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Interuniversity Institute of Myology

# XII IIM-Myology Meeting 1-4 October 2015

## FOCUS ON PATHOGENESIS AND TREATMENT OF NEUROMUSCULAR DISEASES

### TOPICS

- Stem Cells and therapy
- Genetic and epigenetic alterations in muscle dystrophies and myopathies
- Satellite cells and muscle regeneration in healthy muscle and in dystrophies/myopathies
- Biophysics and E-C coupling in the physiopathology of neuromuscular diseases
- Signalling in muscle homeostasis and diseases
- Metabolic alterations and muscle diseases
- Muscle wasting and cachexia
- Therapeutic approaches for muscle diseases

**Scientific Committee:**
Musarò A, Puri PL, Sampaolesi M, Gabellini D, Barbieri E, Protasi F, Blaauw B, Fulle S, Grassi F, Sorci G, Mammucari C, Sandri M.

**With the support of:**

**Plenary Lecture-Stefano Ferrari:**
Michele De Luca (Centre for Regenerative Medicine; Universtity of Modena-Reggio Emilia)

**Main Lectures**
Pura Munoz-Canoves (UPF, Barcelona, Spain)
Rossella Tupler (University of Modena-Reggio Emilia)
Paolo Bonaldo (University of Padua)
Laurent Schaeffer (University of Lyon, France)
Roger Cooke (University of California USA)

**Venue:** Hotel Matilde di Canossa (Reggio Emilia)

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# SESSION 1. SATELLITE CELLS AND MUSCLE REGENERATION IN HEALTHY MUSCLE AND IN DYSTROPHIES / MYOPATHIES

### S100B protein in skeletal muscle regeneration: regulation of myoblast and macrophage functions

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Repair of damaged muscle tissue upon acute injury requires the coordinated action of infiltrating inflammatory cells and activated muscle stem cells known as satellite cells to restore homeostasis. S100B protein regulates myoblast proliferation and differentiation, and its effects depend on its concentration, the myoblast density, the presence of bFGF, and the duration of exposure of myoblasts to the protein (1,2). S100B is abundantly released during the first few days after acute muscle injury and its release rapidly declines thereafter (2). The role of myofiber-released S100B is not known. Here we report that following injury with BaCl2 injection myofiber-released S100B expands the myoblast population, attracts macrophages to the site of damage, and promotes macrophage polarization into M2 (pro-regenerative) phenotype. Also, S100B is transiently expressed in and released by macrophages in response to cytokines. S100B effects are mediated by RAGE (receptor for advanced glycation end-products) in the early phase of muscle regeneration, however during the myoblast proliferation phase S100B also activates the bFGF-FGFR1 complex to stimulate myoblast proliferation and macrophage M1/M2 transition. Thus, S100B controls macrophage phenotypic transition and myoblast activity following acute muscle damage. Our data point to S100B as to a physiological player required for correct timing of skeletal muscle regeneration. However, high amounts of S100B at damage sites early after injury are detrimental causing prolongation of the myoblast proliferation phase at the expense of myoblast differentiation/fusion, larger infiltration with M1 macrophages, prolongation of the macrophage M1 phase, and deposition of fibrotic tissue. Thus, if present in high amounts S100B might contribute to the pathophysiology of muscle degenerative diseases characterized by chronic inflammation and/or deregulated muscle regeneration.

*1. Riuzzi F. et al., J. Cell Sci 2011;124:2389-400.*
*2. Riuzzi F. et al., PLoS ONE 2012; 7: e28700.*

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### Phosphorylation and alternative splicing of MEF2C, a dual switch function in muscle regeneration

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Muscle regeneration is a multistep process that is regulated by a restricted number of transcription factors whose activity is modulated at multiple levels. However, how different layers of regulation are coordinated to promote adult myogenesis is not yet understood. Here we show that the MEF2C transcription factor controls multiple steps of muscle regeneration, including myogenic progression of satellite cells and muscle maturation of newly generated myofibers, exhibiting multiple functions that depend on alternative splicing and post-translational modifications. Inclusion of the α1 exon in *Mef2c* transcripts is upregulated in proliferating mouse satellite cells and in the early phases of muscle regeneration. The encoded MEF2Cα1 isoform stimulates expansion of primary myoblasts *ex vivo* and *in vivo*. The pro-proliferative activity of MEF2C is mediated by phosphorylation of two phosphoserines located in exon α1. Subsequent terminal differentiation and growth of newly formed myofibers are promoted by dephosphorylated MEF2Cα1 and MEF2Cα2. Our results thus reveal an important role for regulatory interactions between alternative splicing and post translational modifications of a single transcription factor in the control of the multilayered regulatory programs required for adult myogenesis.

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### HDAC4 is necessary for satellite cell differentiation and muscle regeneration

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Skeletal muscle exhibits a high capacity to regenerate, mainly due to the ability of satellite cells to replicate and differentiate in response to stimuli. Epigenetic control is effective at multiple steps of this process. The chromatin remodeling factor, HDAC4, is up-regulated in skeletal muscle upon injury, suggesting a role for this protein in muscle regeneration. With the aim to elucidate the role of HDAC4 in satellite cells and skeletal muscle regeneration, we generated inducible mice lacking HDAC4 in Pax7+ cells

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(HDAC4 KO mice). Despite having similar amount of satellite cells, HDAC4 KO mice show impaired muscle regeneration in vivo, and compromised satellite cell proliferation and differentiation in vitro. HDAC4 deletion in satellite cells is sufficient to block their differentiation, not acting via soluble factors, and possibly through the inhibition of Pax7 expression. The molecular mechanisms underling compromised muscle regeneration in HDAC4 KO mice are currently under investigation. All together, these data delineate the importance of HDAC4 in satellite cell differentiation and suggest a protective role of HDAC4 in response to muscle damage.

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**The potential of mass cytometry to reveal the complex interplay between muscle resident mononuclear cells during regeneration**

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The adult skeletal muscle has the ability to self-renew and repair in response to increased workload, stress conditions or limited damage. These properties rely on an array of different progenitor cell populations. While satellite cells play a central role in muscle regeneration, a variety of other mononuclear progenitor cells, either resident in the muscle or recruited from the blood stream, contribute to the complex crosstalk leading to muscle repair. In pathological conditions or with aging, the relative abundance and the activation stage of the different cell populations in the myogenic stem cell compartment vary. The ability to probe the heterogeneity and the dynamic of the muscle tissue is fundamental to achieve a complete understanding of muscle regeneration. To this end we have invested in a novel approach exploiting mass cytometry technology (CyTOF2 platform). CyTOF technology enables probing single cell events, by labelling intracellular and cell surface markers with up to 40 antibodies tagged with stable heavy metal isotopes. The sharp mass peaks obtained by TOF inductively coupled plasma mass spectrometry eliminates the problems of spectra overlap typical of fluorescence based flow cytometry. I will describe the panel of tagged antibodies that I have developed to characterize the heterogeneous muscle mononuclear cell populations and the advantages and limitations of mass cytometry. In addition I will present preliminary data on the dynamic of cell populations under different conditions and stimuli.

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**NF-Y splice variants differentially affect skeletal myogenesis**

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The mechanisms that regulate skeletal muscle development involve the coordinated activity of transcription factors (TFs) and a precise timing of gene expression patterns. NF-Y is a heterotrimeric TF with a pioneer role in the transcriptional and epigenetic regulation of promoters containing the CCAAT-box. NF-Y activates the expression of various genes related to the cell cycle, particularly genes of the G2/M phase. NF-YA, the regulatory DNA-binding subunit of the complex, is expressed in proliferating myoblasts and down-regulated during terminal differentiation. The NF-YA gene encodes for two alternatively spliced isoforms, namely NF-YAs and NF-YAl, which are not functionally identical. Using mouse C2C12 cells, we provide evidence of a different role for NF-YA variants in the myogenic program. While NF-YAs enhances myoblasts proliferation, NF-YAl boosts their differentiation by up-regulating the transcription of novel target genes, among which Mef2D, Sixs and Cdkn1C, which are known to be involved in the differentiation program. We further demonstrate that NF-YA is expressed in resident stem cells (SCs) and the two isoforms are transcribed at different levels during SCs activation and differentiation. The inhibition of NF-Y activity impairs both proliferation and differentiation of SCs and the overexpression of the two NF-YA isoforms differentially affects their fate.

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**Role of the oxidative stress in the alteration of muscle homeostasis age-related**

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Sarcopenia is the age-related loss of muscle leading to loss of muscle power, which in the end results in frailty and disability. At molecular level, sarcopenia is characterized by insufficient antioxidant defense mechanism, increased oxidative stress and altered function of respiratory chain. It has been hypothesized that the accumulation of oxidative stress is also related to an impaired regeneration cooperating to the atrophic state that characterizes muscle ageing. To the purpose, we investigated the myogenic process in satellite cells, the skeletal muscle stem cells, as myoblasts and myotubes collected by human Vastus Lateralis skeletal muscle of young and old subjects through needle-biopsies. To measure both the $O_2$- and ROS level we used NBT and H2DCF-DA assays revealing higher concentration in elderly myoblasts compared to young ones. To evaluate if mitochondria are damaged by ROS we measured mitochondrial transmembrane potential after an oxidant insult as $H_2O_2$. We found that in elderly myoblasts mitochondrial transmembrane potential decreases much more than in young ones probably due to their lower endogenous antioxidant abilities. Specifically, MitoSOX™ Red reagent for direct measurements of $O_2$- in mitochondria revealed that in elderly myoblasts $O_2$- production is increased respect to young ones and the result is worsened in myotubes. Furthermore, the upregulation of the atrophic and ubiquitin-proteasome pathways together with a dysregulation of the proliferative one revealed an alteration at gene expression level in elderly myoblasts vs young ones. Overall our data confirm that oxidative stress impairs muscle regeneration in elderly subjects.

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### Single-cell based analysis of functional populations in aged and dystrophic muscle

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Skeletal muscle is a complex structure endowed with extreme regenerative capability; this ability relies on the orchestrated interplay between different muscle populations that reside within the tissue. Functional changes occurring at the microenvironmental level during aging or pathological conditions however interfere with this ability leading to fibrosis and fat infiltration. Despite a large body of work still we are far from completely understanding these changes; even when genetic cause is known (e.g. Duchenne muscular Dystrophy) we are still unable to pin-point the steps that lead from the molecular cause to the outcome of the disease. The main reason for this bottleneck is that our knowledge has been limited so far by the lack of technical tools to dissect the heterogeneity of these populations. The use of bulk-scale methods able only to provide averaged information has frustrated our effort to characterize those pathological changes leaving those dysfunctional, disease-specific subpopulation to remain hidden within the bulk. Here we present a novel approach based on single cell mass spectrometry to study the populations that reside in the muscle. Using Cytof technology we would profile at single cell resolution the muscle resident populations during aging and in diseased state. This would allow us to identify dysfunctional subsets involved in the regeneration defect. This study would not only shed light on the mechanisms underpinning muscle regeneration but would provide a solid ground for the future identification of diagnostic biomarkers through the study of disease specific subpopulations.

