# Session 5: Regulation and kinetics of thick-thin filament interaction

## Lectures

## L5.1

### The interaction between thin and thick filament-based regulation in skeletal and cardiac muscle

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It is now established that the regulation of contractility in striated muscle involves a dual-filament mechanism in which calcium-dependent structural changes in the thin filament control the access of the myosin motors to the actin-binding sites, whereas regulatory structural changes in the thick filament modulate the number of motors available for the actomyosin interaction. However, it remains unclear how the calcium dependence and dynamics of the regulatory structural changes in thin and thick filaments determine the calcium dependence and rate of force generation, respectively, in muscle cells under physiological conditions. Here we investigated the steady-state and dynamic calcium-dependence of the *in situ* regulatory structural changes in troponin and myosin in demembranated fibres from rabbit psoas muscle and in cardiac trabeculae from rat hearts, using bifunctional rhodamine probes labelling troponin C and the regulatory light chain of myosin at near-physiological temperature (T=27°C) and lattice spacing. Our results show that in both cardiac and skeletal muscle the calcium-sensitivity and cooperativity of force is largely determined by the calcium-dependence of the activation of the thick filament, rather than by that of the thin filament as classically assumed. Moreover, the sequence of events in the activation of troponin and myosin in response to a stepwise-increase in calcium concentration induced by photolysis of caged-calcium (NP-EGTA) reveal that the calcium-induced structural changes in troponin in skeletal muscle are faster than those in cardiac muscle. However, in both muscle types the rate of force generation is mainly controlled by the speed at which the myosin motors are released from their inhibited folded conformation on the filament surface. These results elucidate the interaction between the thin and thick filament-based signalling pathways which underlie the physiological control of contractility and its adaptation to the different mechanical tasks of skeletal and cardiac muscle.

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## L5.2

### Thick filament regulation in cardiac muscle

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In the cardiac muscle during diastole myosin motors are packed in helical tracks on the surface of the thick filament, folded toward the centre of the sarcomere and unable to bind actin and hydrolyse ATP (OFF state; Woodhead *et al*., 2005, *Nature* **436**: 1195–1199; Stewart *et al*., 2010, *PNAS* **107**: 430–435). The OFF state of both motors and thick filament is marked by characteristic signals in the X-ray diffraction pattern (Reconditi *et al*., 2017, *PNAS* **114**: 3240–3245). In electrically paced trabeculae from rat ventricle, during diastole none of the signals related to the OFF state of the myosin motors are affected by inotropic interventions like increase in sarcomere length from 1.95 to 2.25 μm or addition of $10^{-7}$ M isoprenaline, which increase by twofold the peak force, indicating that the inotropic mechanism acts downstream with respect to $Ca^{2+}$-activation, likely modulating the gain of the mechanosensing-based thick filament activation (Caremani *et al.* 2019, *J Gen Physiol,* **151**: 53–65). The effects on the thick filament structure in diastole of other interventions able to alter the OFF state of thick filament in cardiac demembranated trabeculae in relaxing solution, like lowering temperature below the physiological range (Xu *et al*., 2006, *Biophys J* **91**: 3768–3775) or addition of the cardiac activator omecamtiv mecarbil (Kampourakis *et al*., 2018, *J Physiol* **596**: 31-46), are defined here by collecting the X-ray diffraction pattern from intact trabeculae and small papillary muscles.

# L5.3

## A new view of cardiac thick filament structure: It's all mixed up

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Myosin and myosin-binding protein C (MyBP-C) molecules are exquisitely organized into thick filaments within cardiac muscle sarcomeres, yet these proteins are continually replaced *in vivo*. To test the hypothesis that the replacement of myosin and MyBP-C involves the exchange of individual molecules between thick filaments and the cytosol, adult mice were fed a diet that that marked all newly synthesized proteins with a stable isotope. The abundances of the unlabeled myosin and MyBP-C in the heart prior to labeling, and the newly synthesized proteins, were quantified at by high-resolution mass spectrometry over an 8-week period. The rates of change in the abundances of these proteins were well described by analytical models in which protein synthesis occurred *via* a zero-order process and degradation was stochastic. To test whether the newly synthesized proteins were rapidly mixed with preexisting molecules, myosin was biochemically extracted from the muscle using a gradient of ionic conditions to selectively depolymerize the thick filaments. The ratio of labeled to unlabeled molecules were similar between the cytosolic pool and filamentous fractions, as expected from rapid mixing. These data support a model in which the thick filament structure is a dynamic macromolecular assemblies to allow for the replacement its components, as required for intracellular maintenance.

