OTKA PD116558 FINAL REPORT

The aim of this project was to reveal the molecular details of titin's folding from a nanomechanical point of view. Particularly to demonstrate that the molten globule state of its globular domains is part of titin's folding trajectory. To identify such hypothesized contracted intermediate of the folding pathway, I have planned to manipulate individual titin domains with force-clamp optical tweezers. My experimental strategy was to crosslink individual recombinant immunoglobulin and fibronectin type domains from titin with a 2.5 kbp long double stranded DNA handle for efficient nanomechanical manipulation.

The project started with cloning the I28, I27 and I55-62 immunoglobulin type domains from human titin soleus cDNA template. The inserted gene sequence to the expression plasmid contained a C-terminal cysteine codon insertion. As a result, the recombinant proteins expressed in *E. coli,* contained an N-terminal His-tag and a C-terminal cysteine modification. The domain was purified using Ni-affinity chromatography under native conditions (Fig 1.a,b,c.). To create a molecular handle, I have synthesized a 2.5 kbp long DNA in polymerase chain reaction using lambda phage DNA as template. The oligonucleotides used contained 5' biotin and 5' thiolgroup modifications. As a result, the product DNA contained an SH group and a biotin at its termini (Fig. 1. d.). Conjugation of DNA handle to the I28 domain was carried out with the thiol specific irreversible cross-linker bismaleimidohexane (BMH). The reaction was done according to the manufacturers protocol. The constructs after the conjugation reaction were visualized with atomic force microscope, to demonstrate that the 2.5 kbp DNA handles contain a small globular structure at one of their termini. The yield of the reaction was very low, we could not identify the cross-linked constructs (Fig. 1. e.).

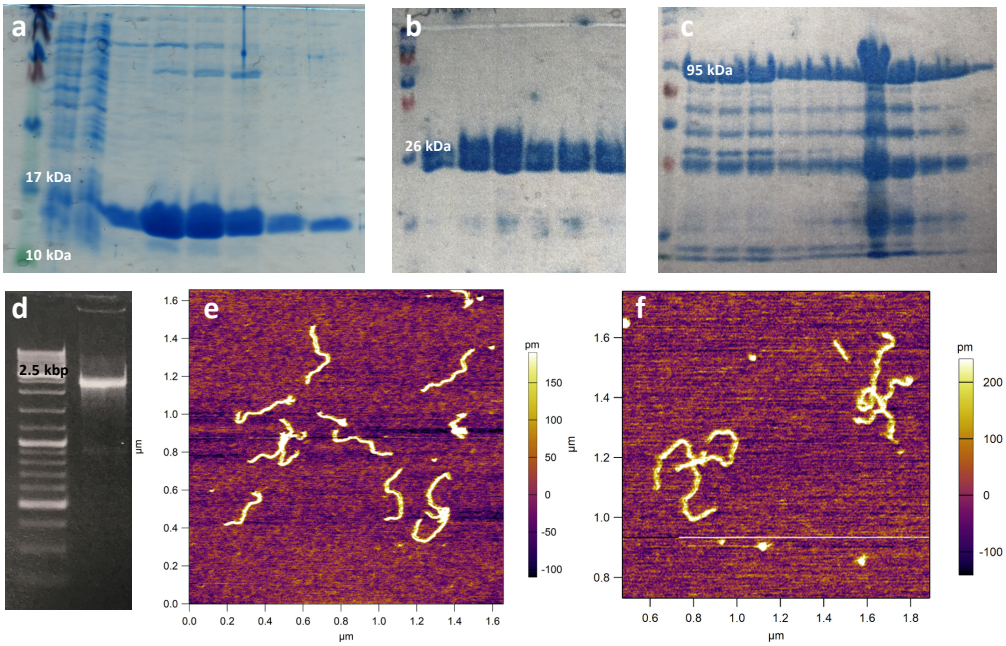

Figure 1. Production of recombinant DNA-protein constructs. **a.** Expression of I28 domain. **b.** Expression of I27-I28 domain dimers. **c.** Expression of I55-62 domain octamers. Gel shows the purification steps during Ni-affinity chromatography. From left to right after flow through, the imidazole concentration was increased stepwise from 5mM up to 250 mM. **d.** Purified PCR product of the SH and biotin modified dsDNA handle. **e.** AFM topography after I28-dsDNA cross linking reaction with BMH for 2 hours. Constructs do not show globular structures at their terminus. **f.** AFM topography after I28-dsDNA overnight cross-linking reaction with BMH. Constructs of cross-linked DNA strands appear, probably because the buried cysteines of the globular domain were activated by the BMH.

