Supplementary Information

A HaloTag-TEV genetic cassette for mechanical phenotyping of proteins from tissues

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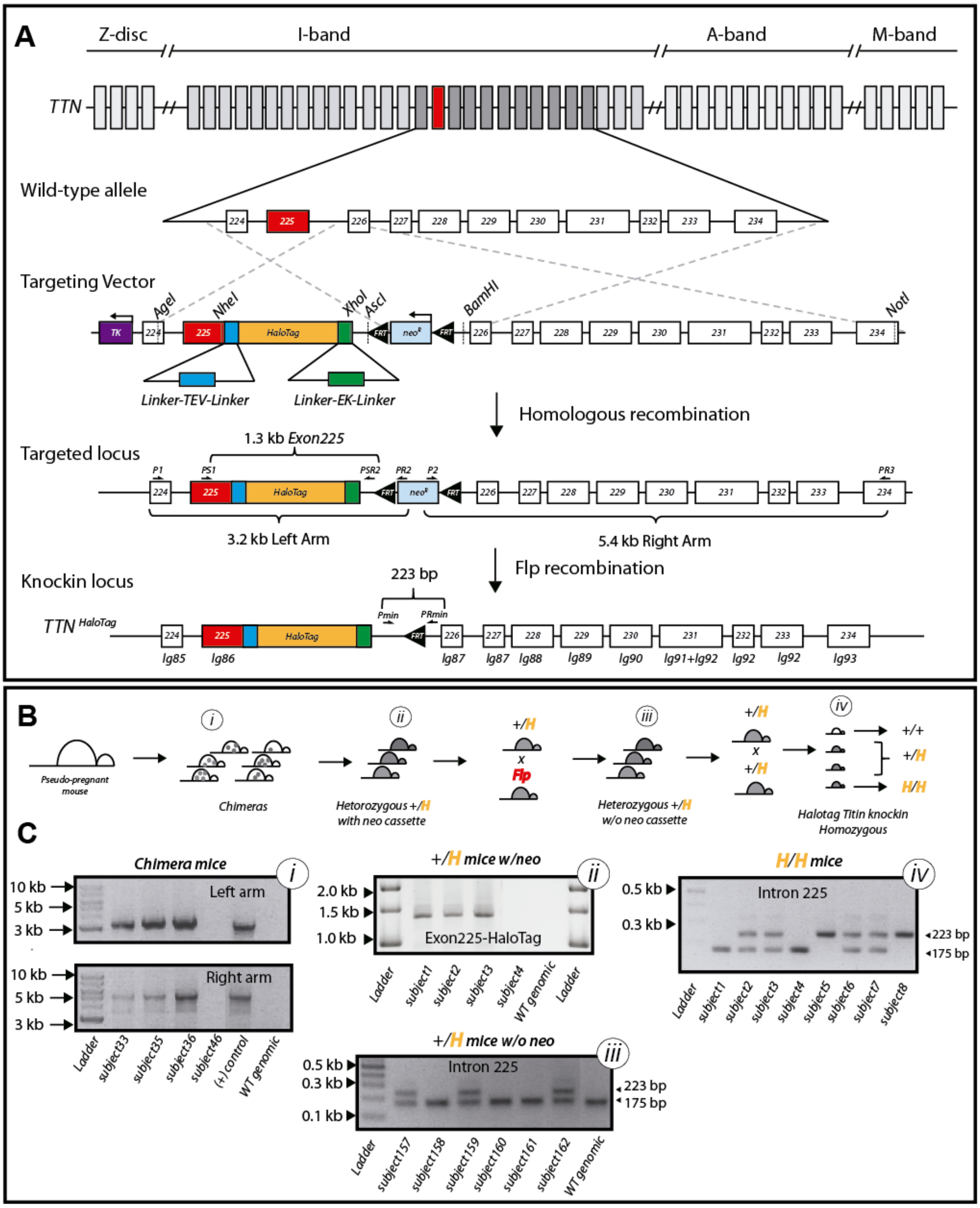
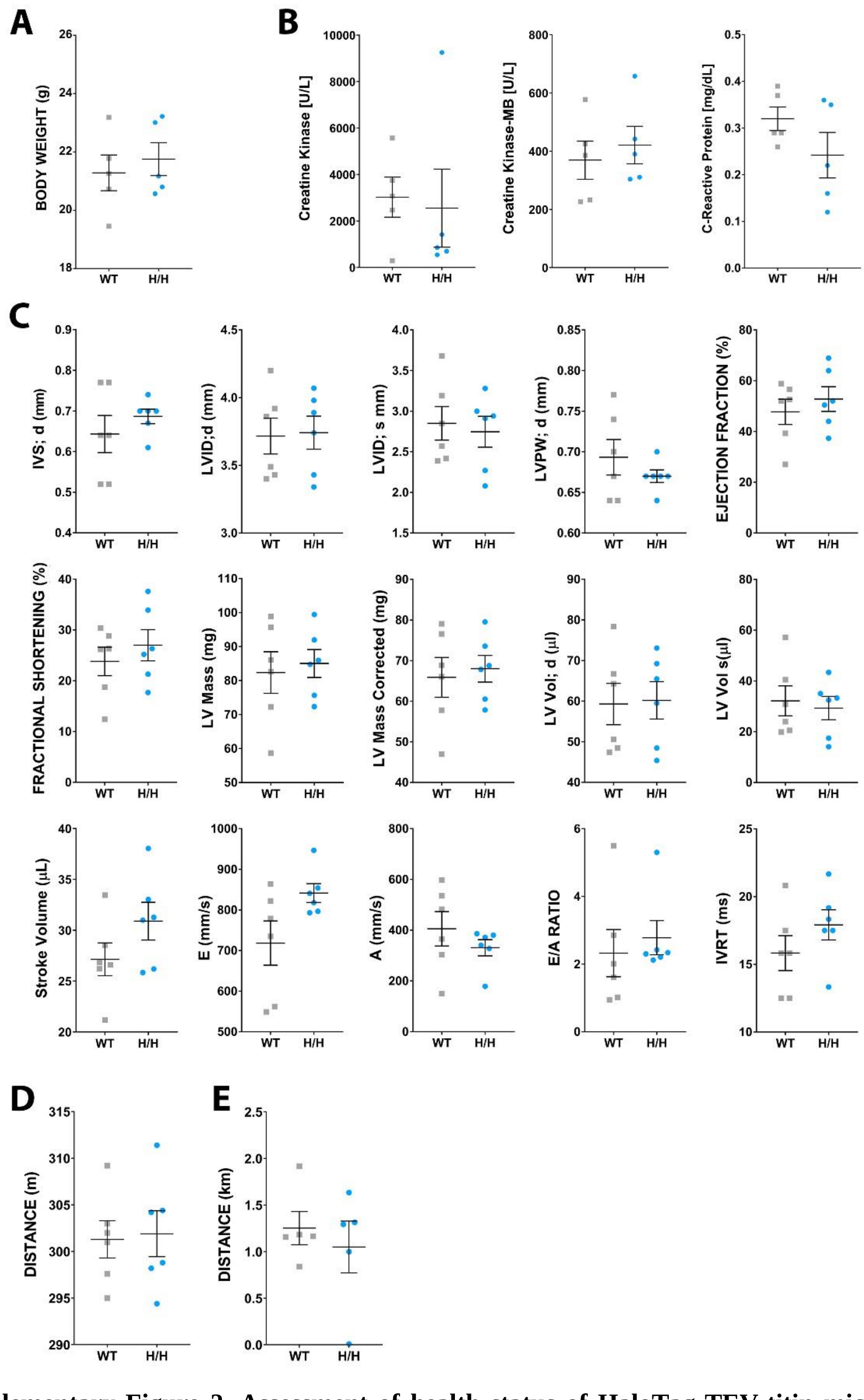
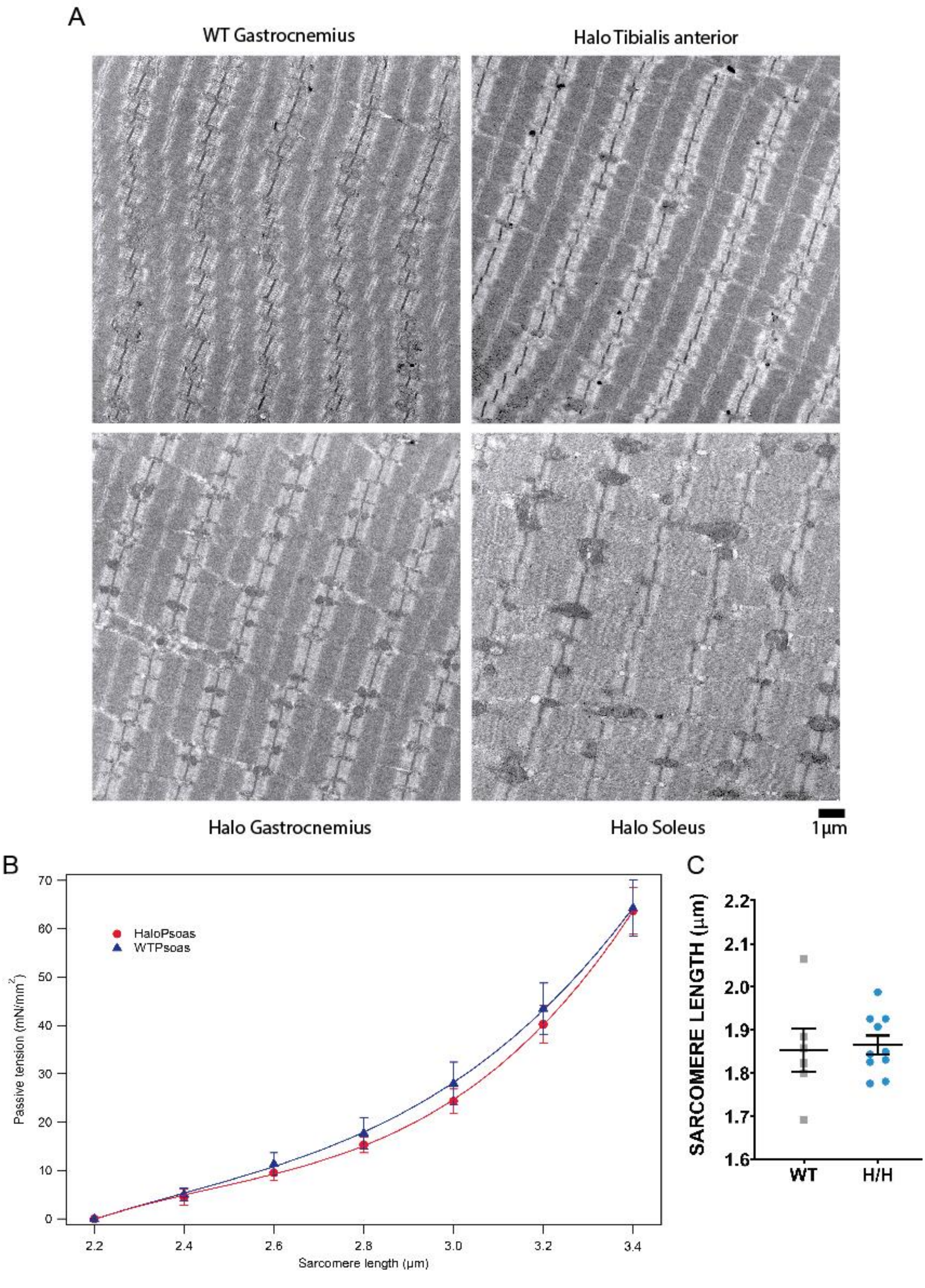


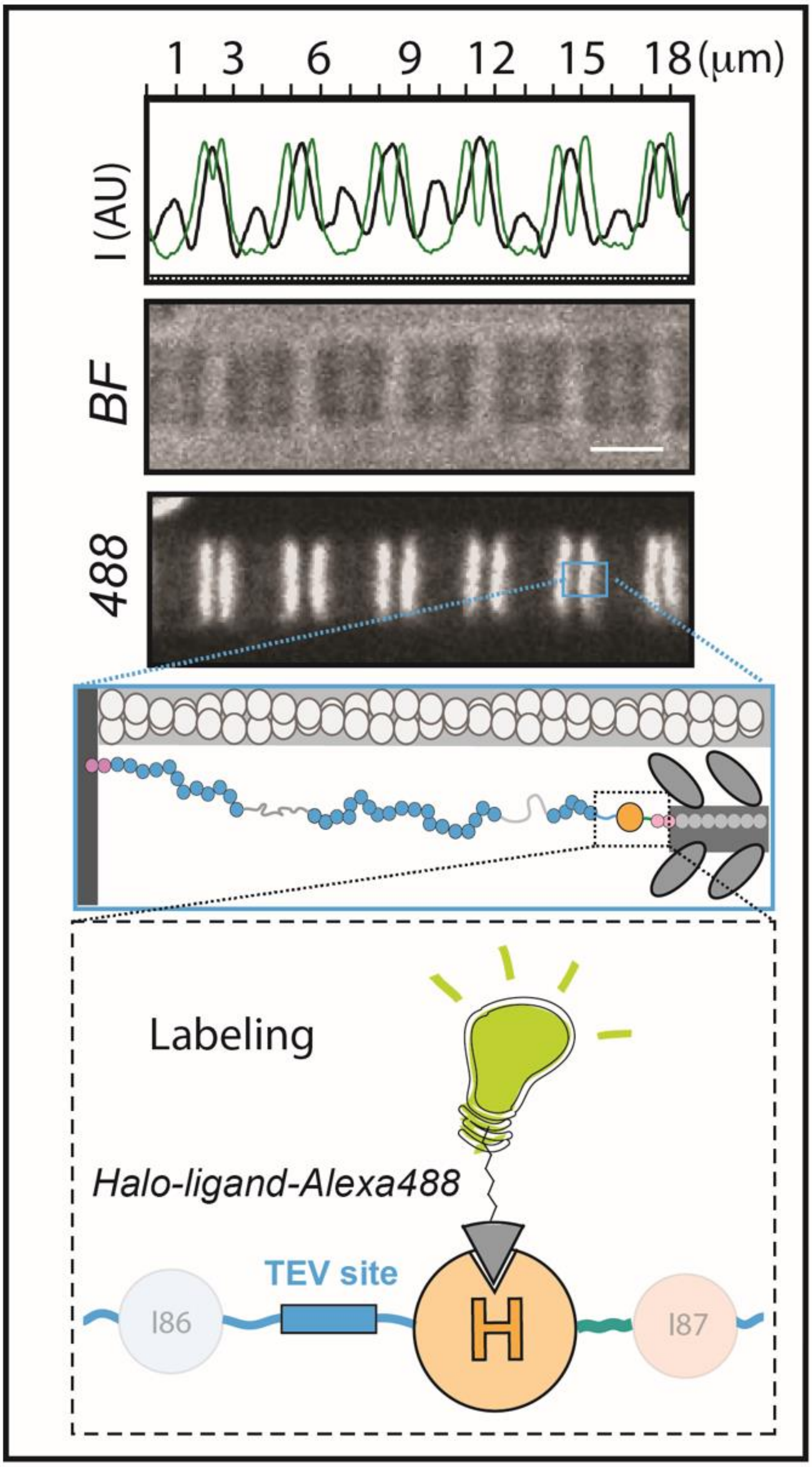
Supplementary Figure 1. Generation of knock-in mice with the HaloTag-TEV cassette inserted in the titin gene. (**A**) The targeting vector contains the *TTN* sequence between exons 224 and 234. The HaloTag gene is inserted downstream of exon 225, flanked by linkers including TEV and EK sites. A Neo resistance gene flanked by FRT elements was inserted in intron 225. Restriction sites and primers used during the different genetic engineering steps are shown, together with the size in base pairs (bp) of relevant fragments. (**B**) Strategy followed to get homozygous knock-in mice (H/H) from recombinant ES cells. The different experimental stages are labeled i-iv, see below. (**C**) We used PCR amplification of genomic DNA to confirm (i) the presence of the HaloTag in the TTN gene in chimera mice (primers *P1* and *PR2, left arm;* primers *P2* and *PR3*, *right arm*), (ii and iii) the presence and subsequent removal of the neo resistance by crossing with Flp mice (primers *PS1* and *PSR2*, Exon225-HaloTag*; Pmin* and *PRmin*, Intron225), and (iv) the generation of the homozygous H/H titin mouse (*Pmin* and *PRmin*). We used the vector construct and wild-type genomic DNA as controls. Sequences of primers are provided in Supplementary Table 1.