# Oral Presentations

# O5.1

## The structure and function of the human cardiac actomyosin complex

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We aim to solve the high-resolution structure of the human cardiac actin-myosin complex and correlate our findings with kinetics data of the actomyosin ATPase. Our overarching goal is to compare the behavior of wild-type and mutant sarcomeric proteins on the assembled thin filament, using human cardiac-specific proteins. Furthermore, we will dock small compounds into pockets associated with the thin filament-myosin interface as a means of mitigating effects of mutations. To date, human β-cardiac myosin subfragment 1 (M2β-S1, amino acids 1-843) was expressed with C-terminal Avi and FLAG tags in C2C12 cells. The ATPase activity ($k_{cat}$ = 8.1±1.7 $s^{-1}$ and $K_{ATPase}$ = 58.3±17.7 μM) and in vitro motility ($V_{avg}$ = 2060±132 nm/s) of purified wild-type M2β-S1, containing endogenous murine skeletal muscle light chains, was similar to our previous results. In complementary structural work, F-actin-tropomyosin filaments were saturated with the human cardiac S1 in the presence and absence of ADP and cryogenically preserved. Cryo-EM images of the complexes were collected at the Purdue Cryo-EM Facility, yielding a 3.7 Å resolution reconstruction, with even higher resolution at the actin-myosin interface, thus increasing the level of detail that we had previously achieved. As shown before by us and others, we find that the myosin-head loop 4 penetrates between actin and tropomyosin to interact with charged residues of the tropomyosin coiled coil and thus is likely to modulate tropomyosin position on thin filaments. In addition, the CM loop and HLH motif of myosin make extensive, well-known contacts with the actin surface and the interaction between myosin loop 2 and actin subdomain 1 is partially resolved.

# O5.2

## Tropomyosin and stretch activation of insect flight muscle

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Asynchronous insect flight muscles are activated by periodic stretches at a priming concentration of calcium. Troponin bridges between thick and thin filaments have been observed in relaxed fibres and identified as possible stretch sensors. The bridges are at the position of troponin on thin filaments; they are spaced at 40 nm and are midway between the target sites for force producing crossbridges on actin. There are two isoforms of tropomyosin (Tm1 and Tm2) in the tropomyosin-troponin complex (Tm-Tn) of Lethocerus flight muscle. Tm-Tn and isolated Tm1 bind to thick filaments, but Tm2 does not. The two Tm isoforms are homodimers in the fibres and could have different functions. Both Tm1 and Tm2 bind to actin with micro-molar affinity. Tm1 alone binds to myosin-S1 with nano-molar affinity. Electron micrographs showed a helical arrangement of Tm-Tn binding to isolated thick filaments, with clear projections spaced at ~40 nm. Tm1 binding to thick filaments was observed, but any helical arrangement was less clear. Thick filaments were often crosslinked by Tm-Tn or Tm1. The arrangement of crossbridges in crowns on relaxed thick filaments was seen by atomic force microscopy (AFM) used in tapping mode. Crowns spaced at ~43 nm were ~30 nm above the mica surface and intermediate crowns were ~19 nm above the surface. The higher crowns maybe the ones that interact with Tm-Tn on thin filaments. Troponin bridges in which myosin-S1 is bound to Tm1 could displace Tm from an inhibitory position on thin filaments after a stretch; strain on thick filaments would convert force-producing crossbridges to the ON state. During oscillatory contractions, this process would be rapidly reversible and occur at a constant concentration of calcium.