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### Exosome-bore microRNAs in muscle hypertrophic and dystrophic conditions

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Hypertrophy and dystrophy are distinct, yet linked processes that remodel both skeletal and cardiac muscles in physiological or pathological settings. Not only is hypertrophy important during development, but it also plays major role upon acute or chronic damage. Muscular dystrophies (MDs) cause progressive degeneration and loss of functionality in both striated muscle types. In MD patients and animal models, an initial hypertrophic response occurs, with contrasting effects on skeletal and cardiac muscle. Recently it has been established that muscle fibers secrete exosomes, whose cargo acts as endocrine signals during myogenesis. We aim at deciphering the exosomal information guiding hypertrophy/dystrophy in both muscle in order to establish a new strategy based on miRNA modulation for novel myogenic regeneration. We performed ex vivo exosome analysis comparing age-matched WT, Sgcb-null (dystrophic), and MAGIC-F1+/+ (hypertrophic) mice. We detected several differentially regulated miRNAs, virtually relevant for striated muscle remodeling and de-/re-generation. We have preliminary results on the effects of ex vivo exosomes on cell types relevant for skeletal and cardiac muscle analysis. Moreover we are currently investigating the uptake routes of exosomes in both muscle types. In the future we will rely on miRNA-sequencing of ex vivo exosomes, to identify key mRNA/miRNA distinctive signatures by means of an high-throughput approach and place our ongoing results into a genome-wide setting. As a final goal, the hypertrophic/dystrophic signatures and tissue-specific information will further be integrated to establish skeletal- and cardiac-enhancing cocktails to selectively improve the regenerative outcome of patient-specific progenitors in vivo, into a xenograft-permissive murine model.

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### Development of a 3D implantable niche of hiPSC-derived satellite cells

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Duchenne muscular dystrophy (DMD) is the most common, lethal, inherited myopathy, which results in muscle degeneration. In this work, we aimed at developing an innovative 3D satellite cell niche derived from human induced pluripotent stem cells (hiPSC) within their native sublaminal position in an engineered human skeletal muscle myofiber. One of the main limitations of cell therapy for DMD is the high number of myogenic cells required and the efficiency of engraftment in vivo. hiPSC ensure large amount of cells and the possibility of derive patient-specific cells, but obtaining myogenic cells in vitro from hiPSC is difficult and the yield is low. In this work, we induced the myogenic differentiation of hiPSC through multiple transfection of modified mRNA of the master transcription factors MYOD, PAX3 and PAX7. To this aim, we exploited a microfluidic platform that allows the downscaling of the process for performing cost-effective, multiparametric and highthroughput experimental investigations. We optimized the protocol for transfecting hiPSC colonies leading to a transfection efficiency of 60% per single transfection. After multiple transfections, exogenous MyoD is expressed in 95% of the cells and endogenous expression of desmin and myosin heavy chain was observed (4 days after the last transfection). Ongoing experiments are extending these results to Pax3 and Pax7. Another key factor for a successful cell therapy is the cell delivery. In this sight, we developed a 3D poly-acrilammide/hyaluronic acid hydrogel (HY) scaffold and optimized its biochemical and mechanical properties in order to sustain the myogenic differentiation of human primary myoblasts and to reproduce the protective microenvironment of the satellite cell niche. The scaffold was designed in order to control the cell topology: 3D parallel micro-channels (80-160 μm in diameter, 10-15 mm long) were produced inside the scaffold and functionalized with ECM proteins. To reproduce the physiological

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mechanobiology, HY chemical composition was optimized in order to obtain a soft scaffold with physiological elastic modulus, E≈12±4kPa. Human primary myoblasts were used to optimize the seeding, culture and differentiation protocols. At 10 days, we observed tightly packed myotubes bundles, expressing myosin heavy chain, α-actinin and dystrophin. We are now integrating hESC-derived myoblast and we observed differentiation into myoubes and expression of myosin heavy chai, α-actinin and desmin.We hypothesize that such engineered niche will provide, upon in vivo implantation, satellite cells able to regenerate the damaged muscle of DMD patients, and reconstitute the stem cell pool for future muscle damages. On the other hand, our 3D niche could be exploited as an in vitro tool to study the biology of the niche itself, the mechanism of the pathology or as a tool for testing new drugs and therapies in a personalized manner.

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### A stem cell niche in the dish

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Skeletal muscle regeneration is mediated by a complex crosstalk between various resident mononucleated cell populations. These cell interactions after fiber damage or stress are finely regulated in time and space. Satellite cells, skeletal muscle stem cells, play a pivotal role during regeneration being the main source of new myoblasts. However, their activation, proliferation and differentiation relies on environmental cues shaped by cell populations such as macrophages, pericytes, and fibro-adipogenic progenitors (FAPs). FAPs have a leading role in the regeneration process since they promote myotube formation by positively regulating satellite cell differentiation. However, in pathological conditions, such as muscular dystropies, these cells play a negative role since they are responsible for fibrosis and fatty tissue accumulation. In in vitro experiments we have observed an improvement in the maturation of myotubes derived from satellite cells, when co-cultured with FAPs. Furthermore, we have also observed that direct contact of these two cell populations inhibits adipogenic differentiation of FAPs while in the transwell system this inhibition does not occur. Even though there is a clear interaction between these two populations, it has not been thoroughly characterized yet. Thus exploiting Luminex technology we are aiming at identifying molecules affecting the differentiation process of these two cell types focusing on cytokines, chemokines and growth factors. In addition we are planning to include in these studies macrophages and pericytes in order to obtain a more complete picture of molecular networks involved in myogenesis and finally build a cell-cell interaction model of skeletal muscle regeneration.

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### Extracellular stimulation with human “noisy” electromyographic patterns facilitates myotube activity

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Electrical stimulation (ES) of skeletal muscle has been proposed to mimic the beneficial effects of physical training and to counteract the muscle atrophy associated with reduced motor activity. If properly used, it can be a potent tool to increase strength and endurance in patients affected by muscle weakness due to ageing or prolonged debilitating illness. However, classical ES exhibits several limitations, such as the unpleasant symptoms due to pulse strength and the occurrence of muscle fatigue. The most appropriate parameters of stimulation, such as intensity, frequency and pulse duration, are still under debate. Field ESs were given to mouse skeletal myotubes in culture. Changes in membrane potential were detected by perforated patch recordings and calcium dynamics was followed using fluorescent indicators. Different patterns of ES were tested. Tetanic high frequency stimulation at 45 Hz induced voltage changes invariably characterized by failures, and discontinuous firing preceding the complete disappearance of the electrical activity, whereas low-frequency stimulations at 1 Hz more efficiently elicited single action potentials. An innovative “noisy” waveform ES pattern was tested, obtained from a segment of electromyogram recording, sampled from a limb muscle of adult volunteers during the execution of a rhythmic motor activity. Using half of the intensity of stimulation employed for more stereotyped ES patterns, it was found to be more efficient in inducing repetitive cell firing, calcium transients and cell twitching. We suggest this approach as a new strategy for the design of new electrical devices able to provide a therapy option for injured muscles in human patients.

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## SESSION 2. BIOPHYSICS AND E-C COUPLING IN THE PHYSIOPATHOLOGY OF NEUROMUSCULAR DISEASES

### Oscillatory behaviour in muscle myosin

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Muscle contraction is generated by cyclical interactions of myosin heads with actin filaments to form the actomyosin complex. The stable configurations of the actomyosin complex have been described in detail, but whether in vivo, at physiological temperatures, these configurations are fixed to the ones observed in cryomicroscopy (at low temperature) or undergo thermal oscillations is unknown and not generally considered in mathematical modeling. By comparing three

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mathematical models, we analyze how this intrinsic property of the actomyosin complex affects muscle contraction at three level; namely, single cross-bridge, single fiber and organ levels, in a ceteris paribus analysis. We observed that state fluctuations allow the lever arm of myosin to easily and dynamically explore all possible minima in the energy landscape, generating several backward and forward jumps between states during the lifetime of the actomyosin complex, whereas the rigid case is characterized by fewer force generating events. Therefore, dynamical oscillations enable an efficient contraction mechanism, in which a higher force is sustained by fewer attached cross-bridges.

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### The molecular machinery for pretzel formation at the neuromuscular junction

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Mammalian neuromuscular junctions (NMJs) undergo a postnatal topological transformation from a simple oval plaque to a complex branch-shaped structure often called a “pretzel”. Although abnormalities in NMJ maturation and/or maintenance are frequently observed in neuromuscular disorders, such as congenital myasthenic syndromes (CMSs), the mechanisms that govern synaptic developmental remodeling are poorly understood. It was reported that myotubes, when cultured aneurally on laminin-coated surfaces, form complex postsynaptic machinery, which resembles that at the NMJ. Interestingly, these assemblies of postsynaptic machinery undergo similar stages in developmental remodeling from “plaques” to “pretzels” as those formed *in vivo*. We have recently demonstrated that podosomes, actin-rich adhesive organelles, promote the remodeling process in cultured myotubes and showed a key role of one podosome component, Amotl2. We now provide evidence that several other known podosome-associated proteins are present at the NMJ in vivo and are located to the sites of synaptic remodeling. Additionally, we identified proteins that interact with Amotl2 in muscle cells. We show that two of them: Rassf8 and Homer1, together with other podosome components, are concentrated at postsynaptic areas of NMJs in the indentations between the AChR-rich branches. Our results provide further support for the hypothesis that podosome-like organelles are involved in synapse remodeling and that Rassf8 and Homer1 may regulate this process.
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### Discovery of junctions mediating store-operated calcium entry in muscle

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**These authors contributed equally to this work.*

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Depletion of calcium ($Ca^{2+}$) from intracellular stores (endoplasmic reticulum, ER), triggers $Ca^{2+}$ entry across the plasma membrane, a process known as store-operated $Ca^{2+}$ entry (SOCE). SOCE is mediated by the interaction between STIM1 (stromal interaction molecule 1), which functions as the Ca2+ sensor in the ER, and $Ca^{2+}$ permeable Orai1 channels in the external membrane. In skeletal muscle, SOCE is the primary mechanism of $Ca^{2+}$ entry during repetitive activity, a crucial step that prevents/delays fatigue. Despite the importance of this mechanism for proper muscle function during sustained activity, the subcellular sites for SOCE in skeletal fibers have not been identified. Here we show that prolonged muscle activity (treadmill running in mice) drives the formation of previously unidentified intracellular junctions between the sarcoplasmic reticulum (SR) and extensions of the external transverse tubule (TT) membrane. The activity-dependent formation of these unique SR-TT junctions reflects a striking and unexpected remodeling of the existing sarcotubular system at the I band of the sarcomere. Using immunochemistry and immuno-gold labeling we also demonstrate that these newly formed, activity-driven junctions contain the molecular machinery known to mediate SOCE in muscle: STIM1 $Ca^{2+}$ sensor proteins in the SR, already present in the I band in control conditions, and Ca2+-permeable Orai1 channels, which move into the I band with TTs during prolonged muscle activity. Thus, we refer to these junctions as $Ca^{2+}$ Entry Units, the first new, molecularly defined subcellular structure in skeletal muscle in over 30 years.

