ESRF	**Experiment title:** Structural bases of regulation of skeletal muscle	**Experiment number**: LS-3212
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Report: Introduction. In the sarcomere, the structural unit of the striated (skeletal and cardiac) muscle, force generation and shortening are due to the cyclical interactions of myosin motors extending from the thick filaments and the nearby actin-containing thin filaments. The aim of this project is to investigate the structural bases of the regulation of contraction which involves also interfilament signaling by means of proteins other than the contractile proteins myosin and actin. Previous X-ray diffraction experiments at ID02 [1,2,3] demonstrated that, beyond the classical Ca^{2+}-dependent thin filament activation that makes the actin available for interaction with the myosin motors, a mechano-sensing mechanism in the thick filament recruits myosin motors from their resting (OFF) state as a function of the load. To investigate the details of the molecular mechanism of thick filament regulation, research has focused on two sarcomeric proteins, (i) the giant protein titin and (ii) the myosin binding protein C (MyBP-C). Titin runs from the midpoint of each thick filament to its tip in the A-band and then extends from the thick filament tip to the end of the sarcomere in the I-band. I-band titin acts as a spring in parallel with the myosin motors and is responsible for passive tension in the resting muscle and likely provides the load to prevent development of large inhomogeneity in serially linked half-sarcomeres with different force capability in the contracting muscle [4]. However, due to its large extensibility at the physiological sarcomere length (SL) titin looks inadequate to transmit external stress to the thick filament and account for its mechanosensitivity. MyBP-C is bound with its C-terminal to the backbone of the thick filament in the central one-third of each half-thick filament (C-zone) and may take dynamic interactions with the thin filament with its N-terminal [5]. Both MyBP-C and titin axial repeats allow periodic interactions with the myosin motors in the OFF state [6,7] suggesting a role in the regulation of the OFF-ON transition of the motors during contraction. A detailed description of the dynamics of the interactions between myosin, titin and MyPB-C underlying thick filament regulation is of fundamental interest because mutations in specific sites of these proteins are the principal causes of myopathies. We combine sarcomere-level mechanics and low-angle interference X-ray diffraction in intact fibres of frog muscle to record the structural changes in the filaments and myosin motors accompanying activation of contraction and stepwise change of force on the thick filament. During LS-2721 we have completed the experiments started with LS-2514, in which rapid changes in force were imposed on the resting intact fibre of frog muscle to determine the stress sensitivity of the myosin-based reflections at low [Ca^{2+}]. Then, we have established a new protocol during Ca^{2+}-activation to verify (i) the putative MyBP-C links responsible for the fast-communicating path between thin and thick filament and (ii) the possibility that titin is responsible for the stress-dependent OFF-ON switching of motors. For this it is necessary to record the structural changes in the filaments following a stepwise change in force imposed on the stimulated

fibre without the confounding effects of myosin motors. After testing the effectiveness of ParaNitroBlebbistatin (PNB, [8]) for 100% inhibition of motor activation (LS-2721 and part of LS-2791), we have established (visits LS-2992and LS-3085) that: (i) 20 μM PNB arrest myosin motors in the OFF state during tetanic stimulation; (ii) the imposition of force steps of 0.13-0.25 T_0 (the isometric force during tetanic contraction in control) reveal a 100 times increase in the I-band titin stiffness upon stimulation; (iii) A-band titin under these conditions moves azimuthally myosin motors on the surface of the thick filament toward the lattice planes of the nearby actin filaments in a load dependent manner. This detailed description of the role of the titin as the mechanical link controlling in a load-dependent manner the OFF-ON switching of the myosin motors, published in PNAS in Feb 2023 [9], sets the stage for future studies in demembranated cells from both mammalian models and human biopsies, aimed at defining the genotype-phenotype relation of titin variants in health and disease and developing specific therapeutical strategies. The next step, started with the experimental visit LS-3150, pursued the aim of defining the time course of the mechanical and structural signals marking the state of the thick and thin filaments following the rise of internal [Ca^{2+}] and their relations with the switch in titin stiffness and in myosin motors. During the experimental visit LS-3212 we have improved the statistics to resolve with the adequate S:N ratio the weaker myosin-based "forbidden" reflections (due to the axial pertur-bation of the myosin periodicities along the tick filament produced by the MyBP-C), and actin-based layer lines.

Muscle fibre preparation and protocols. Frogs (Rana esculenta), cooled to 2-4 °C, were killed by a percussive blow to the head followed by destruction of the spinal cord in accordance with EU official regulations on Use of Laboratory Animals, and of the University of Florence Ethical Committee (in compliance with the rules of the Decreto Legislativo of Italian Government 4 marzo 2014, n.26). Small bundles of 2-3 fibres were dissected from tibialis anterior muscles, taking care at minimising the length of the tendon attachment at the two ends. The bundles were then transferred to an experimental chamber containing Ringer's solution and mounted vertically at beamline ID02 between a capacitance force transducer and a loudspeaker-coil motor, carried by micromanipulators for adjustment of the bundle length and position in the X-ray beam path. Two mylar windows were moved as close as possible to the preparation to reduce the X-ray path in water. 2D diffraction patterns were collected in series of 29 time-windows of 2 ms exposure time and up to 200 Hz frequency (at temperature 4 and 14 °C, SL 2.15 μm) with the EIGER2-4M detector (2068x2162 pixels, active area 155x162 mm^2) first in control, during the rise of the isometric tetanus and during relaxation, and then in 20 μM PNB with the same temporal sequence. To mitigate the radiation damage from the high photon flux (10^{13} photons/s) at the ESRF-EBS, the bundle was shifted axially by 200 μm between contractions. Fast shutters are used to limit the exposure to the acquisition time. 4.8 m camera length allowed the spatial resolution adequate to resolve the fine structure of the reflections marking the state of the thick filament, up to the M6. 31 m camera length was used to record the SL.

One or two diffraction patterns per bundle were recorded with 31 m camera length to collect the sarcomeric reflections and check for SL changes and homogeneity during the protocol.

Results. An example of data collected during the LS-3212 visit is shown in the figure, that reports the time course of the intensities of the "forbidden" M4 myosin-based reflection accompanying tetanic stimulation both in control (black symbols) and with 20 μm PNB (gray symbols).

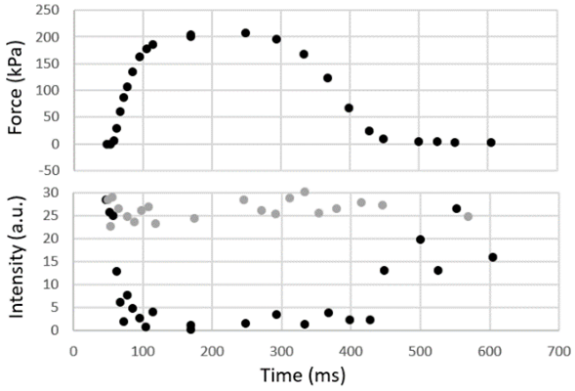

Figure. Upper panel, time course of the tetanic force elicited by a train of electrical stimuli in control solution. Lower panel, time course of the intensity of the M4 "forbidden" reflection in control solution (black symbols) and in presence of 20 μM PNB (gray symbols) that keeps the myosin motors in their OFF conformation and prevents the rise of force. Data from bundle of 3 fibres from tibialis anterior muscle of *Rana esculenta*. Temperature14°C, SL 2.15 μm.

Conclusions. The protocols used will allow full description of the sequence of the structural changes undergone by thin and thick filaments during the whole activation-relaxation cycle.

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