Session 3. Cytoskeleton and (mechano)signalling

Lectures

L3.1

Microtubules orchestrate local translation to enable cardiac growth

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Cardiomyocytes are terminally differentiated cells that respond to stress by regulating their size. During hypertrophy, it is well established that cardiomyocytes activate a hypertrophic transcriptional profile and increase protein translation rates, yet how these changes in transcription and translation are coupled to sarcomerogenesis remains unclear. Concomitantly, the microtubule network of cardiomyocytes proliferates and is dramatically altered by post-translational modifications and decoration by microtubule-associated proteins, but how the microtubule network may regulate hypertrophy is also unknown. Here, we show that microtubules are indispensable for cardiac growth via spatiotemporal control of the translational machinery. We find that the microtubule motor Kinesin-1 distributes mRNAs and ribosomes along microtubule tracks to discrete domains within the cardiomyocyte. Upon hypertrophic stimulation, microtubules redistribute mRNAs and new protein synthesis to sites of growth at the cell periphery. Upon microtubule network disruption, mRNAs and ribosomes collapse around the nucleus, resulting in mislocalized protein synthesis, the rapid degradation of new proteins, and a failure of growth, despite normally increased translation rates. Together, these data indicate that mRNAs and ribosomes are actively transported to specific sites to facilitate local translation and assembly of contractile units and suggest that properly localized translation – and not simply translation rate – is a critical determinant of cardiac hypertrophy.

Oral Presentations

O3.1

Localisation and mobility of nebulin in adult skeletal muscle sarcomeres

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To date, it is unknown how giant muscle proteins are integrated and replaced in continuously contracting muscles. A prime example is nebulin (800 kDa), which binds F-actin and spans the length of the sarcomeric thin filament, with its C-terminus located in the z-disc and the N-terminus near the thin filament pointed-end. To establish the *in vivo* localization and mobility of nebulin in adult sarcomeres, we designed a mouse with photoconvertible Dendra2 inserted at the nebulin N-terminus (Dendra2-KI).
Individual nebulin molecules were visualized by PALM. Dendra2 blinks were fitted with a precision of ~12nm. ~89% of the blinks were located near the pointed-end of the thin filament (i.e. incorporated nebulin; 11% was unincorporated). Individual nebulin N-termini were normally distributed ~1060nm from the Z-disk, with a width at half-maximum of ~120nm.
The mobility of nebulin was studied in mature skeletal muscle fibers of Dendra2-KI mice using *intra-vital* (imaging window) or *in vitro* FRAP microscopy. Additionally, we measured nebulin mobility in myotubes, an immature muscle system. Dendra2 was converted from green to red fluorescent state and fluorescence was followed over time.
Data indicate that, in both in vivo and in vitro mature muscle, nebulin turnover follows a two phase dynamic: recovery of ~30% of green Dendra2 in the first 24 hours, followed by a slower recovery of another ~20% in the next 11 days. After 19 days, converted Dendra2 was still present in the ROI. Recovery in myotubes was ~3 times faster. Our data indicate that nebulin turnover in mature muscle is slow compared to other sarcomeric proteins studied previously.

O3.2

Novel role of drebrin in the AChR clustering and the organization of cytoskeleton at the postsynaptic machinery

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Neuromuscular junctions (NMJs) allow for the effective synaptic transmission essential for voluntary and involuntary movements and respiration. Importantly, the proper function of NMJs requires their postnatal maturation to topologically complex structures. At NMJs, chemical signals are received by the postsynaptic acetylcholine receptors (AChRs) which are clustered by the scaffold protein rapsyn, linking them to the actin cytoskeleton and cytoskeleton-organizing proteins. Molecular mechanisms of the postsynaptic machinery maturation and the rapsyn crosstalk with the cytoskeleton are still poorly understood. Drebrin interacts with actin and microtubules and is crucial for the functioning of the synapses in the central nervous system, but its role at NMJs remains elusive. To explore the role of drebrin at the muscle postsynaptic machinery, we downregulated drebrin expression with siRNAs, inhibited its interaction with actin with BTP2, and used immunofluorescence and biochemical methods. We identified drebrin as a postsynaptic protein colocalizing with the AChRs both *in vitro* and *in vivo*. We also show that drebrin is enriched at synaptic podosomes, actin-rich structures involved in remodeling of AChR clusters. Knockdown of drebrin or blocking its interaction with F-actin in cultured myotubes impairs the organization of AChR clusters and the cluster-associated microtubule network. Finally, we demonstrate that drebrin interacts with rapsyn and a drebrin interactor, plus-end-tracking protein EB3. Our results reveal an interplay between drebrin and AChR cluster-stabilizing machinery involving rapsyn, actin cytoskeleton, and microtubules.