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### Muscle-Nerve communication in murine models of Amyotrophic Lateral Sclerosis

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The loss of connection between muscle and nerve is a crucial biological mechanism involved in Amyotrophic Lateral Sclerosis (ALS), a neurodegenerative disease associated with motor neuron degeneration, muscle atrophy and paralysis [1]. Recent studies showed the primary role of the skeletal muscle in the pathogenesis of the disease, pointing out the key role of the communication between muscle and nerve.
In this context, we developed a protocol to measure, *ex vivo*, the neuromuscular junction (NMJ) functionality [2]. The experimental technique is based on the comparison between the muscle contractile response elicited by membrane

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stimulation and the response evoked by nerve stimulation. Since this latter stimulation bypasses the neuronal signalling, any difference between the two responses may be related to NMJ alterations. In particular, we started studying the Soleus-sciatic nerve preparation of one of the most studied ALS animal models, the $SOD1^{G93A}$ mouse [3], with the particular aim of following the pathology's progress. We observed that the first functional alterations begin at 90 days of age, with an intrinsic damage of the muscle and defects in NMJ functionality who increase until the end-stage of the disease. Subsequently, we approached the study of the $MLC/SOD1^{G93A}$ mouse model, in which superoxyde dismutase-1 mutated gene is expressed exclusively in the skeletal muscle [4]. Our preliminary results highlighted defects in soleus muscle and NMJs functionality in $MLC/SOD1^{G93A}$ mouse model, compared to the wild type, suggesting a direct muscle impairment. Further analysis on this model will provide useful information about the NMJ alterations directly related to oxidative stress on skeletal muscles.

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### Design of a high throughput screening: disrupt the super relaxed state of myosin to cure obesity and diabetes

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Myosin is an abundant ATPase protein. It is estimated that 10% of muscle tissue weight is myosin. Due to its abundance, myosin can be a good target to raise basal metabolic rate in animals. A new low-ATP-consumption state of myosin has recently been proposed (1, 2). This new state has been called the "Super Relaxed State (SRX)" of Myosin. Structural evidence for the SRX state have recently been published showing a close complex formed by the two-myosin heads (3). It is characterized by an ATPase time constant in the order of 300 seconds versus the 15 seconds for the so-called "Disordered Relax State" (DRX)(1,2). The idea is that behind that large number of "dormant" ATPases, there is the key to raise basal metabolism in a physiological way. The amount of myosin in the SRX state is estimated to be approximately 60% of the total. Switching of the myosin heads from the SRX state to the DRX state is regulated by phosphorylation in a cooperativeness-driven-equilibrium. Controlling this equilibrium may lead to an increase in basal metabolism that would consume an additional energy of up to 1000 Kcal/day. We studied the effect of several Regulatory Light Chain mutants on the SRX state and we applied this information to the development of a high throughput screen. We are searching a molecule that is able to destabilize the SRX state in skeletal muscle fibers. We screened 2000 compound of an FDA approved library. Potential lead compounds will be discussed

*1. Cooke. Biophysical Reviews. 2011.*
*2. Stewart et al. Proc Natl Acad Sci 2010.*
*3. Padrón et al. J. Mol. Biol. 2008.*

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### Realization of a sarcomere-like machine based on muscle myosin

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The muscle cell is a biological machine where steady force and shortening are generated by arrays of the motor protein myosin II pulling the actin filament towards the centre of the sarcomere (the ~2 µm long structural unit of muscle) during cyclical ATP-driven interactions. The fraction of the time of the ATP hydrolysis cycle that myosin II spends attached to actin depends on the sarcomere load and at low load can be as small as 0.02. The array-type arrangement of the motors enhances and makes steady the production of force and shortening, but has so far limited the investigation of mechanics, energetics and structural dynamics of this collective motor to top-down approaches, such as single-cell mechanics and X-ray diffraction (Piazzesi et al. Cell 131:784-795, 2007). The laser trap technique in the Three Bead Assay (TBA) configuration allowed the recording of single actin-myosin interaction in vitro, but only when the duration of attachment was increased by reducing the ATP concentration to a few tens of micromolar (two orders of magnitude lower than that in situ in physiological conditions). In this project we use an alternative approach consisting in assembling molecular motor proteins on a nanostructured support to generate a synthetic sarcomere-scale machine, the mechanical output of which is measured with a double laser optical tweezers apparatus (Bianco et al. Biophys. J. 101:866-874, 2011). The shape, the material and the coating of the support carrying the motor array have been optimised using a preliminary version of the machine consisting of an ensemble of motor proteins randomly adsorbed on a flat surface and brought to interact with an actin filament attached to the trapped bead with the correct polarity. Tests on the density and the disposition of the myosin motors on the surface have been done using AFM. The most reproducible results have been obtained when the support for the motor ensemble is the lateral surface of a chemically etched single mode optical fibre (diameter 4 µm). In solution with physiological [ATP] (2 mM), the ensemble drives 350 nm of actin filament sliding developing a steady force of 50 pN.

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## SESSION 3. MUSCLE WASTING AND CACHEXIA

### Involvement of S1P/ S1PR axis in skeletal muscle atrophy

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Skeletal muscle atrophy is caused by several and heterogeneous conditions, such as cancer (cachexia), neuromuscular disorders and aging. In most types of skeletal muscle atrophy overall rates of protein synthesis are suppressed, protein degradation is consistently elevated and atrogenes, such as the ubiquitin ligase Atrogin-1/MAFbx, are up-regulated. Sphingolipids represent a class of bioactive molecules capable of modulating the destiny of many cell types, including skeletal muscle cells. In particular, we and others have shown that sphingosine 1-phosphate (S1P), formed by sphingosine kinase (SphK), is able to act as trophic and morphogenic factor in myoblasts. Here, we report that the inhibition of SphK1 by specific gene silencing or pharmacological inhibition drastically reduced myotube size and myonuclei number, and increased Atrogin-1/MAFbx expression. Notably, the atrophic phenotype of C2C12 myotubes treated with dexamethasone and of muscle fibers obtained from cachectic mice inoculated with C26 adenocarcinoma, was characterized by increased expression of Atrogin-1/MAFbx and reduced levels of active SphK1. In addition, we found that C2C12 muscle cell atrophy was accomplished by changes in the pattern of expression of S1P receptor subtypes (S1PRs) and treatment of myotubes with S1P was able to prevent Dexa-induced atrophic marker expression. Finally, by using specific S1PR agonists and antagonists, we extended the investigation on the role played by S1PRs in the control of Atrogin-1/MAFbx expression. Altogether, these findings provide the first evidence that S1P/SphK1/S1PR axis acts as a molecular regulator of skeletal muscle atrophy, thereby representing a new possible target for therapy in many physiological and pathological conditions.

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### Interstitial cell activation during acute muscle denervation

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Skeletal muscle is a dynamic tissue that can respond to external stimuli through both anabolic and catabolic processes. In a variety of conditions, including immobilization, AIDS and neuromuscular disorders, skeletal muscle mass is decreased (atrophy). Upon denervation or disuse, skeletal muscle undergoes atrophy, leading to reduced size of myofibers, impaired contractile and metabolic activities. Previous studies have identified key molecular pathways leading to protein breakdown and degradation of sarcomeric proteins; yet, it remains a gap of knowledge on whether muscle resident cell populations can regulate the response of muscle to atrophic stimuli. Indeed, the recent identification of muscle-derived interstitial cells, named fibro-adipogenic progenitors, that can adopt multiple lineages and contribute, either directly o indirectly, to muscle regeneration (Joe et al,2010; Uezumi et al,2010) indicates a previously unrecognized complexity in the regulation of muscle homeostasis (Saccone et al,2014). We have discovered an unexpected key role of specific muscle-derived mononuclear cells in the pathogenesis of muscle atrophy. The characterization of the mechanism by which these cells contribute to the loss of muscle mass may lead to the identification of new therapeutic targets to counteract muscolar atrophy.

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### PGC-1α overexpression promotes myogenesis: relevance to cancer-induced muscle wasting?

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PGC-1α overexpression is able to protect skeletal muscle from fasting or denervation-induced atrophy (1) and to improve sarcopenia in old mice (2). Consistently, in the skeletal muscle of cachectic tumor-bearing mice, PGC-1α expression is reduced (3), in association with the accumulation of PAX7+ cells, which is suggestive of an impairment of myogenesis (4). Preliminary observations obtained in mice overexpressing PGC-1α specifically in the skeletal muscle show that the number of CD34+/Sca1+ cells, both integrin-α7 positive (satellite cells) and negative (other myogenic precursors), was higher in the muscle of transgenic (TG) mice than in those of wild-type (wt) animals. Not only, myotubes originating from TG-derived myogenic precursors were increased in both number and size in comparison to those obtained from wt progenitors. Aim of the present study was to investigate if PGC-1α overexpression can improve the regenerative capacity in the muscle of tumor (C26)-bearing animals after chemically-induced injury. BaCl2 (30 µl, 1.2% w/v) was injected in the tibialis anterior muscle the day after tumor implantation. Thirteen days after injury, both wt and TG controls almost completely recovered the initial myofiber cross sectional area (CSA; 70% of uninjured muscle). By contrast, CSA recovery was markedly delayed in wt or TG tumor-bearing mice (30% of uninjured muscle). Such a lack of CSA rescue in TG C26 hosts occurred despite TG mice constitutively possess a number of myogenic precursors higher than wt animals. As an estimate of mitochondria number, cytochrome c expression was evaluated. The results show that cytochrome c levels were significantly reduced in the regenerating muscle of wt C26 hosts, while remained comparable to those of uninjured muscle in BaCl2-treated

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TG tumor bearers. Previous observations showed that inhibition of ERK activity improved muscle wasting and myogenesis in the C26 hosts (4). In this regard, muscle pERK levels were significantly lower in TG tumor bearers than in wt C26 hosts. In conclusion, the present study shows that PGC-1α overexpression in the regenerating muscle of tumor hosts resulted in improved mitochondrial mass, and likely, oxidative capacity, and in reduced pERK levels, however without obtaining a significant CSA rescue. These observations suggest that while PGC1α overexpression exerts positive effects on tumor-induced derangements at the molecular level, it does not appear able to impinge on the multifactorial nature of muscle wasting.

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*2. Wenz et al., Proc Natl Acad Sci U S A. 2009 Dec 1;106(48):20405-10.*
*3. Penna, Bonetto et al., Int J Cancer. 2010 Oct 1;127(7):1706-17.*
*4. Penna et al., PLoS One. 2010 Oct 27;5(10):e13604.*

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### The activation of the SDF1/CXCR4 pathway retards muscle atrophy during cancer cachexia

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Cancer cachexia is a life-threatening syndrome that affects most patients with advanced cancers and involves severe body weight loss, with rapid depletion of skeletal muscle. No effective treatment is available. We analyzed microarray datasets to identify a subset of genes whose expression is specifically altered in cachectic muscles of Yoshida hepatoma-bearing rodents, but not in those with diabetes, disuse, uremia or fasting. By Ingenuity Pathways Analysis, we found three genes belonging to the CXCR4 pathway downregulated only in muscles atrophying because of cancer: *SDF1*, *PAK1* and *ADCY7.* Consistently, we show that the expression of all SDF1 isoforms declines also in Tibialis Anterior from cachectic mice bearing colon adenocarcinoma or renal cancer and anti-cachexia drugs such as sunitinib restore it. Overexpressing genes of this pathway (i.e. *SDF1* or *CXCR4*) in cachectic muscles increases the fiber area by 20%, partially protecting them from wasting. The mechanisms behind this muscle preservation during cachexia include both reduced degradation of long-lived proteins, by either SDF1α or SDF1β on atrophying myotubes, and increased protein synthesis, mainly by SDF1α. However, inhibiting CXCR4 signaling with the antagonist AMD3100 does not affect protein homeostasis in atrophying myotubes at all, whereas normal myotubes treated with AMD3100 display decreased diameter in a time- and dose-dependent manner, until a plateau. This further confirms the involvement of a saturable pathway (i.e. CXCR4). Overall, these findings support the idea that activating the CXCR4 pathway in muscle suppresses the deleterious wasting associated with cancer.