# O5.3

## Evidence for changes in cross-bridge cycling kinetics at steady-state following active shortening but not active stretching

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The mechanisms responsible for the decrease (FD) and increase (RFE) in force observed at steady-state isometric contractions following active shortening and stretching, respectively, remain unknown (Herzog J, 2017, *Neuro Eng Rehab*). It has been suggested that FD and RFE might be the result of a change in cross-bridge cycling kinetics following active shortening and stretching (Rassier & Herzog, 2004, *J Appl Physiol*). The purpose of this study was to investigate cross-bridge cycling kinetics in muscle fibres following active shortening and stretching, and thus gain insight into the mechanisms responsible for FD and RFE.

Experiments were performed in skinned muscle fibres (n=12) isolated from rabbit psoas muscle. We determined the rate of force redevelopment ($K_{TR}$), stiffness and force at steady-state isometric contractions following active shortening and stretching to sarcomere lengths of 2.4 μm and 3.0 μm, respectively, and compared them to those obtained at steady-state after purely isometric reference contractions at the same final lengths. We then used a two-state cross-bridge model to determine the rates of cross-bridge attachment (f) and detachment (g) (Brenner, 1988, PNAS). Our results showed that FD was accompanied by a decrease in $K_{TR}$, stiffness, f and g, suggesting a change in cross-bridge kinetics at steady-state following active shortening compared to the corresponding purely isometric contraction. Active stretching did not lead to changes in $K_{TR}$, stiffness, f and g, thus confirming previous findings that RFE does not necessarily originate from altered cross-bridge kinetics (Leonard & Herzog, 2010, *Am J Physiol Cell Physiol*).

# Virtual Posters

## P5.1

### Conditional knock-out of cardiac myosin light chain kinase ameliorates hypertrophic cardiomyopathy phenotype in a murine model

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Hypertrophic cardiomyopathy (HCM) is the most commonly inherited cardiac disorder estimated to affect up to 1 in 200 individuals. It is associated with sarcomeric protein mutations in thin and thick filament proteins, including cardiac troponin C (cTnC). Within the thick filament, each myosin heavy chain subunit contains one cardiac regulatory light chain (cRLC). The phosphorylation of the cRLC has been found to increase cardiac muscle force production, myofilament $Ca^{2+}$-sensitivity, and rate of force redevelopment. Our group established a successful HCM knock-in mouse model bearing the cTnC A8V point mutation. The goal of this study was to establish the efficacy of decreased phosphorylation levels of cRLC through the conditional knock-out of cardiac myosin light chain kinase (cMLCK) for reversal of HCM. Echocardiographic, heart weight/tibia length, histopathology, fibrosis quantification, pressure-volume loop, protein quantification, and RNA sequencing measurements were performed before and after cMLCK knock-out in cTnC-A8V mouse KI hearts. This study reports on overall improvement post cMLCK knock-out in the murine HCM hearts. Western Blot analysis demonstrated normalization of $Ca^{2+}$ handling protein expression levels and RNA sequencing demonstrated significant changes in gene profile towards the WT hearts. Our data strongly support cMLCK inhibition and/or reduction in cRLC phosphorylation levels as a unique treatment opportunity for the reversal of the HCM phenotype.