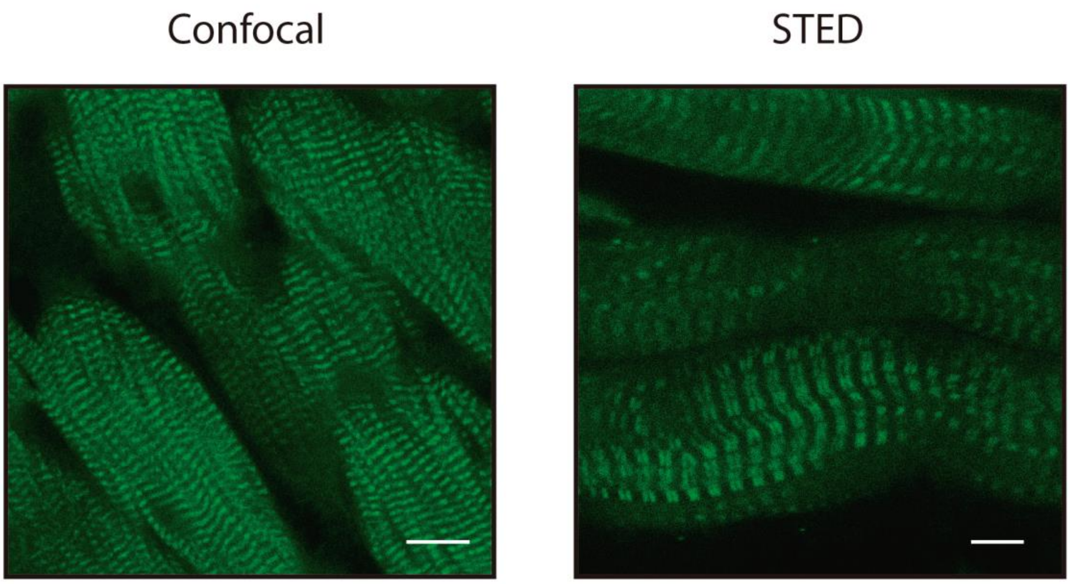
Supplementary Figure 2. Assessment of health status of HaloTag-TEV-titin mice. (A) Body weight of wild-type (WT) and homozygous (H/H) HaloTag-TEV-titin mice. **(B)** Serum levels of creatine kinase (marker of striated muscle damage), creatine kinase-MB (more sensitive to myocardial damage) and C-reactive protein (inflammation marker). **(C)** Cardiac function assessed by echocardiography. “s” and “d” indicate parameters obtained in systole and diastole, respectively. IVS, inter ventricular septum. LVID, left ventricular internal diameter. LVPW, left ventricular posterior wall thickness. Stroke volume, difference between endsystolic and end-diastolic volumes. E, A, early and late diastolic peak velocity waves, respectively. IVRT, isovolumic relaxation time. **(D)** Distance run by the mice during the six last training sessions. Each data point is the average value for all mice of the same genotype for a given training session. **(E)** Distance run by mice in the endurance session. In all plots, error bars represent SEM. No statistically significant difference between the groups was found for any parameter (unpaired t-test). Source data are provided as a Source Data file, including results from statistical significance tests. Results in (A), (B), (D) and (E) were obtained with five 13-15-week-old female mice per group. Results in (C) were obtained with six 10-week-old male mice per group.



Supplementary Figure 3. Ultrastructural and mechanical characterization of HaloTag-TEV-titin muscle fibers. (A) The ultrastructure of HaloTag-TEV-titin muscles is equivalent to wild-type (WT). Representative images of n= 20 (WT Gastrocnemius), n=40 (Halo Gastrocnemius), n=15 (Halo Tibialis anterior), and n=25 (Soleus Halo) images. **(B)** Passive tension generated by WT and HaloTag-TEV-titin bundles of fibers isolated from psoas muscle (n = 6 for both genotypes; error bars represent SEM and the solid lines are polynomial fits). Source data are provided as a Source Data file. **(C)** Resting sarcomere length. n = 6 cells (WT); n = 10 cells (HaloTag-TEV-titin homozygous mice, H/H). Error bars represent SEM.



Supplementary Figure 4. Spinning disk microscopy. Under bright field illumination, the A- and I-band sections are easily distinguished along the myofibril as dark and clear regions, respectively (middle panel, BF). The fluorescent signal coming from Alexa488-labeled HaloTag-TEV titin appears as doublets at the I/A band interface (bottom panel, 488). The top panel shows intensity profiles (Alexa488 fluorescence in green and bright-field intensity in black). *Insets:* Cartoons showing the location of the HaloTag-labeled titin. Scale bar, 2.5 μm (also valid for Alexa488 channel). Equivalent results were obtained in a replicate.