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O3.3

Tubulin acetylation increases cytoskeletal stiffness to regulate mechanotransduction in striated muscle

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Mechanotransduction is central to how striated muscles sense, respond and adapt to a change in their mechanical load environment. Our lab has identified and characterized a mechanotransduction pathway by which the mechanics of contraction or stretch activates membrane bound NADPH oxidase 2 (Nox2) to elicit reactive oxygen species (ROS) signals that regulate calcium (Ca^{2+}) channels at the triad junction. Central to this pathway are microtubules (MTs) whose density and properties impact cytoskeletal stiffness and the magnitude of Nox2-ROS and Ca^{2+} signals. Our initial works identified that the level of detyrosinated tubulin, a post translational modification (PTM) to tubulin, regulated cytoskeletal stiffness and mechanotransduction through Nox2-ROS. Our recent works have focused on α-tubulin acetylation at lysine 40 within the lumen of polymerized MTs. Using pharmacologic and genetic strategies to increase α-tubulin acetylation independent of detyrosination, we show that microtubules enriched in acetylated α-tubulin increase cytoskeletal stiffness and viscoelastic resistance. We further showed that these changes slow rates of contraction and relaxation during unloaded contractions and increase the activation of Nox2 by mechanotransduction. Extending our work to intact muscle in vitro we show that acetylated MTs contribute very little to the passive mechanics and have no impact on the magnitude or kinetics of isometric contractions. Together, these findings implicate MT's enriched in acetylated tubulin as regulators of mechanotransduction through Nox2-ROS which adds to growing evidence that microtubules contribute to the mechanobiology of striated muscle.

O3.4

Stretch-induced unfolding of titin-like kinases as *in vivo* mechanosensing mechanism

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Stretch-induced unfolding of titin and titin-like proteins is thought to be a key mechanosensing mechanism in muscle. Yet, little evidence of its existence *in vivo* has been gathered. Prominently, stretch is postulated to regulate twitchin/titin sarcomeric kinases, which are autoinhibited by mechanosensory regulatory tails and functionally linked to muscle stress responses. To test stretch-unfolding in living muscle, we generated transgenic *C. elegans* expressing twitchin containing FRET moieties flanking the kinase domain and used computer vision algorithms to simultaneous measure fluorescence and muscle contraction in freely moving worms [1]. The data revealed a periodic change in kinase conformation during muscle activity supporting the proposed mechanism. We next investigated the functional outcome of such stretch-unfolding using human titin kinase (TK) [2]. We found that, in the absence of stretch, TK is robustly ubiquitinated by MuRF1 and that ubiquitination promotes the assembly of the autophagy receptors Nbr1/p62 onto TK, putatively leading to degradation upon inactivity. In active muscle, unfolding of TK's regulatory tail distances TK from the MuRF1-docking site on titin, seemingly down-regulating ubiquitination and, thereby, titin targeting by Nbr1/p62. This illustrates how mechanical signals regulate sarcomere targetability and subsequent remodelling.

References:

1. Porto *et al.* (2021) Conformational changes in twitchin kinase *in vivo* revealed by FRET imaging of freely moving *C. elegans*. *BioRxiv*. doi: 10.1101/2021.03.01.433414.

2. Bogomolovas *et al.* (2021) Titin kinase ubiquitination aligns autophagy receptors with mechanical signals in the sarcomere. *EMBO Rep*. In press.