## P5.2

### Low-load shortening at the start of stimulation delays thick filament activation in intact mouse EDL muscle

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The thick filaments of skeletal muscle regulate force development via series of structural changes in myosin motor conformation that precede force development (Linari *et al.*, 2015, *Nature*; Hill *et al.*, 2021, *eLife*). In amphibian muscles, the imposition of rapid low-load shortening (LLS) at the onset of muscle activation delays both force generation and the structural changes in the thick filament associated with its activation, suggesting that thick filaments may be activated by mechano-sensing (Linari *et al.*, 2015, *Nature*). However, this mechanism has not been investigated in mammalian muscles contracting in near-physiological conditions. We, therefore, recorded X-ray diffraction patterns at 5-ms intervals during tetanic contractions of intact mouse EDL muscles at 28ºC, using a Pilatus P2-2M detector at the I22 beamline of the Diamond Light Source (DLS, Didcot UK), in either fixed-end conditions or with 10ms of low-load shortening applied at the start of stimulation. LLS delayed the half-time of force development by about 5 ms, and also delayed the decrease in intensity of the first-order myosin layer line signalling the helical folded conformation of myosin motors characteristic of their OFF state in resting muscle. Similar delays were observed in the increase in the periodicity of the thick filament backbone linked to mechano-sensing, and in the intensities of the equatorial reflections and actin-based layer line that signal motion of motors towards, and binding to, actin. The results indicate that the mechano-sensing mechanism operates in mammalian muscle in physiological conditions, and provide further information about its molecular structural basis.

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# P5.3

## Dual roles of phosphate in cross-bridge kinetics studied in rabbit psoas single myofibrils

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We aimed to study the role of inorganic phosphate (Pi) in the production of oscillatory work and cross-bridge (CB) kinetics of striated muscle. We applied small-amplitude sinusoidal length oscillations to rabbit psoas single myofibrils and muscle fibers, and the resulting force responses were analyzed during maximal $Ca^{2+}$ activation (pCa 4.65) at 15°C. Three exponential processes, A, B and C, were identified from the tension transients, which were studied as functions of Pi concentration [Pi]. In myofibrils we found that process C, corresponding to phase 2 of step analysis during isometric contraction, is almost a perfect single exponential function compared to skinned fibers, which exhibit distributed rate constants, as described previously. The [Pi] dependence of the apparent rate constants $2\pi b$ and $2\pi c$, and that of isometric tension, was studied to characterize the force generation and Pi release steps in the CB cycle, as well as the inhibitory effect of Pi. In contrast to skinned fibers, Pi does not accumulate in the core of myofibrils, allowing sinusoidal analysis to be carried out nearly at [Pi]=0. Process B disappeared as [Pi] approached 0 mM in myofibrils, indicating the significance of the role of Pi rebinding to CBs in the production of oscillatory work (process B). Our results suggest that Pi competitively inhibits ATP binding to CBs, with an inhibitory dissociation constant of approximately 2.6 mM. Finally, we found that the sinusoidal waveform of tension is mostly distorted by second harmonics, and that this distortion is closely correlated with production of oscillatory work, indicating that the mechanism of generating force is intrinsically nonlinear. A nonlinear force generation mechanism suggests that the length-dependent intrinsic rate constant is asymmetric upon stretch and release, and that there may be a rachet mechanism involved in the CB cycle.

# P5.4

## Mutations E41K and N202K in tropomyosin Tpm2.2 impair $Ca^{2+}$-dependent activation of acto-myosin interactions

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In the three state model of the striated muscle thin filament activation, binding of myosin is required for switching the filament to the fully active open state, which is accompanied by shifting tropomyosin (Tpm) chains on actin. The sequence of striated muscle Tpm is divided into seven actin-binding periods. Recent studies suggested that myosin heads directly bind to Tpm within the first halves of actin-binding periods (Doran *et al.*, 2020, *Biophys J* **119**: 821-830), however other studies showed that the second halves are involved (Barua *et al.*, 2012, *PNAS* **109**: 18425-18430). To verify the importance of the second halves, we examined effects of two myopathy-causing mutations in Tpm2.2 isoform on actin regulation of actomyosin interactions. We selected mutations E41K and N202K located in the second half of the first and fifth actin-binding period of Tpm2.2, respectively. We found that in the presence of troponin (Tn) and $Ca^{2+}$ both mutations decreased the ability of Tpm2.2 to activate the actomyosin ATPase activity, but the inhibition in the absence of $Ca^{2+}$ was not affected. The affinity of both Tpm2.2 mutants to actin in the presence of weakly binding myosin heads (+ATP) and Tn ($\pm Ca^{2+}$) was slightly reduced. Under conditions that were unfavorable for Tpm2.2 binding to F-actin, strongly bound myosin S1 was able to induce binding of wild type and both mutant binding to F-actin, but unlike Tpm2.2-E41K, Tpm2.2-N202K required higher filament occupancy with S1 to saturate actin.

