated proteins was unique for different groups of skeletal muscles in ambulatory rats, which could correspond to their distinct functional properties. Following the literature, where SUMO-2/3 conjugation is stress related (Saitoh and Hinchey, 2000) the band intensities of SUMO-2/3 conjugates were much lower compared to that of SUMO-1 among the observed muscles.

To understand the variations in the conjugation profile of SUMOylated proteins among different groups of muscles, we performed a transcriptome analysis of the SUMO components using total mRNAs isolated from the nine groups of skeletal muscles. The transcripts of the SUMO enzymes exhibited a differential expression pattern across the skeletal muscle groups. These results explained the differences in the SUMO protein profiles mainly due to variations in the abundance and stoichiometry of SUMO components machinery.

These results together with the profiles of the SUMOylated proteins suggested a strong correlation between the different sets of SUMO enzymes within the analyzed skeletal muscles that make unique the associated functions in the body.

7.20 SUMOylated proteins are high in oxidative muscle fibers from ambulatory rat (paper IV)

To discover the distribution of SUMOylated proteins within the skeletal muscle groups, we performed immunofluorescence coupled with two histochemical assays (NADH-TR and ATPase pH 10.8 stains) on consecutive cryosections muscles from the ambulatory rat. Base on the heterogeneity of the fiber types in the skeletal muscles, the SUMOylated proteins demonstrated a distinctive distribution among the muscle groups. By combining histochemical stains with SUMO-1 and SUMO-2/3 immunofluorescence assay, we showed that SUMOylated proteins are localized predominantly in oxidative type I and type IIA fibers compared to the in glycolytic type IID/X and IIB fibers. This result also supports our previous observation, paper I, where SUMOylated proteins are mainly localized in oxidative fibers in control rat diaphragms.

7.21 Muscle wasting in soleus altered the proteome and transcriptome of SUMO network (IV)

Until now, we have found a connection between stoichiometry of SUMOylation and muscle function. Next, we questioned if the process of muscle wasting might induce any perturbations in the overall process of protein SUMOylation. To address this, we adopted Wistar-Han rats where limb muscle wasting was induced by tail-suspension from one to four days. We anticipated the emergence of new SUMOylation patterns in response to the non-physiological state of the muscle. To test this, we probed the soleus muscle lysates from soleus of control and muscle-wasted rats for SUMO-1, SUMO-2/3 and ubiquitin targets by Western blots. Interestingly, the results suggested an immediate increase in the conjugation of substrates by SUMO-1, and SUMO-2/3 already in 1 day of unloading. On contrary, the protein ubiquitination was unaffected until 2 days of unloading and can be also attributed to a no significant upregulation of MuRF1 observed. These results suggest that the early intervention of the SUMO network may contribute to delay the activation of the ubiquitin-proteasome system mediated by MuRf1 and the progression of atrophy in unloaded soleus muscles.

7.22 PAX6 as a regulator of Ubc9 transcription in unloaded limb muscles (IV)

In both our first studies concerning diaphragms (paper I) and different skeletal muscle groups (paper IV), we detected the distribution of SUMOylated proteins to be predominant in oxidative fibers from ambulatory rats. The readouts from immunofluorescence assay using anti SUMO-1, and anti SUMO-2/3 antibodies suggested an increase in the conjugation of SUMOylated proteins in tail-suspended rat soleus muscles compared to the controls. Besides, immunofluorescence staining with mitochondrial marker MTCO1 antibody and NADH-TR staining suggested a change in fiber type phenotype from oxidative to glycolytic in unloaded rat soleus muscles.

Therefore, we wanted to understand why there is an increase of SUMOylated proteins in all fibers of unloaded soleus muscle, despite an overall enrichment in glycolytic. To answer this question, we focused on UBC9, the unique SUMO E2 conjugating enzyme, which is critical in regulating SUMO reactions. Transcription analysis revealed upregulation of Ubc9 mRNA levels as an early event, like after 12 hours of unloading compared to control soleus muscles. We confirm that this was not related to an atrophy response since there was no change in the mRNA levels of MuRF1 compared to the ambulatory samples. By using ENCODE software, we discovered PAX6 as one of the potential transcriptor factor bindings to the promoter of Ubc9 gene. A common read out from immunofluorescence assay using PAX6 antibody on both tail-suspended rat soleus and bed rest human vastus lateralis muscle tissues, suggested a translocation of PAX6 into myonuclei from the cytoplasm. This translocation of PAX6 into myonuclei increased the transcription of Ubc9 gene during the early phase of the inactive state of the muscle in the analyzed rat and human muscle biopsies. These results explain the possible mechanism of how the PAX6 contributed to promoting UBC9 that enhances the accumulation of SUMOylated proteins during muscle wasting in both rat and human models.

7.23 Concluding remarks of paper IV

Through this study, we demonstrate that there is a strong correlation between the muscle fiber composition and functional activity with the abundance of SUMO components machinery. We show that a change in the physiological state could alter the stoichiometry and abundance of SUMO network and contribute in the onset of atrophy in rat soleus muscle. We show UBC9, the SUMO conjugating enzyme responsible for regulating the increase of SUMOylated proteins muscle wasting induced rat soleus muscle. We emphasize that alterations in SUMO pathway and its components machinery can be considered as possible biomarkers to predict muscle disease, which can be targeted by drugs to alleviate atrophy. We propose, PAX6 as an attractive therapeutic target in regulating SUMOylation, which may benefit to control atrophy in bed-ridden patients.