The incubation time for crosslinking proteins with DNA has been increased up to overnight (in contrast, the recommended incubation of the manufacturer was 1-2 hours) and as a result several DNA molecules were attached to one I28 domain, possibly because the domain contains extra cysteine residues buried inside its beta-barrel structure, that have become accessible to BMH during the long incubation (Fig. 1. f.). Regardless of incubation or molar ratio parameter we could never achieve the correct conformation of the construct.

To overcome the difficulties in cross-linking the DNA with the recombinant domain, we have decided to manipulate full length titin molecules with optical tweezers. In these experiments we were hoping to demonstrate the molten globule intermediate during the folding of the protein chain. Native titin molecules were isolated from rabbit back muscle (*m. longissimus dorsi*) according to routinely used protocols established by our lab (Fig 2 a,b). The isolated native titin molecules were manipulated with optical tweezers. Briefly, the molecule was captured between two microbeads, one recognizing the C-terminus I1-I2 domains of titin through antibodies, the other was a specifically attached to the N-terminal part of the molecule via a photochemical cross-linker. One of the beads was captured with the laser trap and the other was moved by a piezo controlled micropipette in force-clamp and in constant pipette position experiments (Fig. 2c)

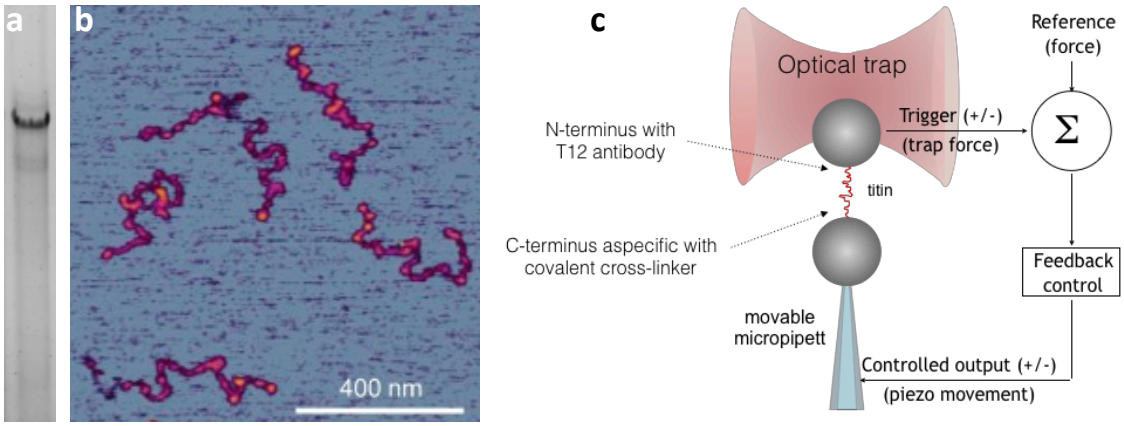

Figure 2. Isolation and manipulation individual titin molecules with force-clamp optical tweezers. **a.** 1% agarose gel profile of isolated titin. **b.** AFM image of isolated individual titin molecules. **c.** Experimental layout of titin manipulation and feedback control.

In these experiments titin was stretched with constant velocity (250 nm/s) to reach a force above 100 pN to trigger domain unfolding. After this initial stretch phase, force was instantaneously quenched to 0 pN by the rapid movement of the micropipette (50 μm/s) and held at a constant position for 20–40 s. During the constant-pipette-position phase force was measured on the trapped bead with a sampling rate of 5 kHz. Force gradually increased on the time scale of a few seconds, indicating that the trapped bead was pulled in by the folding titin molecule. Forces up to 2 pN was generated in this experiment (Fig. 3) at the expense of a mere 10 nm contraction. Fluctuations of force with peak-to-peak amplitude exceeding 0.5 pN, and easily discernible from thermal fluctuations, could be identified during this process (Fig. 3a inset), suggesting that titin domains fluctuate between contracted and extended conformational states. The force-generation process could be followed in force versus extension graphs as well (Fig. 3b). In titin which completely refolded during the waiting period of the experiment, the force generation process transferred the molecule from a long-contour-length state to a short-contour-length one (Fig. 3b inset). We analyzed the kinetics of force generation as a function of initial force by fitting the force versus time traces with a single-exponential function. Force generation was observed in the initial force range of 0–8 pN, and its rate decreased with the initial force (Fig. 3c). The calculated force generation rate at an apparent zero force is 1.5 s^{-1}.