# P5.5

## A Z-disc pathogenic variant alters force production in human myocardium

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*ACTN2* gene encodes for the alpha-actinin 2 protein, which has an actin-binding role. In cardiac muscle, alpha-actinin 2 is located in the Z-disc of the sarcomere, where it anchors myofibrillar actin filaments. A 48-years old female patient presented with ventricular fibrillation arrest was successfully resuscitated. Coronary angiography exhibited normal coronary arteries. Cardiac MRI did not show myocardial scar tissue or arrhythmogenic right ventricular dysplasia (ARVD). Echocardiogram showed normal left ventricular ejection fraction, stage II diastolic dysfunction and elevated right ventricular systolic pressure. Genetic testing identified a missense variant of unknown significance in *ACTN2* (a gene previously associated with dilated cardiomyopathy/ARVD), and in *SCNB2* (encodes sodium voltage-gated channel beta subunit 2, a gene previously associated with long QT syndrome). We investigated the effects of the *ACTN2* variant on cardiac muscle contraction, providing an inside view of the importance of *ACTN2* in sarcomere function. Left ventricular free wall samples were obtained from the patient's explanted heart at the time of cardiac transplantation. Mechanics of contraction were measured in permeabilized cardiac muscle preparations (CMPs) in the presence of 3% dextran T-500 (to restore the myofilament lattice to physiological dimensions) at 30°C. The experiments were performed at sarcomere lengths 2.1 μm and 2.3 μm. CMPs containing *ACTN2* mutant protein displayed increased myofilament $Ca^{2+}$ sensitivity of force and decrease maximum steady-state isometric force compared to control CMPs. Sinusoidal stiffness in *ACTN2* mutant protein CMPs presented decreased cross-bridge population at all levels of activation when compared to control CMPs. The rate of tension redevelopment in *ACTN2* mutant protein CMPs was faster at all levels of $Ca^{2+}$ activation and isometric force exhibited a faster $k_{TR}$ at similar force levels when compared to control. Alpha-actinin 2 is an important sarcomeric protein that regulates the kinetics of cardiac muscle contraction. This work establishes the importance of alpha-actinin 2 in the Z-disc mechanotransduction to thin and thick filament interactions.

# P5.6

## Group comparisons of specific force output of permeabilized muscle fibres are more confounded by absolute differences in fibre cross-sectional area than relative differences in cross-sectional area

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Muscle force output is commonly normalized to the cross-sectional area (CSA) of that muscle, and the resulting value is referred to as specific force. Maximal specific force of permeabilized muscle fibres declines as the CSA of muscle fibres increases. This can be confounding when comparing force output of different groups of fibres. The purpose of this study was to use simulated data sets to compare the confounding effects of absolute and relative differences in fibre CSA. Two groups of 25 fibres were simulated and compared. Mean CSAs of group 1 were set to 1000-8000 $\mu m^2$ in 1000 $\mu m^2$ increments with standard deviations (SD) of 20% of mean CSA. Mean CSA of group 2 was set to ±0, ±10, ±20, ±30, and ±40% of the mean CSA of group 1 with SD of 20% of mean CSA. The mean force of fibres at matched CSAs was set to be equal, or 13% lower in group 2 than group 1 across all CSAs. SD of force output was one sixth of the mean force output at each CSA. Specific force was then calculated for each group and compared using 2-tailed t-tests for independent samples. Each scenario was simulated 1000 times. The imposed relative differences in CSA caused greater rates of statistical error when mean CSA was large (8000 $\mu m^2$) compared to when mean CSA was small (1000 $\mu m^2$). These simulations suggest that large absolute differences in CSA are more likely to be confounding than large relative differences in CSA when comparing maximal specific force of groups of permeabilized fibres.