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### Role of the apelinergic pathway in colon adenocarcinoma-induced muscle wasting

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Cancer cachexia is a systemic syndrome that consists of a dramatic weight loss with rapid muscle depletion due to enhanced protein degradation, irrespective of food intake. Remarkably, 50% of advanced cancer patients are affected by cachexia, which accounts for approximately 20% of cancer deaths. No therapy is available. Interestingly, females are more resistant to cancer cachexia than males. We analyzed previous microarray datasets to identify genes whose expression is specifically altered in cachectic muscles of Yoshida hepatoma-bearing male rodents. Among these genes, we found that apelin was drastically downregulated to 8% of controls in cachectic gastrocnemius muscles (with 14% of weight loss) from male rats bearing Yoshida hepatoma for 5 days. We confirmed by Q-PCR that apelin was downregulated to 45% and 2% of controls also in Tibialis Anterior (TA) muscles in Lewis Lung Carcinoma and in Colon Adenocarcinoma 26 (C26)-bearing mice, respectively. Moreover, in TA from C26-bearing mice also the expression of apelin receptor (*APJ*), a member of G-protein coupled receptors, was reduced to 16%. Q-PCR analysis further confirmed that apelin downregulation occurred at all stages of cancer cachexia of C26-bearing male mice, while the expression of *APJ* was significantly reduced to 30% of controls only in early cachectic mice with less than 14% of body weight loss. Since apelin is expressed on X chromosome both in humans and mice and it is not downregulated in muscles from C26-bearing female mice, we believe that apelin could be a good candidate to explain the gender difference of cancer cachexia.

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### Tumor-derived microvesicles: new players in cancer-induced muscle wasting

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Cachexia is a syndrome frequently occurring in cancer patients. It is characterized by body and skeletal muscle wasting and by metabolic abnormalities. These latter are mediated, partially at least, by humoral factors. Energy balance perturbations also contribute to the onset of cachexia.

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In this regard, impaired mitochondrial functions and altered energy expenditure likely play a role. Recent observations suggest that in addition to classical humoral factors such as hormones or cytokines, also tumor-derived microvesicles (MVs), circulating particles containing different molecules such as proteins, mRNAs and microRNAs, may contribute to derangements associated with cachexia (1). MVs were isolated by differential ultracentrifugation from the conditioned medium of LLC (Lewis Lung Carcinoma) cells and were quantified by a NanoSight apparatus. After five day culture in differentiation medium, C2C12 myotubes were treated for 24 h with LLC-derived MVs. In C2C12 myotubes tumor-derived MVs induce a reduction of PGC-1α, the master regulator of oxidative metabolisms and mitochondrial biogenesis, as well as of Cyt-c mRNA expression. These results are in agreement with previous observations showing decreased PGC1α expression in the skeletal muscle of cachectic mice. In myotubes oxygen consumption is significantly decreased while lactate levels in the culture medium are increased after treatment with MVs. BNIP3 mRNA expression is significantly increased, while no differences can be observed as for myotube size and mRNA expression of both Atrogin1 and MuRF-1, two muscle-specific ubiquitin ligases. These results suggest that tumor-derived MVs affect mitochondria in C2C12 cultures. The reduction of mitochondrial mass (decreased Cyt-c mRNA expression) and function is associated with down-regulation of PGC-1α expression and enhancement of selective autophagy (mitophagy). On the whole, MV-induced alterations could contribute to muscle wasting during cancer cachexia.

*1. He WA, Calore F, Londhe P, Canella A, Guttridge DC, Croce CM. Microvesicles containing miRNAs promote muscle cell death in cancer cachexia via TLR7. Proc Natl Acad Sci U S A. 2014 25;111(12):4525-9.*

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## SESSION 4
## METABOLIC ALTERATIONS AND MUSCLE DISEASES

### Drp1 overexpression in skeletal muscle leads to growth defects, mitochondrial stress and translational impairment

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High mobility group box 1 (HMGB1) is a nuclear protein that acts extracellularly as an alarmin to modulate inflammation and tissue repair by recruiting cells and promoting their migration and activation. Recently, we showed that HMGB1 orchestrates both processes by switching among mutually exclusive redox states. Fully reduced HMGB1 acts as a chemoattractant, whereas a disulfide bond makes it a proinflammatory cytokine and further cysteine oxidation by reactive oxygen species (ROS) abrogates both activities. The fully reduced HMGB1 is prevalent in the extracellular environment immediately after acute muscle injury, and disulfide- HMGB1 appears a few hours later. Thus, the generation of ROS during muscle damage might modulate the redox status of the protein and eventually limit its lifespan and functions. We created a mutant (3S-HMGB1) not susceptible to redox modifications and we evaluated its regenerative activity in a model of acute muscle injury induced by cardiotoxin. We demonstrated so far that HMGB1 has beneficial effects in skeletal muscle regeneration after acute injury by dramatically increasing the number of healthy fibers and the number of satellite cells and M2c macrophages, two cell types essential for muscle repair. Moreover, HMGB1 acts directly on primary myoblasts by inducing their migration and their fusion to form large myotubes. Remarkably, 3S-HMGB1 behaves as a superagonist of HMGB1 in vivo, suggesting that it is a promising candidate for muscle repair therapies. Our study will be extended to other models of muscle damage, in particular dystrophies, in order to evaluate the therapeutic potential of 3S-HMGB1 in chronic conditions.

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### Role of the new E3 ubiquitin ligase Asb2β in skeletal muscle

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Atrophy is an active process controlled by specific signaling pathways and transcriptional programs. The identification of the precise signaling cascades that regulate muscle wasting remains poorly understood. The Ubiquitin Proteasome System (UPS) is one of the major systems that control protein breakdown during muscle wasting. The specificity of ubiquitin-dependent degradation derives from many E3s that recognize specific substrates. This work is focus on a novel muscle-specific circadian-rhythm dependent ubiquitin ligase named Asb2β. To dissect its role, we have generated muscle specific and tamoxifen-inducible muscle specific knock-out mice. We have characterized these knockout mice in physiological and in catabolic conditions. Asb2β defective muscles show normal muscle morphology and mitochondrial content but muscles display a fiber type switch and glycogen accumulation. Glucose tolerance test revealed an improved glucose uptake in knockout mice. Moreover, glycogen content dramatically decreased in Asb2β knockout mice during fasting. The changes in glucose homeostasis are Akt independent but TBC1D1and AS160 dependent. However, absence of nutrients triggers necrotic degeneration and appearance of abnormal mitochondria in Asb2β-null muscles. We have also started to characterize the tamoxifen-inducible knockout mice. Preliminary data show that acute inhibition of Asb2β induces a time dependent muscle growth. In conclusion, we have identified a novel muscle specific ubiquitin ligase, Asb2β, that plays an important role in glucose homeostasis and muscle hypertrophy.

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### Perilipin 2 downregulation induces skeletal muscle hypertrophy

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Aging is characterized by loss of skeletal muscle mass and function, condition known as sarcopenia. The mechanisms underlying sarcopenia are not completely understood, however a role for ectopic fat accumulation has been proposed. Skeletal muscle accumulates lipid in form of triglycerides within lipid droplets (LDs). LDs are characterized by the presence of Perilipins (Plins), that control lipid accumulation and metabolism under physiological and pathological conditions. In skeletal muscle one of the most representative is Plin2, particularly involved in lipid storage. However, the exact role of Plin2 is not still clear. We found that in human muscle the expression of Plin2 increases with aging and it is inversely associated with muscle mass and strength. Moreover, Plin2 expression is associated with atrophy-related genes, MuRF-1 and Atrogin, suggesting a role for Plin2 in muscle aging and atrophy. We also analysed the expression of Plin2 in adult mice where muscle atrophy was induced by denervation. Denervation of tibialis anterior muscle was compared with the contralateral intact side. After denervation, beside the expected increase of MuRF-1 and Atrogin, also Plin2 expression actually increases. This suggested that Plin2 expression is somehow associated with muscle atrophy. To support this hypothesis, we performed muscle-specific *in vivo* silencing experiments of Plin2. After 7 days from injection, a decrease of Plin2 was observed, and most interestingly the cross-sectional area (CSA) of Plin2-negative fibres resulted increased of about 30% with respect to Plin2-positive ones. As a whole, these data suggest that in skeletal muscle Plin2 is involved not only in muscle atrophy, but also in hypertrophy. Further studies are ongoing to better clarify this new role of Plin2 in skeletal muscle.

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### Investigating the cell origin and heterogeneity of embryonal rhabdomyosarcoma

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The rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in people under 20 years of age. It can commonly arise anywhere in the body but the head and neck, the extremities and the genitourinary tract are predominant sites. Based on histology, RMS tumors are classified into two major subtypes, embryonal and alveolar, which also differ in the molecular pathogenesis of development. Despite these differences, the origin of aRMS and eRMS seems to be the same but the precise cell type that triggers RMS is still unclear. Some evidence supports the notion that skeletal muscle precursors, probably satellite cells, may initiate RMS. Alternative theories propose mesenchymal stem cells, or even cells belonging to the adipocyte lineage, as possible tumor-initiating cells. In order to shed light on the origin of eRMS, we adopted the KrasG12D/+Trp53Fl/Fl conditional mouse model. This model allows us to generate eRMS in the hind limb of mice by infecting them with an adenovirus vector carrying the CRE recombinase. In a first approach we want to describe and rationalize the changes in the tumor mass cell populations by analyzing the tumor at different stages of development by using flow and mass cytometry techniques. In a second approach we aim at identifying the cell population(s) that are responsible for initiating the tumor. To this end we induce the gene mutations that are responsible for rhabdomyosarcoma development by infecting, with the CRE recombinase adenovirus, isolated muscle mononucleate cell populations and monitor their ability to develop rhabdomyosarcoma tumorigenic properties *in vitro*.

