Report:

Muscle contraction is generally considered in terms of the interaction between two proteins, myosin and actin, polymerized in their respective filaments that overlap in the structural unit of muscle called the sarcomere. However the physiological performance of muscle in vivo, and in particular its regulation and response to external mechanical conditions is an integrated response of the whole sarcomere involving proteins other than myosin and actin. In particular, increasing interest focuses on two components of the thick filament- the giant protein **titin**, that runs from the midpoint of each thick filament to its tip, then extends from the thick filament tip in parallel with the thin filaments to the end of the sarcomere, and the **myosin binding protein C** (**MyBP-C**), a thick filament component that can bridge thick and thin filaments in resting muscle. The present experiments were aimed at elucidating the contribution of these two proteins to the recently discovered stress-sensing mechanism that controls the load-dependent recruitment of myosin motors (Linari et al., accepted for publication in Nature), by specific interaction with the contractile proteins and how their action is modulated by other regulatory factors like degree of overlap between the filaments, [Ca^{2+}] and stress in the thick filament. For this time-resolved X-ray diffraction patterns were collected at ID-02 from resting mammalian muscles subjected to stepwise rise in the passive force (isotonic stretch protocol), starting from different sarcomere length (sl).

Muscle preparation and protocol. Mice aged 4–6 weeks were sacrificed by cervical dislocation after inhalation of anaesthetic (isoflurane) 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). The Extensor Digitorum Longus (EDL) muscle was dissected from the hind limb using scissors and forceps under a stereomicroscope and mounted in a temperature controlled trough containing physiological solution. The tendns of the muscle were fixed one to the through and the other to the lever of a motor/force transducer system (300C-LR, Aurora Scientific Inc.) carried by a micromanipulator for adjustment of the muscle length. The trough was sealed with a Perspex cover and mounted vertically at the beamline. Two mylar windows close to the muscle reduced the X-ray path in water. The solution was kept at 30°C and continuously saturated with carbogen (95% O_2, 5% CO_2, pH 7.4). The viability of the muscle was periodically checked by eliciting the isometric contraction with electrical stimulation through two electrodes running parallel to the muscle. The experiment was terminated when the isometric tetanic force (T_0), initially 268±31 kPa (mean±SD), was reduced by more than 15 % below the value at the start of the experiment.

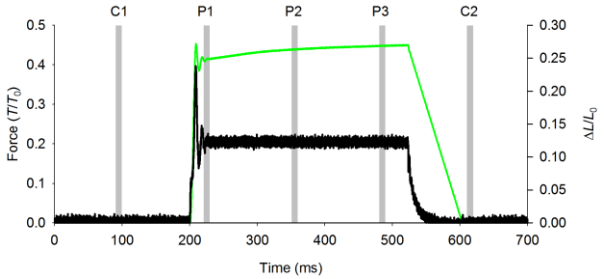

Fig. 1. Change in length ($\Delta L/L_0$, green) in response to a stepwise increase in force of 0.22 T_0 (black) imposed on a resting muscle at sarcomere length 2.4 µm**.** X-ray exposure time-windows (recorded by a pin diode) are shown by the gray bars.

Force steps of ca 0.25 T_0 were imposed on the resting muscle (Fig. 1) at the initial muscle length L_0 (the length at which the maximum isometric force is developed, corresponding to sl 2.4 µm). The response consisted of a simultaneous lengthening to 1. 25L_0 (which, taking into account the tendon contribution, corresponds to sl ~2.9 µm) and a slow further increase by 2-3% L_0 (attained 300 ms after the force step). 10 ms-frames were recorded just before the force step (C1) and at 20, 150 and 280 ms after it (P1, P2 and P3 respectively). At 300 ms after the step the original resting length was recovered with a ramp shortening and a final 10 ms frame was recorded at 410 ms (C2), when force had returned to zero. The protocol was repeated increasing L_0 to have an initial sl of 2.9 µm, at which the static passive force is <0.05 T_0. In this case the lengthening in response to the 0.25 T_0 force step was only 5% L_0, due to the increase in titin contribution to passive stiffness. The relation

between muscle length and sarcomere length, which is influenced by the tendon contribution, was determined by exploiting the beamline capability to perform direct sarcomere length measurements on the X-ray patterns collected with 30m camera length both at rest and during isometric contraction.