Virtual Posters

P3.1

Nestin expression in the hypertrophic myocardium of the spontaneously hypertensive rats

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Nestin, a remarkable intermediate filament, transiently expressed during heart development, occurs in various cells in the adult myocardium, particularly in cardiac diseases. In this study we described expression and distribution pattern of nestin and its co-polymerizing proteins desmin and vimentin in the rat unaffected myocardium and in the hypertrophic myocardium of the spontaneously hypertensive rats. Samples from one-year-old and one and half-year-old animals were processed for immunohistochemistry. Nestin was not detected in any cells in the unaffected myocardium. But in the hypertrophied myocardium nestin immunostaining was observed in rare desmin$^+$ vimentin$^-$ cardiomyocytes, mildly more frequent in the older animals, where they were more often found near the larger blood vessels and in region of the apex of the heart. Furthermore, nestin was detected in some vimentin$^+$ interstitial cells with different intensity of desmin immunoreactivity mostly forming groups and very seldom in the myocardial capillaries and in the wall of the larger blood vessels. Complex structural changes in the hypertrophic myocardium of the pressure over-loaded hearts are accompanied by nestin re-expression in the different cell types indicating their cytoskeletal remodelling. Our findings suggest noticeable involvement of nestin re-appearance not only in mechanisms of myocardial hypertrophy but likely also in cardiac regeneration.

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P3.2

Involvement of myosin VI in inflammatory process in skeletal muscles and myogenic cells

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Myosin VI (MVI) is an unique unconventional actin-based motor, its diverse roles are mediated via interactions with a number of different binding partners present in multiprotein complexes. Results obtained on myoblasts derived from mice not synthesizing MVI (*Snell's waltzer, SV,* a natural MVI knockout) showed disturbances in the adhesion and fusion of myoblasts resulted in the formation of aberrant myotubes. These changes were accompanied by a significant increase in the level of reactive oxygen species (ROS) in myogenic cells. The lack of MVI in skeletal muscles led to a significant increase in the level of proteins involved in the inflammatory response, such as IL-6 and FOXO1. Threre are three main pathways that are involved in pro-inflamatory response: NF-κB, MAPK and JAK-STAT. The increased levels of phospho-NFκB, phospho-p38 and phospho-SAPK/JNK proteins in the hindlimb muscles derived from MVI-KO mice indicate a disturbance of these pathways, suggesting that MVI could be involved in the development of inflammation. We hypothesize that one of the mechanisms that could be behind this observation is the aberration in mitochondria followed by the changes in a redox status of the MVI-KO skeletal muscles, leading to chronic inflammation. In-depth studies on the MVI role in regulation of redox status of skeletal muscles are in progress.

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P3.3

The effects of various tropomyosin isoforms on the properties of tropomodulin-2 and cofilin-1

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Tropomodulin-2 (Tmod2) and cofilin-1 (Cof1) are actin-binding proteins that regulate the dynamics and properties of the actin filaments. In our work, we investigated how various tropomyosin (Tpm) isoforms affected the function of Tmod2 and Cof1 and revealed that their properties directly depend on Tpm isoforms.

Using fluorescent microscopy, we estimated the length of actin filaments after incubation with Cof1. Tpm1.5, Tpm1.6, Tpm1.7, Tpm3.2 and Tpm4.2 prevented the depolymerization of actin filaments by Cof1, while Tpm1.12, Tpm3.1, Tpm3.4, Tpm3.5 and Tpm 3.7 had no effects on the length of actin filaments. We also tested whether cofilin can displace Tpm from the surface of actin filaments. Tpm isoforms that effectively protected the actin filaments from cofilin-mediated depolymerization had significantly worse displacement kinetics.

We use the surface plasmon resonance to quantify the interaction of Tpm and Tmod2. The lowest equilibrium dissociation constant was observed for the Tpm1.5 ($6.18 \cdot 10^{-5}$ M), and the highest for the Tpm1.12 ($1.17 \cdot 10^{-8}$ M). Tpm1.12, Tpm3.7, and Tpm3.2 had sufficiently high equilibrium dissociation constants ($\approx 10^{-8}$), which characterizes the interactions of Tmod2 and Tpm as highly affine. We also estimated the ability of Tmod2 to accelerate actin polymerization in the presence of Tpm. Tpm1.6, Tpm1.12, Tpm3.5 and Tpm3.7 accelerated the actin polymerization rate in the presence of Tmod2; Tpm1.5, Tpm4.2 didn't change the polymerization rate as well as Tpm3.1 and Tpm1. 7 slowed it down.