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### MURC/cavin-4 is co-expressed with Caveolin-3 in rhabdomyosarcoma tumors and its silencing prevents myogenic differentiation in the human embryonal RD cell line

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The purpose of this study was to investigate whether MURC/cavin-4, a plasma membrane and Z-line associated protein exhibiting an overlapping distribution with Caveolin-3 (Cav-3) in heart and muscle tissues, may be expressed and play a role in rhabdomyosarcoma (RMS), an aggressive myogenic tumor affecting childhood. We found MURC/cavin-4 to be expressed, often concurrently with Cav-3, in mouse and human RMS, as demonstrated through in silico analysis of gene datasets and immunohistochemical analysis of tumor samples. In vitro expression studies carried out using human cell lines and primary mouse tumor cultures showed that expression levels of both MURC/cavin-4 and Cav-3, while being low or undetectable during cell proliferation, became robustly increased during myogenic differentiation, as detected via semi-quantitative RT-PCR and immunoblotting analysis. Furthermore, confocal microscopy analysis performed on human RD and RH30 cell lines confirmed that MURC/cavin-4 mostly marks differentiated cell elements, colocalizing at the cell surface with Cav-3 and labeling myosin heavy chain (MHC) expressing cells. Finally, MURC/cavin-4 silencing prevented the differentiation in the RD cell line, leading to morphological cell impairment characterized by depletion of myogenin, Cav-3 and MHC protein levels. Overall, our data suggest that MURC/cavin-4, especially in combination with Cav-3, may play a consistent role in the differentiation process of RMS.

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### Cavin-1 and Caveolin-1 are both required to support cell proliferation, migration and anchorage-independent cell growth in rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is a childhood soft tissue tumor with broad expression of markers that are typically found in skeletal muscle. Cavin-1 is a recently discovered protein actively cooperating with Caveolin-1 (Cav-1) in the morphogenesis of caveolae and whose role in cancer is drawing increasing attention. Using a combined in silico and in vitro analysis here we show that Cavin-1 is expressed in myogenic RMS tumors as well as in human and primary mouse RMS cultures, exhibiting a broad subcellular localization, ranging from nuclei and cytosol to plasma membrane. In particular, the coexpression and plasma membrane interaction between Cavin-1 and Cav-1 characterized the proliferation of human and mouse RMS cell cultures, while a downregulation of their expression levels was observed during the myogenic differentiation. Knockdown of Cavin-1 or Cav-1 in the human RD and RH30 cells led to impairment of cell proliferation and migration. Moreover, loss of Cavin-1 in RD cells impaired the anchorage-independent cell growth in soft agar. While the loss of Cavin-1 did not affect the Cav-1 protein levels in RMS cells, Cav-1 overexpression and knockdown triggered a rise or depletion of Cavin-1 protein levels in RD cells, respectively, in turn reflecting on increased or decreased cell proliferation, migration and anchorage-independent cell growth. Collectively, these data indicate that the interaction between Cavin-1 and Cav-1 underlies the cell growth, migration and differentiation grade in myogenic tumors.

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## SESSION 5.

## SIGNALLING IN MUSCLE HOMEOSTASIS AND DISEASES

### Mitochondrial protein import is regulated by CK2-dependent phosphorylation of outer mitochondrial membrane protein Tom22 in mouse skeletal muscles

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Recently, it was shown that in yeast CK2-dependent phosphorylation of the mitochondrial import receptor Tom22 promotes biogenesis of the TOM translocase and is required for import of mitochondrial proteins. We asked whether CK2-dependent phosphorylation of TOM proteins also occurs in mammals. Using CK2β-deficient skeletal muscle lysates, we observed less phosphorylation of Tom22. Moreover, we confirmed CK2 phosphorylating residues serine 15 and threonine 43 of murine Tom22. Further, CK2-dependent phosphorylation of Tom22 changes its binding affinity for proteins need to be imported into mitochondria. In the absence of CK2 mitochondrial protein import is impaired in muscle fibers and mitochondria are dysfunctional. Pink1, a mitochondria health sensor and involved in Parkinson s disease, accumulates within mutant muscle cells, and labels removal of dysfunctional mitochondria by mitophagy and involvement of autophagic adaptor protein p62/SQSTM1. Consequently, the metabolism of oxidative muscle fibers in mutant muscles shifted towards glycolytic. As proof of concept, removal of aggregated p62/SQSTM1 by muscular in vivo electroporation of phosphomimetic Tom22 was successful. This is the first evidence for both, regulated protein import into mammalian mitochondria, and muscle weaknes due to a mitochondrial protein import defect.

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### Canonical Wnt/-catenin signaling through Axin2, YAP/TAZ and TEAD1, is essential for myotube formation and small diameter adult fiber types

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Canonical Wnt/β-catenin signaling plays a role in myogenic differentiation, but its role in adult muscle fibers is completely unknown. We approached canonical Wnt signaling in adult myofibers by well-known reporter Axin2-lacZ mice, monitoring X-Gal staining in muscle stem cells, in adult muscle fibers and at their neuromuscular synapse. In muscle stem cells, canonical Wnt signalling is absent in quiescent cells and 72 h proliferating cells. In adult muscle fibers, canonical Wnt signaling is strongly detectable by Axin2- and β-catenin-positive skeletal muscle fibers, where it is expressed only in fast fiber types with small cross-sectional areas. In these fibers, canonical Wnt signaling is active together with Hippo signaling members, YAP/TAZ and TEAD1. During differentiation of C2C12 cells, Axin2 increases together with the expression of TEAD1-target genes: *CTGF*, *Ankrd1* and *Cyr61*. In cultured primary muscle

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cells, we observed Axin1 and Axin2 being involved in proliferation and myotube formation in a Wnt1 and Wnt3a dependent manner. We present a model how canonical Wnt/β-catenin signaling, together with YAP/TAZ and TEAD1, influences muscle fiber diameter in fiber-type specific manner.

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### The Mitochondrial Calcium Uniporter controls skeletal muscle trophism *in vivo*

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Muscle atrophy contributes to the poor prognosis of many pathophysiological conditions, but pharmacological therapies are still limited. Muscle activity leads to major swings in mitochondrial [$Ca^{2+}$] which control aerobic metabolism, cell death and survival pathways. We have investigated in vivo the effects of mitochondrial $Ca^{2+}$ homeostasis in skeletal muscle function and trophism, by overexpressing or silencing the Mitochondrial Calcium Uniporter (MCU). The results demonstrate that both in developing and in adult muscles MCU-dependent mitochondrial $Ca^{2+}$ uptake has a marked trophic effect that does not depend on aerobic control, but impinges on two major hypertrophic pathways of skeletal muscle, PGC-1α4 and IGF1-AKT/PKB. In addition, MCU overexpression protects from denervation-induced atrophy. These data reveal a novel $Ca^{2+}$-dependent organelle-to-nucleus signaling route, which links mitochondrial function to the control of muscle mass and may represent a possible pharmacological target in conditions of muscle loss.

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### Role of atypical PKCs in skeletal muscle homeostasis

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PKC (protein kinase C) family is composed by 3 subgroups: conventional, novel and atypical PKC. These kinases are involved in a large number of biological processes (such as protein synthesis, glucose metabolism, apoptosis). PKCzeta and PKClambda/iota belong to the atypical PKC subgroup and differ from conventional and novel PKCs for their activation mechanism. Indeed atypical PKCs are calcium and diacylglycerol (DAG) insensitive, while classical PKCs are activated by calcium and DAG, and novel PKCs are activated by DAG but not by calcium (1,2). Little is known on the role of PKCzeta on skeletal muscle homeostasis. Thus, we overexpressed this kinase by *in vivo* transient transfection. We observed a marked hypertrophy in PKCzeta positive myofibers compared to surrounding not transfected fibers. In addition PKCzeta overexpression protected muscle from denervation-induced atrophy. Next, we studied the effects of 3 different PKCzeta mutants on fiber size: 1) PKCzeta-DN (a dominant negative isoform carrying a point mutation on the ATP-binding site); 2) PKCzeta-ΔNPS (a costitutive active mutant); 3) PKCzeta-InLoop (a dominant negative isoform mutated in the activation loop). Surprisingly all these mutants cause muscle hypertrophy and protect from denervation-induced atrophy suggesting a possible kinase-independent mechanism of PKCzeta on skeletal muscle trophism.

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### PAK1 positively modulates p38 activation during myogenic differentiation and muscle regeneration

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P38 mitogen activated protein kinases (MAPKs) are required at several stages during differentiation of muscle progenitor cells. P38 phosphorylation initially accompanies satellite cells activation and triggers asymmetric division. At a later stage, it orchestrates myoblast differentiation promoting myotube formation. The signals that trigger or modulate p38 activation during the differentiation process are still debated. Both cellJtoJcell contact and TNFα prime p38α/β phosphorylation and activation during myogenesis. Cdo, a multifunctional surface protein has been implicated in myogenesis. Following cellJtoJcell contact and ligation to cadherin, Cdo binds JLP and BnipJ2 which act as scaffolds for recruitment of p38α/β and Cdc42. The formation of the complex leads to activation of Cdc42, which is fundamental to promote p38α/β phosphorylation and myogenic differentiation. However, the phosphorylation cascade leading to p38α/β activation has not been elucidated. We focused on Pak1, a member of the p21 activated kinase family, which is activated by Cdc42. We have observed that treatment of differentiating myogenic progenitors (mesoangioblasts) with the Pak1 inhibitor IPAJ3 negatively modulates p38α/β phosphorylation and myogenin expression without affecting cell proliferation. This inhibition of the myogenic differentiation program results in a lower efficiency of myotube formation. We followed these observations *in vivo* by monitoring regeneration efficiency in mice treated with IPA-3 and we observed that mice treated with IPA-3 displayed a delayed recovery after cardiotoxin injury. These results suggest the Pak1 contributes to myogenic differentiation of progenitor cells *in vitro* and participates in muscle regeneration *in vivo*.

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### Dissecting the role of Ambra1 in developing and adult skeletal muscle

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Ambra1 (activating molecule in Beclin 1-regulated autophagy) is an adaptor protein involved in a plethora of cellular processes. Studies in mice with a randomly mutated Ambra1 locus (Ambra1$^{gt/gt}$) showed that this gene is essential for the development of the central nervous system. A recently published work by our team suggests that Ambra1 may also play a key role for muscle development in zebrafish and mouse. Indeed, Ambra1$^{gt/gt}$ E13.5 mouse embryos display severe defects of neck, tongue, dorsal and limb muscles, being characterized by increased cellularity and a marked disorganization of myofibers. To better clarify the role of Ambra1 in skeletal muscles, we generated mice with a floxed Ambra1 allele (*Ambra1*$^{flox/flox}$). *Ambra1*$^{flox/flox}$ mice were then crossed with a CAG-Cre transgenic line, which express Cre recombinase in the oocytes, thus obtaining Ambra1$^{+/-}$ mice. Here we show that *Ambra1*$^{-/-}$ mice die at late developmental stages and display severe morphological defects, similar to Ambra1$^{gt/gt}$ embryos. Adult *Ambra1*$^{+/-}$ mice show an increased percentage of centrally nucleated fibers and a decreased proportion of oxidative fibers. *Ambra1*$^{flox/flox}$ mice were then bred with MLC-1f-Cre transgenic animals, which only express Cre recombinase in mature myofibers. Our preliminary data in adult *Ambra1*$^{flox/flox}$;MLC-1f-Cre mice show a significant increase of centrally nucleated fibers, although we did not observe any overt defect of oxidative fibers. Altogether, our data suggest that Ambra1 is important for the development of skeletal muscle. Further studies in different muscles of *Ambra1*$^{flox/flox}$;MLC-1f-Cre mice under different stress conditions will allow elucidating the role of this adaptor protein in myofiber homeostasis