Results. In resting muscle the relation between muscle length and sl is linear up to sl 3.0 μm, at which the passive force starts to rise, and deviates downwards at larger lengths, due to the increasing fraction of the imposed lengthening being taken by the tendon. The passive force attains ~0.25 T_0 at sl ~3.5 μm, close to the point of intersection with the descending limb of the active force – length relation. A 0.25 T_0 force step imposed on the resting muscle at the initial length L_0, which is accompanied by an increase in sl to 2.9 μm, induces the following changes in the X-ray signals: (**1**) A decrease in the spacing of the 1,0 equatorial reflection ($d_{1,0}$) from 35 nm at C1 to 32 nm at P1, without any further change at times P2 and P3. The same reduction in $d_{1,0}$ occurs if sl is increased by the same amount under static conditions, and no further significant reduction is produced by a 0.25 T_0 force step applied at sl 2.9 μm. (**2**) A reduction by ~60% (after correction for the change of the muscle mass interrogated by the X-ray beam, monitored by the intensity of 1,0 equatorial refection) of the intensity of the M2, M4 and M5 reflections associated with the triplet perturbations of the myosin heads on the surface of the thick filament. A similar reduction is obtained by static increase in sl by the same amount , and no further significant reduction is produced by the application of 0.25 T_0 force. (**3**) The spacing and the fine structure of the M3 reflection remain constant with a dominant peak at ~14.35 nm that reflects the ordered helical disposition of myosin motor along the axis of the thick filament in the OFF state (Linari et al., accepted for publication in Nature), independently of the increase in both sl and passive force. After correction for the change of the muscle mass interrogated by the X-ray beam and for the increase in the radial width of the reflection, the intensity of M3 (I_{M3}) is reduced by 40% following the 0.25T_0 force step (and the consequent sl increase to 2.9 μm), while it is reduced only by 22% at the static 2.9 μm sl. This reduction with the static increase in sl is not observed in frog muscle (Reconditi et al., 2014 *J Physiol* **592**:119-1137). Intensity changes similar to those of M3 are shown also by the 1st myosin layer line (ML1), which originates from the helical symmetry of myosin heads on the thick filament. (**4**) The spacing of the M6 reflection (S_{M6}), a parameter that measures the extension of the thick filament, increases by 0.4% following the 0.25T_0 force step (and the accompanying sl increase), while, when the same 2.9 μm sl is attained in static conditions, S_{M6} increases only by 0.15%. The 0.25T_0 force step imposed at this length produces a further increase in S_{M6} to a value similar to that attained when the same force step is imposed at the initial sl 2.4 μm.

Conclusions. These results show that increase in sarcomere length per se induces (*i*) reduction in the filament lattice as expected from the constant volume constraint; (*ii*) reduction in the intensity of forbidden reflections as expected from the reduction in the overlap between the MyBP-C region of thick filament and the thin filament (Reconditi et al., 2014), in support of the idea that the axial perturbations of the myosin heads on the surface of the thick filament is due to resting links between the MyBP-C and the actin filament. These effects of lengthening appear independent of the actual force on the thick filament, indicating that MyBP-C links do not participate in the stress-sensing mechanism. Instead, independent of sarcomere length, force has a specific effect on the spacing of the M6 reflection (and thus the extension of the thick filament) and on both the intensity, but not on the fine structure, of the M3 reflection and the intensity of the ML1. These effects can be explained if a 0.25 T_0 increase in passive force, which causes 0.4% extension of the thick filament, triggers a stress-sensing mechanism that rapidly releases a significant fraction (~20%) of myosin motors from their ordered helical disposition on the thick filament toward a disordered conformation.