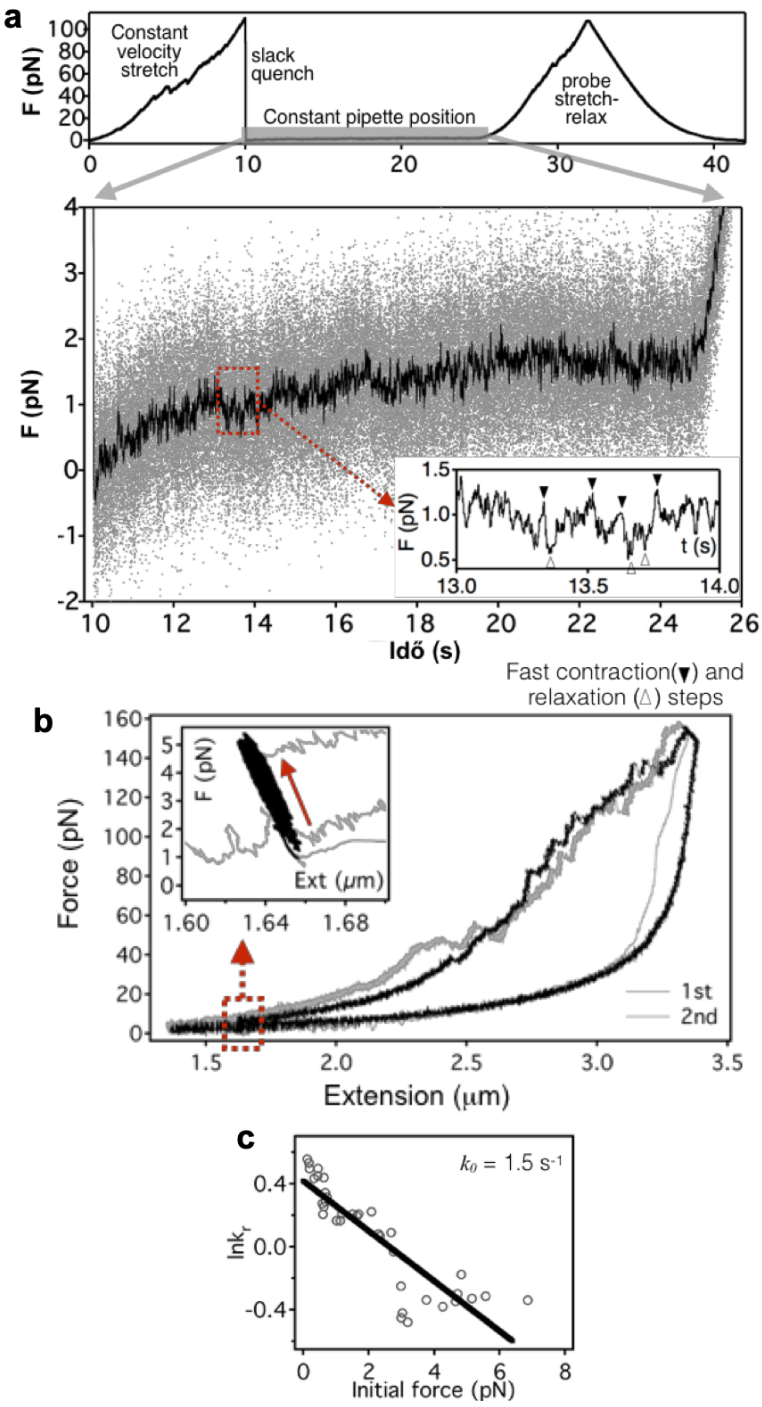

Figure 3. Force generation by titin folding. **a.** Position clamp experiment. Upper trace, Force versus time plot displaying the experimental protocol. Following a stretch period at constant velocity, force is rapidly quenched by quickly moving the pipette back to its initial position. Then the pipette is held in a constant position while the force on the trapped bead is monitored. The protocol ends with a complete stretch-relaxation cycle to monitor refolding. Lower trace, Force versus time trace recorded during the position-clamp phase. Grey and black traces correspond to raw and 100-point median-filtered data, respectively. Inset, Enlarged view of the framed area showing force fluctuations consisting of contraction (filled arrowheads) and relaxation steps (empty arrow- heads) with sub-pN amplitude. **b.** Force versus extension curves reconstituted from a position clamp experiment. Gray and black traces correspond to the first and second nanomechanical cycles, respect-tively. In between the two cycles the pipette position was clamped so that the initial force was 1.2 pN. Inset shows the gradual increase in force (~4 pN, red arrow) during the 25 s waiting period in between the mechanical cycles. **c**. Rate of force generation (shown as ln k_r) as a function of initial force during the waiting time in position clamp experiments. Data obtained on 38 molecules are shown. Force generation rates were obtained from single- exponential fits to the force versus time data. The rate data were locally averaged with a smoothing window (width 20 points). Black line is linear fit to the ln k_r versus force data. From the y-intercept a zero-force rate of 1.5 s^{-1} was calculated.