8 DISCUSSION

Skeletal muscle is the largest organ where maintenance of strength and muscle mass is essential for the wellbeing of the human body (Rivas and Fielding, 2012)(Miljkovic et al., 2015). Several enzymes, proteins, cellular, and molecular mechanisms collectively contribute to maintaining the regular activity of skeletal muscles (Frontera and Ochala, 2015). Skeletal muscles have a remarkable regeneration ability following a muscle injury but many diseases such as diabetes, sarcopenia, cachexia, neurological disorders, induced atrophies, and genetically inherited dystrophies hinder the regeneration capacity of skeletal muscle, making it difficult to recover (Shadrin et al., 2016)(Trovato et al., 2016)(Wagner, 2002). Although skeletal muscles are functionally and anatomically well defined, there is still a lack of proper understanding in molecular mechanisms leading up to severe muscular disorders in humans (Davies and Nowak, 2006) (Corpeno et al., 2014).

When I started my PhD studies, few studies were suggesting the importance of SUMOylation in the skeletal muscle physiology. Considering the presence of still unresolved and untreatable skeletal muscles diseases, there is a strong need for understanding the molecular mechanisms, which regulate muscular health. I felt my studies on the role of SUMO pathway in skeletal muscle pathophysiology would open new avenues to understand and treat severe muscular diseases and make the world a healthy muscle.

The first challenge I faced during the beginning of my PhD research was to handle a dynamic and transient mechanism like SUMOylation in well-evolved and complex tissue i.e. skeletal muscle. The fact that SUMOylation/deSUMOylation is a rapid reaction which only modifies a tiny portion of the total pool of substrates to regulate its functions (Andreou and Tavernarakis, 2009), made it difficult to preserve the SUMO on conjugated protein in our lysate samples. It was a challenging issue to maintain the steady state of the SUMO-substrate complex in the muscle lysates. Later with experience and optimization of perfect denaturing conditions, I could master the art of keeping SUMO unmoved from substrate proteins in my lysate samples and started getting results. Using of a 20 mM of a chemical compound N-Ethylmaleimide in all our lyses buffers with a swift operation of experiments at optimal temperature provided us the results we needed to prove something really important which in future may translate into a significant pharmacological intervention.

Another key point of my studies (paper I) are the immunoprecipitations we prepared for mass spectrometry analysis using monoclonal SUMO-1, SUMO-2/3 antibodies which were developed and purified from the supernatant of hybridoma cell lines. Our mass spectrometry results included previously known SUMO substrates like sarcoplasmic reticulum calcium ATPase (Chen et al., 2016) and the α-skeletal muscle actin (Uda et al., 2015) excluding highly expressed muscle proteins such as rod-shaped cytoplasmic protein dystrophin (427 kDa) or proteins derived from the transmembrane dystroglycan or sarcoglycan complexes. This result further validated the novelty and optimization of our immunoprecipitation approach. From mass spectrometry data, we discovered four classes of muscle proteins related to contractile, mitochondria, remodelling and with calcium homeostasis functions as SUMO substrates in normal and in induced respiration treatment.

In my projects, we demonstrated the localization of SUMOylated proteins to be abundant in oxidative compared to glycolytic fibers in skeletal muscles of healthy rats. We also

established that total amount of SUMOylated proteins were altered in all fibers within skeletal muscles (diaphragm, soleus) with the intervention of CMV or muscle wasting, indicating a relation between the progression of a disease and alterations in SUMO pathway.

There was always a very less chance of error, especially in experiments concerning eukaryotic cell lysates. The putative SUMO substrates were deSUMOylated in the frozen cell lysates, experiments always needed fresh samples in order to obtain expected results. This deSUMOylation of substrates resulted mostly from mishandling of samples and little changes in denaturing conditions such as temperature. It didn't take much time for me to get control on SUMO dynamics in eukaryotic cells, where I could also isolate mitochondria and preserve the not easy to identify conjugated SUMO proteins on this cell compartment.

One of the exciting discoveries of this study was finding the skeletal muscle specific, E3 ubiquitin ligase, MuRF1 as a SUMO substrate in rat diaphragms. We discovered that the PTM mediated by SUMO1 on MuRF1 protein enhances its enzymatic functions and provides translocation into the nucleus. We found that the SUMO conjugation site on lysine 238 residue on MuRF1 sequence is located close to amino acids which the correspondent point mutations T232M, D254N have been associated to cardiac hypertrophy (Su et al., 2014) and the truncated protein Q247* (Olivé et al., 2015) has been linked to cardiac and skeletal muscle protein aggregate myopathies. As a future work, we will be interested to discover correlations between these mutations that might affect the alterations in SUMOylation of MuRF1 protein and the muscle pathologies. This study opens up new possibilities to understand muscular disorders associated with MuRF1 mutations in the context of its stagnation in the cytoplasm due to the mutations on the SUMO site.

As I mentioned in aims of my thesis, this study provides a novel role of SUMO pathway as an important signaling mechanism in regulating normal physiology of skeletal muscle, which is severely altered during muscle disease as VIDD. We provide a new set of SUMO endogenous substrates to develop pharmacological interventions in muscular disorders and improve skeletal muscle functions as well as upstream factors, like PAX6 involved in the remodeling of important SUMO machinery enzymes, as UBC9. This discovery is just a tip of the iceberg. By showing that PAX6 is a transcriptional regulator of UBC9, further studies based on to understand its nuclear translocation during muscle inactivity would add new interesting therapeutic approaches to deal with muscular diseases associated with UBC9 deregulation.

9 ACKNOWLEDGEMENTS

It was a great journey to graduate as a PhD from Karolinska Institute; it was a collective journey of challenges and gaining of lot of experience in the scientific field. When I look back into past four years, I feel privileged to have met and worked with many inspiring, excellent and passionate scientists. I am thankful to everyone who helped me to achieve and grow professionally and personally into what I am today. You all made it a very interesting journey.

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