Our data confirm the hypothesis that the isoform-dependent interaction of Tpm with Cof1 and Tmod2 can be one of the regulatory mechanisms of the dynamics of actin filaments.

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P3.4

Possible roles of mTORC1 and GSK-3 in the regulation of ribosome biogenesis in the atrophying rat postural muscle

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Prolonged disuse results in a significant reduction in the rate of muscle protein synthesis and subsequent skeletal muscle atrophy. However, molecular mechanisms involved in the regulation of ribosome biogenesis under disuse conditions are poorly studied. Both mechanistic target of rapamycin complex 1 (mTORC1) and glycogen synthase kinase-3 (GSK-3) are known to regulate ribosome biogenesis and protein synthesis (PS). The aim of the study was to elucidate possible contribution of mTORC1 and GSK-3 to diminished ribosome biogenesis in rat soleus muscle under disuse conditions. Wistar rats were subjected to 7-day hindlimb unloading (HU) or 7-day HU with daily injections of AR-A014418 (GSK-3beta inhibitor, 4 mg/kg) or rapamycin (mTORC1 inhibitor, 1.5 mg/kg). The unloaded rats were compared with the control animals. The key markers of ribosome biogenesis were assessed by gel-electrophoresis, immunoblotting and RT-PCR. As expected, 7-day HU resulted in a significant decrease in the rate of PS in rat soleus muscle. Treatment of rats with GSK-3 inhibitor partially prevented HU-induced increase in decrease in the rate of PS. Further, inhibition of GSK-3 prevented HU-induced downregulation of c-Myc expression as well as decreases in the levels of 45S pre-rRNA and 18S+28S rRNAs. The use of rapamycin during 7-day HU did not significantly affect such markers of protein synthesis as c-Myc, 45S pre-rRNA and 18S+28S rRNAs. Thus, GSK-3, but not mTORC1, can play a significant role in the regulation of ribosome biogenesis in rat soleus muscle under conditions of 7-day mechanical unloading.

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P3.5

Role of pannexin channels and ATP-dependent pathways in the regulation of signaling during skeletal muscle unloading

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Skeletal muscle unloading results in atrophy. We hypothesized that ATP is involved in response of muscle to unloading. We tested this hypothesis by blocking pannexin channel 1 (PnX1) regulating efflux of ATP from the cytoplasm. Rats were divided into 6 groups (8 rats each): non-treated control for 1 and 3 days of the experiments (1C and 3C, respectively), 1 and 3 days of hindlimb suspension (HS) with placebo (1H and 3H, respectively), and 1 and 3 days of HS with PnX1 inhibitor probenecid (PRB; 1P and 3P, respectively). When compared with 3C group there was a significant increase in ATP in soleus muscle of 3H and 3P groups (32 and 51%, respectively, $p<0.05$). When compared with 3H group 3P group had: 1) lower mRNA expression of E3 ligases MuRF1 and MAFbx (by 50 and 38% respectively, $p<0.05$) and MYOG (by 34 %, $p<0.05$); 2) higher phosphorylation of p70S6k and p-p90RSK (by 51 and 35% respectively, $p<0.05$); 3) lower levels of phosphorylated eEF2 (by 157%, $p<0.05$); 5) higher level of phosphorylated GSK3β (by 189%, $p<0.05$). In conclusion, PnX1-mediated ATP transport may be involved in the regulation of muscle atrophic processes by modulating expression of E3 ligases, and protein translation and elongation processes during unloading.