The amplitude of force generation showed a weak positive correlation with the initial number of unfolded domains. Our findings thus suggest that the magnitude of the force-generation process scales with the number of domains involved and the process can be inhibited by raising the force titin is exposed to. During force generation the overall length of the molecule barely shortens (~10 nm) which suggests that the force generating step is not between the unfolded and native but rather a compact, intermediate (molten globule) and the native structure. We have shown that the force generation can be alleviated by urea, that favors the molten globule state by abolishing the H-bonding required for the consolidation of the structure into the native state.

Our findings support the three-state model of titin folding represented with free-energy minima, corresponding to the folded, molten-globule and unfolded states, in the conformational space. A schematic section of this space, demarcated by the force vector that defines the reaction coordinate, is shown in Figure 4. Conformational equilibrium is determined by the relative free energy levels of the states, and the rate of transition (k) between the states by the height of the barriers separating them. Mechanical force acting along the length (reaction) coordinate tilts the energy landscape, thereby altering the transition rates and the conformational equilibrium. At high forces, transition toward the unfolded state is strongly favored so that the molten-globule state stays unpopulated and is passed unnoticed. By contrast, during refolding, at low (<10 pN) forces the energy landscape becomes less tilted, and even an equilibrium between the molten-globule and unfolded states may occur. In this equilibrium titin domains dynamically repopulate the contracted molten-globule and extended unfolded states, which results in large fluctuations of molecular extension and hence fluctuations of mechanical force measured at the ends of the molecule.

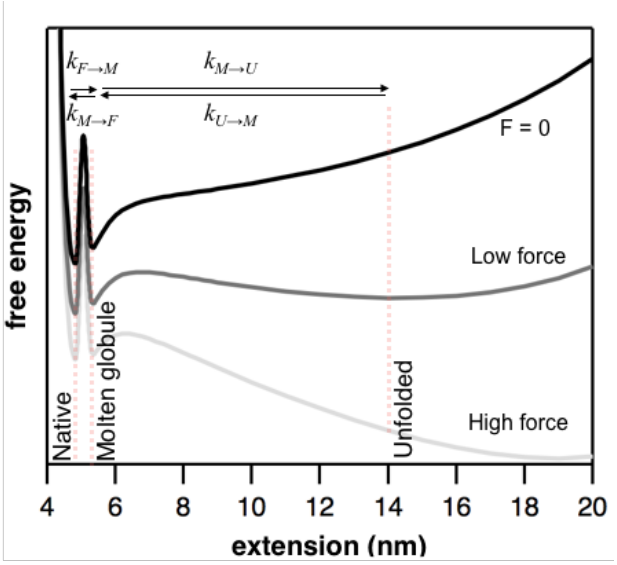

Figure 4. Model and simulation of molten-globule dynamics during force-dependent titin refolding. a. Schematics of the titin folding landscape based on the three-state model. F, M and U correspond to the folded, molten-globule and unfolded states, respectively. Arrows indicate reactions between the states with the force-dependent rate k, the subscript of which refers to the direction of the transition (F→M, M→U, M→F and U→M for folded-to-molten globule, molten globule-to-unfolded, molten globule-to-folded and unfolded-to-molten globule, respectively). The length of the arrows, as shown in the figure, do not correspond to the actual rates. The landscape is tilted as force increases, and at low forces the free energies of the molten-globule and unfolded states become similar.

Although in our work we have used native full-length titin filaments instead of individual globular domains, we could successfully answer the original scientific question of the project – Is the molten globule intermediate part of titin's folding process? Experiments with the native titin is a better model of the sarcomeric structure and behavior of the molecule than its isolated recombinant domains. In our model the molten globule state has a collapsed, compact structure that is indistinguishable by length from the native state in case of single domains, thus the collective mechanical ensemble of titin domains in the native polypeptide was crucial to demonstrate that the molecule explores the molten globule state during folding. This transition would be undetectable by using single domains, since the length separation of the native and molten globule structure for one domain is below the resolution limit (sub-nm) of the method.