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### The role of myopalladin in skeletal muscle growth

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Myopalladin (MYPN) is a striated muscle-specific sarcomeric protein belonging to a small family of actin-associated immunoglobulin-containing proteins. MYPN mutations have been identified in patients with dilated (DCM), hypertrophic, and restrictive cardiomyopathy. Furthermore, we identified three MYPN mutations in limb girdle muscular dystrophy (LGMD) patients with associated DCM. Within the sarcomeric Z-line, myopalladin binds to α-actinin, nebulin, and PDZ-LIM proteins. Furthermore, it is present in the nucleus and the I-band where it binds to the stress-inducible transcriptional cofactor CARP/Ankrd1, which, in turn, binds to the I-band region of titin, suggesting a role of MYPN in mechanosensing. In our preliminary studies, we found that MYPN can bind to and bundle filamentous actin, thereby promoting actin polymerization. Moreover, MYPN interacts with MRTF-A and strongly increases MRTF-A-mediated activation of serum response factor (SRF) signaling. In studies of MYPN knockout (MKO) mice, we found that MKO mice are significantly smaller compared to wildtype (WT) mice and have an about 30% reduction in skeletal muscle cross-sectional area (CSA) at all ages. Consistently, reduced differentiation rate and myotube width was observed in primary skeletal muscle cultures derived from MKO mice. Furthermore, studies of muscle performance in 2-month-old MKO mice showed reduced isometric force and power during isotonic shortening at any loads as a result of the reduced cross sectional area, whereas the force developed by each myosin molecular motor was unaffected. By up- and downhill treadmill running, MKO and WT mice performed similarly. However, while the performance of WT mice was unaffected following four consecutive days of downhill running, the performance of MKO mice decreased progressively and signs of muscle regeneration following muscle damage were observed. Consistent with a higher susceptibility to muscle damage, progressive Z-line widening was observed in MKO skeletal muscle from about 8 months of age. RNAseq revealed downregulation of actin isoforms and other SRF-target genes in MKO muscle both at 2 and 4 weeks of age, suggesting altered SRF signaling as a possible explanation for the reduced CSA in MKO mice.

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### Micro-RNA206 and Exosomes: A retrograde signaling that controls neuromuscular junction and tissue homeostasis

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Impairment of autophagy in muscle leads to precocious ageing1. In particular, autophagy deficient mice are characterized by weakness are atrophy that are associated with alteration in Neuro Muscular Junction (NMJ) and loss of innervation. In order to investigate the cross-talk between muscle and nerve, we found that the expression of FGFBP1, a neurotrophic factor that is critical to preserve muscle-nerve interaction, is suppressed in muscle of autophagy deficient mice. FGFBP1 has been found to be regulated by miRNA206, the muscle-specific miRNA2. When we checked

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the level of miRNA206 expression, we found higher level of miRNA206 in serum of muscle specific autophagy deficient mice than in controls. Importantly, miRNA206 was detected in the heart of those mice. To understand whether autophagy deficient muscles released vesicles containing microRNAs, we analysed exosomes Quantitative RT-PCR analyses confirmed an increased expression of miRNA206 in purified exosomes from both denervated and autophagy deficient fibers. Moreover expression of BDNF in neurons treated with purified exosomes containing miRNA206 was down-regulated. This finding suggests a potential role of exosomes and miRNA206 in modulating synaptic plasticity. In order to mimic autophagy deficient mice condition, we systemically injected exosomes transfected with miRNA206 in wild-type animals. MiRNA206 was found in several tissues, in particular liver and heart. Moreover the treatment was sufficient to induce skeletal muscle atrophy and changes in the expression of several neurotrophic factors. These data support the role of exosome as a signaling mechanism that connects muscle with different tissues including motorneuron, heart and liver.

*Carnio et al., Cell Rep, 2014. Williams et al., Science, 2009.*

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**MicroRNA signature in mdx dystrophic mice overexpressing mIGF-1**

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Duchenne muscular dystrophy (DMD) is a genetic disease in which loss of functional dystrophin protein results in progressive skeletal muscle degeneration. Although the genetic defect is widely known, the mechanisms by which the absence of dystrophin leads to the complex pathophisiology of the disease is not completely understood. MiRNAs are small non coding RNA that are important regulatory elements for muscle development and function [1]. Altered levels of specific miRNAs were found in several muscular disorders, including DMD [2, 3]. In particular it has been identified a specific DMD-signature miRNAs that may serve as a marker for therapeutic purposes [4]. Moreover, in a recent work it has been defined a specific group of miRNAs strictly correlated to dystrophin levels and whose deregulated expression could explain several pathogenetic features of DMD [5]. Previously we have demonstrated that the local expression of mIGF-1 in mdx mice ameliorates the dystrophic phenotype reducing myonecrosis and upregulating survival pathways such as AKT pathway [6]. In this work, we show that a specific group of miRNAs, dystrophin-indipendent, are modulated by mIGF-1 expression. In particular, local expression of mIGF-1 promotes the modulation of miR-206 and miR-24 as well as muscle specific genes associated with maturation of regenerating muscle fibers and differentiation. These results indicates that local overexpression of the anabolic factor mIGF-1 in mdx mice ameliorates the dystrophic microenviroment modulating the expression of a specific group of miRNAs and inducing a partial rescue of the characteristic DMD-signature.

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**Circular RNAs expression and function in myogenesis**

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Circular RNAs have been recently re-discovered as a large class of putative non-coding RNAs with a peculiar structure and poorly understood functions. Although their biogenesis, which proceeds via a back-splicing reaction, has been studied and dissected in the last years, their role in biologically relevant processes is still uncharacterized. Here, we performed expression profiling of circRNAs during in vitro differentiation of murine and human myoblasts, we selected and validated the expression of a subset of highly expressed, conserved circular RNAs and applied a high-content functional genomic screen in order to identify molecules that were able to impact on the differentiation process. We focused on three circRNAs whose down-regulation resulted in important phenotypes and further scrutinized one of them, named circ-ZNF609, with the aim of undestanding its molecular function. We found that circ-ZNF609 contains an open reading frame spanning from the native start codon of its host transcript and terminating at an in-frame stop codon that is created upon circularization. Circ-ZNF609 is associated to heavy polysomes and is translated into a 30-KDa peptide that is able to promote human myoblasts proliferation.

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## SESSION 6.

## SIGNALLING AND THERAPEUTIC APPROACHES FOR MUSCLE DISEASES

**Muscle derived pericytes for artificial skeletal muscle human-like size**

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Stem cells and regenerative medicine have greatly increased the expectations of the scientific community and the public for their potential in applications that aim at recovering or replacing injured, aged and diseased tissues. Nevertheless

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their clinical application is currently hindered by cell survival, inflammatory response, tissue engraftment, vascularization and efficient differentiation. Tissue engineering exploits biomaterials to improve stem cell engraftment and differentiation by mimicking organogenesis. Skeletal muscle tissue engineering is an up-and-coming biotechnology that could offer great potential, in the near future, for muscle repair. Reconstructing the skeletal muscle architecture and function is still a challenge requiring parallel alignment of myofibrils arranged into organized sarcomeres. We show that an "anatomical bioreactor-like" represented by the surface of mouse tibialis anterior muscle (TA), positively influences maturation and alignment of fibers derived from adult muscle stem/progenitor cells embedded into a poly-ethylene-glycol-fibrinogen (PF) hydrogel. This approach leads to the generation of an artificial normal muscle. Furthermore by the same approach we succeeded in replacing a complete mouse TA after massive muscle ablation, recovering morphology and function of the substituting artificial TA. Starting from these observations, we are now developing a novel approach for regeneration and/or reconstruction of skeletal muscle tissue segments human-like size in order to translate this technique to clinical application. For this purpose human derived muscle pericytes have been isolated from muscle biopsies and have been investigated for their myogenic potential. Moreover by exploiting the PF properties, we demonstrated the noteworthy potential of this cell population for human skeletal muscle tissue engineering.

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### Effects of the HDACi Givinostat on muscle regeneration of mdx mice

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Histone deacetylases (HDACs) control the transcriptional networks underlying both muscle differentiation and progression of dystrophy. Considering that, HDAC inhibitors (HDACi) are important candidate drugs for pharmacological interventions in muscular dystrophies. Although the beneficial effects of HDACi in the treatment of muscular dystrophy are known, it remains to dissect the mechanism of action and the cellular mediators of these drugs. The goal of this project is to analyze the molecular mechanisms underlying the role of resident satellite cells and infiltrating macrophages in mediating the activity of HDACi ITF2357 (also referred to as Givinostat) in dystrophic muscle of mdx mice, the best animal model of Duchenne Muscular Dystrophy (DMD). We analyzed the dystrophic phenotype of mdx mice treated with Givinostat at different stages of disease, specifically 6, 12 and 36 weeks, corresponding to necrotic/inflammatory, regenerative and fibrotic stage, respectively. The histopathological and morphometric analyses show an amelioration of dystrophic phenotype with a significant increase of muscle fiber cross-sectional area and a consistent reduction of intramuscular fibrosis, surprisingly also at late stage of disease, suggest a positive outcome also in old mdx mice. Moreover, gene expression analysis of whole skeletal muscle and purified cell populations pointed out a modulation of fibrosis and inflammatory markers and fibroadipogenic differentiation. Overall, these data confirm the beneficial effects of Givinostat on dystrophic muscle and identify the involvement of macrophages in mediating Givinostat activity.

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### An anti-oxidant treatment (NAC) reduces formation of cores and improves muscle function in RYR1$^{Y522S/WT}$ mice

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Central Core Disease (CCD) and Malignant Hyperthermia (MH) are related disorders linked to mutations in the ryanodine receptopr-1 (RYR1) gene, encoding for the sarcoplasmic reticulum (SR) Ca2+ release channel. CCD is a congenital myopathy characterized by amorphous regions lacking mitochondrial activity (cores) in skeletal fibers. In humans, the RYR1-Y522S mutation is associated with MH and formation of structural cores. Skeletal fibers of knock-in RYR1$^{Y522S/WT}$ mice develop mitochondrial damage and cores, caused by excessive oxidative stress (Boncompagni et al. 2009 PNAS). We treated RYR1$^{Y522S/WT}$ mice with an anti-oxidant, N-acetylcysteine (NAC), provided ad-libitum in drinking water (1%w/v) for 2 months and verified reduction of oxidative stress: indeed, level of 3-nitrotyrosine was increased by 1.44-folds in RYR1$^{Y522S/WT}$ mice, but reduced to control levels after NAC treatment. Electron microscopy was used to assess mitochondrial integrity following NAC-treatment: a) mitochondria swelling and frequency of damaged mitochondria were both decreased (-24% and -10%, respectively); b) the number/100 μm2 of mitochondria (25.9 ± 0.7 vs 29.1 ± 0.6) and their proper association with the SR (+22%) were both increased. Using histological analysis, we also verified that NAC was effective in reducing the frequency of cores (-20% *contracture cores*; -30% *unstructured cores*). Finally, we evaluated muscle function in treated mice by grip strength test: NAC was able to improve muscle strength of about 80%. This work provides the bases for clinical trial as it demonstrate that NAC-administration prevents mitochondrial damage, development of cores, and improves muscle function in a mouse model of CCD.