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P3.6

The role of unconventional MVI during myogenic differentiation

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Myosin VI (MVI) is a multifunctional actin-based motor protein present in a number of intracellular compartments including endocytic vesicles, membrane ruffles, the Golgi complex and secretory vesicles. Its divers cellular functions are mediated by interactions with a high variety of binding partners. Our recent data revealed that MVI is highly expressed in striated muscles as well as in myogenic cells, and it is believed to be engaged in muscles and myogenic cells functioning. We have observed altered myogenic program and aberrant myotube formation in primary myoblasts derived from mice not synthesizing MVI (*Snell's waltzer, SV*, natural MVI knockout). The absence of MVI resulted in formation of large and thick myotubes with myosac-like morphology and misaligned (centrally positioned) nuclei. Analysis of fusion efficiency revealed that the percentage of the nuclei in large myotubes (4-10 nuclei or more than 10) was significantly higher in MVI-KO cells compared to control, suggesting that MVI is involved in myonuclear accretion to promote myotube formation. It is well known that fusion of myoblasts is essential for the formation of multinucleated muscle fiber. We have demonstrated alteration in expression pattern of key proteins involved in myoblast membrane fusion in myogenic cells derived from mice lacking MVI compared to control cells. The obtained results provide not only the new information about functions of MVI in myogenic cells but also deepen the knowledge of the mechanisms related to myogenic cell differentiation.

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P3.7

Plantar mechanical stimulation-induced neuromuscular activity attenuates protein synthesis decline in disused rat soleus muscle via maintaining nitric oxide content

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Microgravity and ground-based space analog studies have shown that air pump-based plantar mechanical stimulation (PMS) of cutaneous mechanoreceptors is able to increase postural neuromuscular activity. PMS is known to attenuate unloading-induced skeletal muscle atrophy and impaired muscle function. The aim of the present study was to evaluate the effects of PMS on anabolic signaling in rat soleus muscle following 7-day hindlimb suspension (HS) and to elucidate if the effects of PMS on anabolic pathways would be NO-dependent. Feet were stimulated daily for 4 h with a frequency of 1-s inflation/1-s deflation with a total of 20 min followed by 10 min rest. We observed a decrease in slow-type fiber cross-sectional area (CSA) by 56%, which significantly exceeded a decrease (–22%) in fast-type fiber CSA. PMS prevented a reduction in slow-twitch fiber CSA, but had no effect on fast-twitch fiber CSA. PMS prevented a 63% decrease in protein synthesis as well as changes in several key anabolic markers, such as p70S6k, 4E-BP1, GSK3β, eEF-2, p90RSK. PMS also prevented a decrease in the markers of translational capacity (18S and 28S rRNA, c-myc). Some effects of PMS on anabolic signaling were altered due to NO-synthase inhibitor (L-NAME) administration. Thus, PMS is able to partially prevent atrophy in rat soleus muscle during 7-day HS, affecting slow-type muscle fibers. This effect is mediated by alterations in anabolic signaling pathways and may depend on NO-synthase activity.

P3.8

Lack of myosin VI affects morphology and mTOR pathway in murine hindlimb muscles

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Myosin VI (MVI) is an unique unconventional myosin as it moves towards the minus-end of actin filaments. This property enables MVI to be involved in numerous cellular processes. Our group showed for the first time that MVI is present also in striated muscles, where it localizes to sarcoplasmic reticulum, neuromuscular junction and nucleus as well as Golgi apparatus. In order to understand mechanisms of MVI involvement in skeletal muscles, we examined hindlimb skeletal muscle of *Snell's waltzer (SV)* mice with a knockout of MVI gene.

Preliminary studies showed the increased muscle mass/body mass ratio in soleus and gastrocnemius muscles in adult *SV* mice with respect to wild type animals. Therefore, we examined the skeletal muscle morphology and metabolism. The H&E staining revealed differences in the muscle fiber number and fiber cross sectional area in *SV* mice compared to controls. The biggest changes were visible in *soleus* muscle fibers in 3 and 12 months old mice as well as in newborns (P0). The changes were also observed in the mTORC1 signaling pathway, which is one of the key regulators of cell size and plays a significant role in maintaining skeletal muscle mass.

We also examined the content of muscle myosin isoforms in the fibers of both wild type and MVI-knockout mice. The analysis was performed by means of immunohistochemistry with the use of antibodies against specific MHC isoforms. We noticed changes in the fiber type composition with a clear fast-to-slow transition in 1-year old MVI knockout mice. The question remains whether and how these changes are translated into contractile properties of the examined muscles.

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