Monte–Carlo simulations incorporating a compact molten- globule intermediate in the folding landscape recovered all features of our magnomechanics results (Fig. 5). The ensemble molten-globule dynamics delivers significant added contractility that may assist sarcomere mechanics, and it may reduce the dissipative energy loss associated with titin unfolding/ refolding during muscle contraction/relaxation cycles. As shown on (Fig. 5b), a shift of the domain population from the unfolded state to molten globule provides added contractility so that titin shortens to a length nearly indistinguishable from that of the folded structure. Accordingly, the measured length of a titin section may not fully reveal its structural status.

Even if the length of a canonical structured titin segment appears to reflect a folded state, the component domains may have been unfolded and then collapsed into the compact molten globule state. Thus, our results strongly favor the idea that folding/unfolding dynamics of titin, via the molten-globule state, are present in situ in the sarcomere.

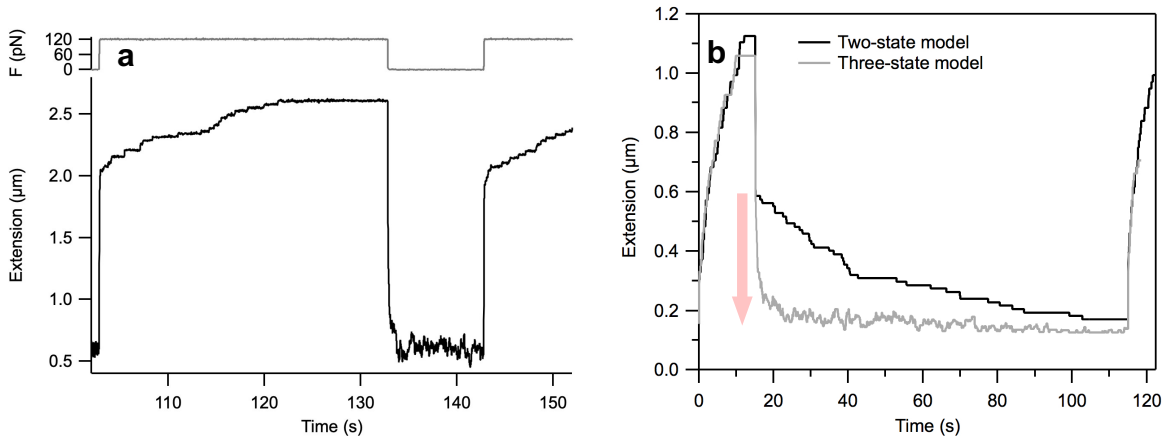

Figure 5. Force clamp nanomechanics of native titin. **a.** Force and extension versus time trace of a single titin molecule recorded in a force-clamp experiment. During the high-force phase force was clamped at 120 pN, whereas during the low-force phase it was 1 pN. Extension steps corresponding to stepwise domain-unfolding at high clamp force. Inset ii, Fluctuation of the molecular extension measured at low clamp force. **b.** Monte–Carlo simulation of a force-clamp experiment based on the classical two-state (folded and unfolded) and the three-state (folded, molten-globule and unfolded) models, shown in black and gray traces, respectively. The time-dependent protocol, similarly to that employed in the optical tweezers experiments, is partitioned into an initial high-force clamp (120 pN), followed by a low-force-clamp refolding phase (3.1 pN) and finally by high- force-clamp monitor phase (120 pN). The simulation with the three-state folding model fully recovered the experimental data. Red arrow indicates the contraction gain in excess of the entropic collapse.

The findings reported in this project was accepted with positive feedback by the scientific communities. It was published in the special issue on molecular machines in Protein Science. This work has been selected twice for oral presentation, at the Hungarian Biophysical Society's XXVI. Congress in Szeged, Hungary and at the 46th European Muscle Conference in Potsdam, Germany. Also, I have won the poster prize with this work at the 47th Membrane Transport Conference in Sümeg, Hungary. In this project I have developed a collaborative connection with prof. Julio Fernandez at Columbia University, where I was investigating the fast folding contraction of native titin molecules using magnetic tweezers. I have spent 80 days at Columbia University, New York in the lab of prof. Fernandez, where I have studied novel molecular cross-linking methods for single molecule experiments, together with the methodology of pulling proteins with magnetic tweezers.