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### Lethal exertional strokes in ryr1y522s/wt and CASQ1-null mice are prevented by drugs used to treat malignant hyperthermia in humans

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In humans, lethal hyperthermic episodes can be trigger by anesthetics (a disorder known as Malignant Hyperthermia, Susceptibility, MHS) and by high temperature and/or strenuous exercise (crises identified as Environmental/ Exertional Heat Strokes, EHSs). The correlation between MHS and EHS is strongly supported by extensive work in animal models: indeed, both RYR1$^{Y522S/WT}$ knock-in and CASQ-1 knockout mice trigger similar lethal crises when exposed to both halothane and heat. Here we tested the following hypotheses: a) strenuous exercise is a stimulus capable to trigger EHS-lethal episodes; b) MHS and EHS share common molecular mechanisms underlying crises. When RYR1 $^{Y522S/WT}$ and CASQ1-null mice were subjected to an exertional-stress (ES) protocol (executed on a treadmill placed in an environmental chamber), which was well tolerated by WT animals (0% of deaths), the mortality rate was dramatically increased (80% and 75%, respectively), with a rise in core temperature (hyperthermia) significantly higher than in WT at the end of the stimulus. During exertional-crises, most fibers from RYR1 $^{Y522S/WT}$ and CASQ1-null mice suffer severe structural damage (~99% and ~64% of fibers, respectively), indication of rhabdomyolysis. Importantly, pre-treatment of animals with azumolene (a more water soluble dantrolene analog, the only drug approved for treatment of MH crises in humans) almost completely abolished mortality rate in RYR1 $^{Y522S/WT}$ and CASQ1-null animals, by reducing hyperthermia, rhabdomyolysis, and $Ca^{2+}$leak from the SR. All these results strongly suggest that EHS share common molecular mechanisms with anesthetic-induced MH episodes and that drugs used to treat classic MH should be considered for treatment of EHS.

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**_Mdx/Ager$^{-/-}$_ mice show reduced muscle necrosis and inflammation compared with _mdx_ mice**

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Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disorder characterized by progressive muscle degeneration due to lack of dystrophin, a protein essential for the integrity of sarcolemma during contraction. In DMD compensative degeneration/regeneration cycles determine a condition of chronic inflammation contributing to progressive muscle wasting. RAGE (receptor for advanced glycation end-products) is a multiligand receptor belonging to the immunoglobulin superfamily involved in physiological and pathological processes including inflammation and myogenesis [1]. RAGE is not expressed in adult muscle tissue, whereas it is expressed in regenerating myofibers during muscle regeneration [2,3], in dystrophic muscles and activated immune cells. To have information about the role of RAGE in the pathophysiology of DMD we generated a double mutant *mdx/Ager$^{-/-}$* mouse lacking dystrophin and RAGE (*Ager*). We analyzed diaphragms and hind-limb muscles (i.e., tibialis anterior and quadriceps femoris) of *mdx*, *mdx/Ager$^{-/-}$*, *Ager$^{-/-}$* and WT mice at different ages (i.e., 2, 3, 4 and 5 weeks, and 3 and 6 months of age). We found that although the absence of RAGE in dystrophic mice did not affect the onset of the pathology, muscles of 5 week- and 6 month-old *mdx/Ager$^{-/-}$* mice showed significantly reduced numbers of necrotic myofibers, and reduced areas of immune cell infiltrate compared with age-matched *mdx* mice. Also, muscles of *mdx/Ager$^{-/-}$*mice showed strongly reduced expression of the marker of activated macrophages, MAC3, compared with *mdx* mice. Moreover, muscles of *mdx/Ager$^{-/-}$* mice exhibited significantly reduced PAX7$^{+ve}$ and myogenin$^{+ve}$ cell numbers, pointing to a reduced recruitment of muscle precursor cells and a more efficient regeneration in dystrophic mice lacking RAGE. Our results suggest that RAGE has an important role in sustaining inflammatory and degenerative processes in dystrophic muscles, and that inhibition of RAGE expression/activity might represent a potential therapeutic approach in DMD patients.

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*2. Riuzzi F, et al. J Cell Sci 2012;125:1440-54.*
*3. Haslbeck KM, et al. Acta Neuropathol 2005;110:247-54.*

*****

**Ageing causes ultra-structural modification to calcium release units and mitochondria in cardiomyocytes**

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Ageing is associated to a dramatic increase in the incidence of heart failure, even if the existence of a real age-related cardiomyopathy remains controversial. As effective contraction and relaxation of cardiomyocytes also depends on $Ca^{2+}$ supply to myofibrils (handled by calcium release units, CRUs) and on efficient ATP production (provided by mitochondria), in this study we performed a morphological study of cardiac cells in hearts from adult and old mice (4 months vs. ≥ 24 months of age) using confocal and electron microscopy. The analysis of CRUs indicates that couplons become shorter with age and that the number of CRUs/50 $\mu m^2$ is decreased of about 24% (adults: 5.1±0.32; old: 3.9±0.19). Also mitochondria present structural modifications, with a significant increase in the percentage of organelles presenting severe alterations (3.5% vs. 16.5%). Importantly, both CRUs and mitochondria undergo a spatial re-organization with respect to sarcomeres/myofibrils: CRUs may be miss-oriented (longitudinal) or miss-placed (found at the A band instead of being correctly placed in proximity of Z-lines), while mitochondria are often grouped in an abnormal fashion. In addition, WB analysis shows that in aged mice, there is a significant reduced expression of junctophilin-2 (JP-2), a membrane protein involved in maintaining stability and morphometry of dyads. These age-related ultra-structural changes may underlie an inefficient supply of Ca2+ and ATP to contractile elements, providing a possible explanation for heart dysfunction associate to age.

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### Chemokines in dystrophic heart and implications for stem cell homing/therapy

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Progressive muscle degeneration followed by dilated cardiomyopathy is a hallmark of muscular dystrophy. Stem cell therapy is suggested to replace diseased by healthy myofibers, although so far we are faced by low efficiencies of migration, engraftment and differentiation of stem cells. Chemokines are signalling proteins guiding cell migration and have been shown to tightly regulate cardiac repair. We sought to determine which chemokines are expressed in a dystrophic heart that is undergoing cardiac remodelling. Therefor we analysed chemokine expression of Sarcoglycan-α (Sgcα) null, Sarcoglycan-β (Sgcβ) null and immunodeficient Sgcβ-null mice. High expression of all three monocyte-chemotactic proteins was observed, especially Ccl8 in both Sgcβ-null models and to a greater extent in Sgcα-null mice. Additionally, Fractalkine (Cx3cl1) was upregulated in both the immunocompetent and immunodeficient Sgcβ-null mice. In addition, we aim to evaluate the migration potential of cardiovascular progenitors derived from pluripotent stem cells in vitro, that have the potential to differentiate with high efficiency towards cardiomyocytes, smooth muscle cells and endothelial cells in vitro. We plan to test these cells for their in vivo differentiation and migration capacity towards the previously mentioned chemokines. This sheds perspective for an approachable mechanism, which could potentially improve stem cell homing towards the dystrophic myocardium.

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### Long and short-term effects of plant derived nutrients on Dystrophic Cardiomyopathy

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Cardiac dysfunction from cardiomyopathy is a frequent manifestation of muscular dystrophy. The primary defect common to most dystrophies involves the disruption of the dystrophin-glycoprotein complex (DGC) with subsequent sarcolemma instability and $Ca^{2+}$ influx, inducing cellular necrosis. Defective $Ca^{2+}$ uptake resulting from decreased expression and reduced activity of calcium-transporting ATPase (SERCA2a) and, recently, SERCA2a gene therapy has been demonstrated to mitigate dystrophic diseases. Our previous studies have demonstrated that the dystrophic phenotype observed in δ-sarcoglycan–null hamster is dramatically improved by a long-term dietary supplementation with flaxseeds (FS) (rich in n3-PUFAs), but the molecular mechanisms have not yet been fully understood. The present study was designed to test the hypothesis that FS enriched diet could regulate DGC and SERCA2a proteins that play an important structural and functional role in cardiomyocytes. Therefore, the levels of these proteins and mRNAs were analyzed in heart dystrophic hamsters fed with FS diet for long (five months) or short time (48 hours). Results showed that α- distroglycan, α-, β, γ-sarcoglycan and SERCA2a proteins were down-regulated in dystrophic hearts and FS-diet restored their normal expression pattern. The RT-PCR analysis showed that α-distroglycan, α-sarcoglycan and SERCA2a were up-regulated at the transcriptional level. Interestingly, the mRNAs increase was observed even when FS was administered for short periods suggesting the involvement of an epigenetic mechanism. Therefore, it seems plausible to consider the administration of plant-originated n-3 PUFAs as an adjuvant strategy for attenuating sarcolemma instability and defective $Ca^{2+}$ uptake that represent major damages associated with dystrophic cardiomyopathies.

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## SESSION 7.

## GENETIC AND EPIGENETIC ALTERATIONS IN MUSCLE DYSTROPHIES AND MYOPATHIES

### HDAC4 modulates the response to oxidative stress in skeletal muscle

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Oxidative stress (OS) is an imbalance between the production of free radicals, in particular reactive oxygen species (ROS), and the ability of the cells to counteract them by antioxidant responses. ROS production in skeletal muscle occurs mainly in mitochondria, both following physiological stimuli (e.g. aging, physical exercise, or at birth) (1-3) and in response to pathological events, such as denervation (4). In all cases, high levels of ROS actively influence the maintenance of muscle homeostasis. Histone deacetylase 4 (HDAC4) is a member the class II of the HDAC superfamily that regulates many cellular processes (5-7). Following denervation, HDAC4 is upregulated in skeletal muscle: it induces muscle atrophy and represses reinnervation (8-9). Increased levels of ROS cause HDAC4 translocation from the nucleus to the cytoplasm, thus inducing the release of genes transcriptionally repressed by HDAC4 (10). However, HDAC4 targets in skeletal muscle have not been discovered yet. In order to investigate the role of HDAC4 in response to OS in skeletal muscle, we use a mouse model with the selective deletion of HDAC4 in myogenin positive cells (HDAC4 mKO mice). HDAC4 mKO mice are viable and do not show gross abnormalities in skeletal muscle. We analyzed mice in two different conditions characterized by elevated OS: at birth and in adult mice following denervation. Molecular responses to oxidative stress are blunted in both newborn and adult HDAC4 mKO compared to control mice. Since elevated ROS contribute to

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mitochondrial damage and are important in redox signaling from the organelle to the rest of the cell, we analyzed mitochondrial ultrastructure. Both newborn and adult HDAC4 mKO muscles presented damaged mitochondria, altered mitochondrial dynamics and defects in myofiber organization. Our results indicate that HDAC4 is important in skeletal muscle to maintain muscle integrity and a proper response upon OS. Current studies are focused on the identification of HDAC4 targets in the OS response in skeletal muscle

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**Collagen VI, a key extracellular matrix protein at the crossroad of skeletal muscle and peripheral nerves**

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Collagen VI (ColVI) is a major extracellular matrix component made of three genetically distinct α chains and abundantly deposited in the basement membrane of both skeletal muscles and peripheral nerves. Mutations in *COL6A1*, *COL6A2* and *COL6A3* genes are known to cause different forms of muscle diseases, including Bethlem myopathy, Ullrich congenital muscular dystrophy and myosclerosis myopathy. ColVI null (*Col6a1*$^{-/-}$) mice display a myopathic phenotype characterized by latent mitochondrial dysfunction, spontaneous apoptosis, defective autophagy regulation and compromised muscle regeneration. We recently demonstrated that the absence of ColVI in peripheral nerves leads to hypermyelination, altered Remak bundles, sensory-motor functional deficits and decreased nerve conduction velocities, thus pointing at ColVI as a crucial molecule for peripheral nerve structure and function. Given the muscle and nerve defects displayed by *Col6a1* null mice, we decided to explore the role of ColVI in the neuromuscular junction (NMJ). Our unpublished studies revealed that ColVI is indeed deposited at the synapse. Immunofluorescence analysis showed ColVI deposition in NMJs. Preliminary results revealed altered expression of synaptic genes and abnormal electrophysiological parameters in Col6a1$^{-/-}$ mice. These findings suggest a potential role for ColVI at the NMJ, and further studies will allow shedding new light on the roles of this extracellular matrix component in the nerve/muscle axis.

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**miR-143 is activated under TSA conditions and helps cells to choose an anti-adipogenic fate**

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Muscular dystrophies are non curable diseases. Recently, new strategies shed light to an increase of muscle regeneration. These strategies focus on epigenetic drugs. TSA (HDACi) achieve to enhance the regeneration rate in both mice and humans. However, new challenges stay on the horizon. Monitoring and controlling the changes of the treatment in muscle without invasive techniques are one of that's. In our research we identified seven microRNAs differential expressed in FAPs population. FAPs are Key players of muscle regeneration under HDACi treatment. From these seven microRNA, miR-143 has been validated with qRT-PCR, and Chip techniques. This miR-143 form part of a cluster with miR-145 that locates into a long non coding RNA non characterized until that moment. The overexpression of this miR-143 turns FAPs into a non adipogenic phenotype, whereas the inhibition of it recovershe adipogenic behavior. Thus, in this work we are trying to characterize the role of this microRNA and their host gene to understand if it could be a good candidate to be used as marker during the treatment.

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**Characterization of a nuclear lncRNA involved in skeletal and cardiac muscle differentiation**

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The central dogma of gene expression is that DNA is transcribed into messenger RNAs, which in turn serve as the template for protein synthesis. In recent years, it has been discovered that genomes of multicellular organisms are characterized by the pervasive expression of different types of non-coding RNAs (ncRNA) and, among them, long non-coding RNAs (IncRNAs). In particular the mammalian genome contains many thousands of lncRNAs, which have been proposed to be fundamental in the regulation of many biological processes such as cellular differentiation and show an aberrant regulation in a variety of diseases. A transcriptome analysis performed during *in vitro* murine muscle differentiation allowed us to identify a subset of new lncRNAs differentially expressed during myogenesis (1). These transcripts were classified on the basis of their expression in proliferating versus differentiated conditions, muscle-restricted activation and subcellular localization. We are now focusing on the characterization of a nuclear lncRNA conserved in human, lnc-405, up-regulated during

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differentiation, whose expression is cardiac and skeletal muscle restricted. To dissect its role in myogenesis, we performed loss of function experiments using LNA-Gapmers followed by a transcriptome analysis. This approach revealed a strong down-regulation of a subset of genes involved in fiber contraction, cell fusion and related to several cardiomyopathies. With the idea to better explain its crucial role during myogenesis, we are now focusing on the molecular mechanism by which lnc-405 exerts its function in the nucleus by RIP, ChIRP and RNA pull-down assays that are on going.

*1. Ballarino M, et al. MCB, 2015.*

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**Muscle expression of SOD1G93A modulates microRNA and mRNA expression pattern in the spinal cord of transgenic mice**

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The functional connection between muscle and nerve is affected in several neuromuscular diseases, including Amyotrophic Lateral Sclerosis (ALS) whose major pathological processes are motor neuron degeneration. However, other cells may be involved in the pathogenesis of ALS and open the possibility that alteration in skeletal muscle homeostasis represents one of the principal mediators of motor neuron degeneration. We have evidences that indicate that muscle selective expression of $SOD1^{G93A}$ mutant gene modulates, at the level of spinal cord of $MLC/SOD1^{G93A}$ mice, relevant mRNA and microRNA associated with myelin homeostasis. Our study provided insights into the pathophysiological interplay between muscle and nerve and supports the hypothesis that skeletal muscle is a source of signals that can affect the nervous system.

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**Characterization of a calsequestrin-1 mutation identified in patients affected by a vacuolar myophaty**

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Calsequestrin (CASQ) is the major protein of the sarcoplasmic reticulum of striated muscle that binds $Ca^{2+}$ with high capacity and moderate affinity. CASQ exist as a monomer and polymers, depending on $Ca^{2+}$ concentration. CASQ switches from an unfolded state to a folded monomer when the ionic strength increases allowing the formation of front-to-front first and then back-to-back interactions in higher $Ca^{2+}$ concentrations. In humans, mutations in the cardiac isoform CASQ2 lead to catecholamine-induced polymorphic ventricular tachycardia. Recently we reported one mutation in the skeletal CASQ1 gene in a group of patients with a vacuolar myopathy characterized by the presence of inclusions containing CASQ1 and other SR proteins. The CASQ1 mutation ($CASQ1^{D244G}$) affects one of the high-affinity $Ca^{2+}$-binding sites of the protein and alters the kinetics of $Ca^{2+}$ release in muscle fibers from patients. Expression of the $CASQ1^{D244G}$ in myotubes and in mouse fibers causes the appearance of SR vacuoles containing aggregates of the mutant CASQ1 protein that resemble those observed in patients. Studies of $Ca^{2+}$ release showed an increase in $Ca^{2+}$ storage in $CASQ1^{WT}$ COS-7 transfected cells whereas no increase was observed in $CASQ1^{D244G}$. Moreover both $CASQ1^{WT}$ and $CASQ1^{D244G}$ were expressed in bacteria, purified and analysed for their ability to polymerize at increasing $Ca^{2+}$ concentrations. The results obtained indicate that the $CASQ1^{D244G}$ protein polymerizes at lower $Ca^{2+}$ levels and more rapidly than $CASQ1^{WT}$. These results suggest that the $CASQ1^{D244G}$ mutation interferes with the correct process of $Ca^{2+}$ -dependent protein polymerization causing altered intracellular calcium storage and the formation of protein aggregates.

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**Functional cross-talk between the different cell populations that contribute to DMD progression**

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Muscle regeneration is dependent upon a complex interplay of different cell types in the muscle stem cell niche. In particular, the recently described population of interstitial fibro-adipogenic progenitors (FAPs) and satellite cells (MuSCs) establish a complex network of interactions to coordinate muscle regeneration. FAPs are able to promote satellite cell differentiation and compensate for muscle necrosis. In our recent studies, we demonstrated that FAPs are the key cellular mediators of the beneficial effect of HDAC inhibitors at early stages of Duchenne Muscular Dystrophy (DMD). Indeed, FAPs, from young mdx mice HDACi, induce myogenesis at expense of adipogenesis and enhance their ability to support MuSCs differentiation. Conversely, FAPs from old mdx mice are resistant to HDACi and repress MuSCs differentiation (Mozzetta et al., 2013; Saccone et al., 2014). Given the importance of the cross-talk between FAPs and MuSCs in DMD progression, we are currently deciphering the role of FAP-released extracellular vesicles (and in particular the exosomes - endosome derived vesicles) as mediators of the functional interactions between mononuclear cell types that contribute to muscle regeneration.

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### Study of physio-pathological mechanisms implicated in sarcoglycanopathies

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Limb Girdle Muscular Dystrophies are rare genetic diseases, characterized by weakness and progressive muscular atrophy. A subfamily of LGMD2 regroups sarcoglycanopathies caused by mutations in genes coding for sarcoglycans. These transmembrane proteins are part of the dystrophin complex that protects muscle fibers against mechanical stress due to contraction. There is no treatment available for these diseases.In order to understand the molecular mechanisms implicated in sarcoglycanopathies and to identify new therapeutic targets, we are conducting two studies:
1 - SG mutants are not present at the muscle fiber membrane because they are retained in the endoplasmic reticulum by the quality control (ERQC) and they are prematurely degraded by the proteasome. To study the ERCQ pathways responsible for sarcoglycan disposal at molecular level, we first generated cell lines expressing clonally one SG mutant. Those clones are now used to investigate the SGs cellular trafficking mechanisms and then to test pharmacological compounds modulating ERCQ pathways.
2 – In these diseases, muscular atrophy affects limb muscles and infrequently head muscles. To investigate mechanisms underlying the fact that some muscles are more affected than other, we analyzed different muscles to search for molecular differences that may sign their relative sensitivity to the genetic defects. The content in micro-RNA of muscles from the limbs and face of Macaca fascicularis was explored. Experiments are in progress to analyze the function of identified micro-RNAs and to evaluate their therapeutic potential for sarcoglycanopathies. These projects will improve the knowledge on physio-pathological mechanisms of sarcoglycanopathies in order to identify new therapies for patients.

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### Molecular characterization of DBE-T lncRNA driving FSHD muscular dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common human myopathies and arises with progressive wasting of facial mimic muscles as well as upper arms and shoulder girdle muscles. In 95% of the cases, FSHD is associated with the copy number reduction of D4Z4 macrosatellite repeats at the subtelomeric region of chromosome 4 (4q35). This change is associated with an epigenetic deregulation of the region that ultimately leads to the de-repression of nearby genes, such as *DUX4* and *FRG1* that have been reported to contribute to the muscular dystrophic phenotype observed in FSHD patients. The chromatin-associated lncRNA *DBE-T*, encoded by the FSHD locus, has been shown to be one of the main players of such event, though the molecular mechanism has not been yet fully elucidated. *DBE-T* is preferentially expressed in FSHD patients where it favors the transcription of the 4q35 genes thanks to the recruitment of the histone methyl transferase of the Trithorax group of epigenetic activators ASH1L. Interestingly, through a structural/functional analysis, we have recognized several *DBE-T* functional domains that can be exploited as new molecular targets for therapeutic purposes. Specifically, we have identified a region and the molecular mechanism required for *DBE-T* tethering to the chromatin. In addition, we have mapped the minimal binding domains in ASH1L and *DBE-T*. Finally, we have highlighted a portion of *DBE-T* required to positively promote transcription. In agreement, a *DBE-T* mutant lacking this region is unable to trigger transcription. Currently, through proteomic approaches, we are investigating *DBE-T* protein partners that are specific for each *DBE-T* functional domain. Our goal is to identify unknown molecular players that, similarly to ASH1L, are recruited by *DBE-T* to the FSHD locus and can play a role in the disease. Overall, our study elucidates the molecular mechanism of *DBE-T* in FSHD and might unveil new therapeutic targets for the treatment of the disease.

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