Supplementary Figure 5. The HaloTag-TEV cassette is correctly inserted in cardiac titin. The heart of a homozygous HaloTag-TEV titin mouse was incubated with HaloTag Oregon Green ligand, fixed and clarified. Although these samples show higher autofluorescence than skeletal preparations (**Figure 1B**), there is strong labeling in bands as expected from the location of the HaloTag insertion in titin (**Figure 1A**). Staining in doublets can also be observed, although to a lesser extent than in skeletal muscle, probably reflecting shorter I-bands in cardiac sarcomeres (n = 1 experiment; > 5 fields of view showed equivalent results). Scale bars: 10 μm (Confocal) and 5 μm (STED).

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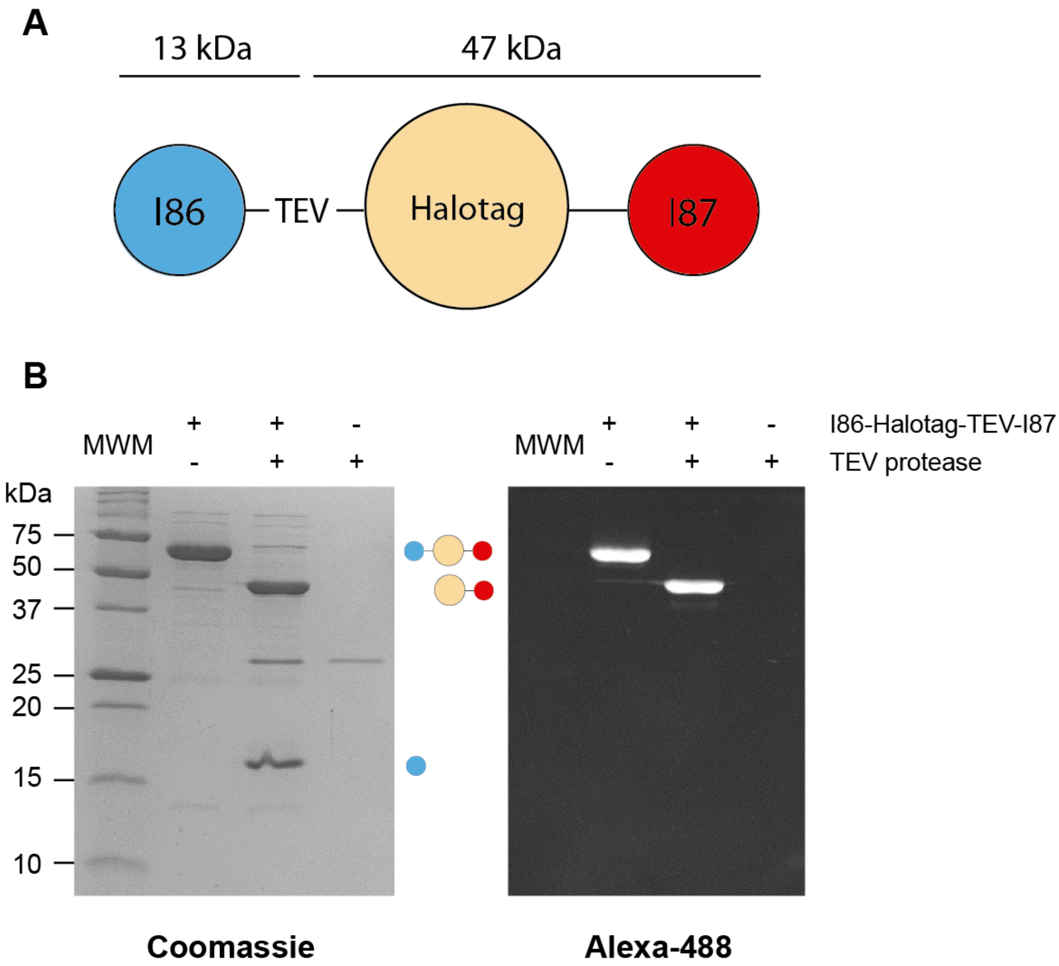
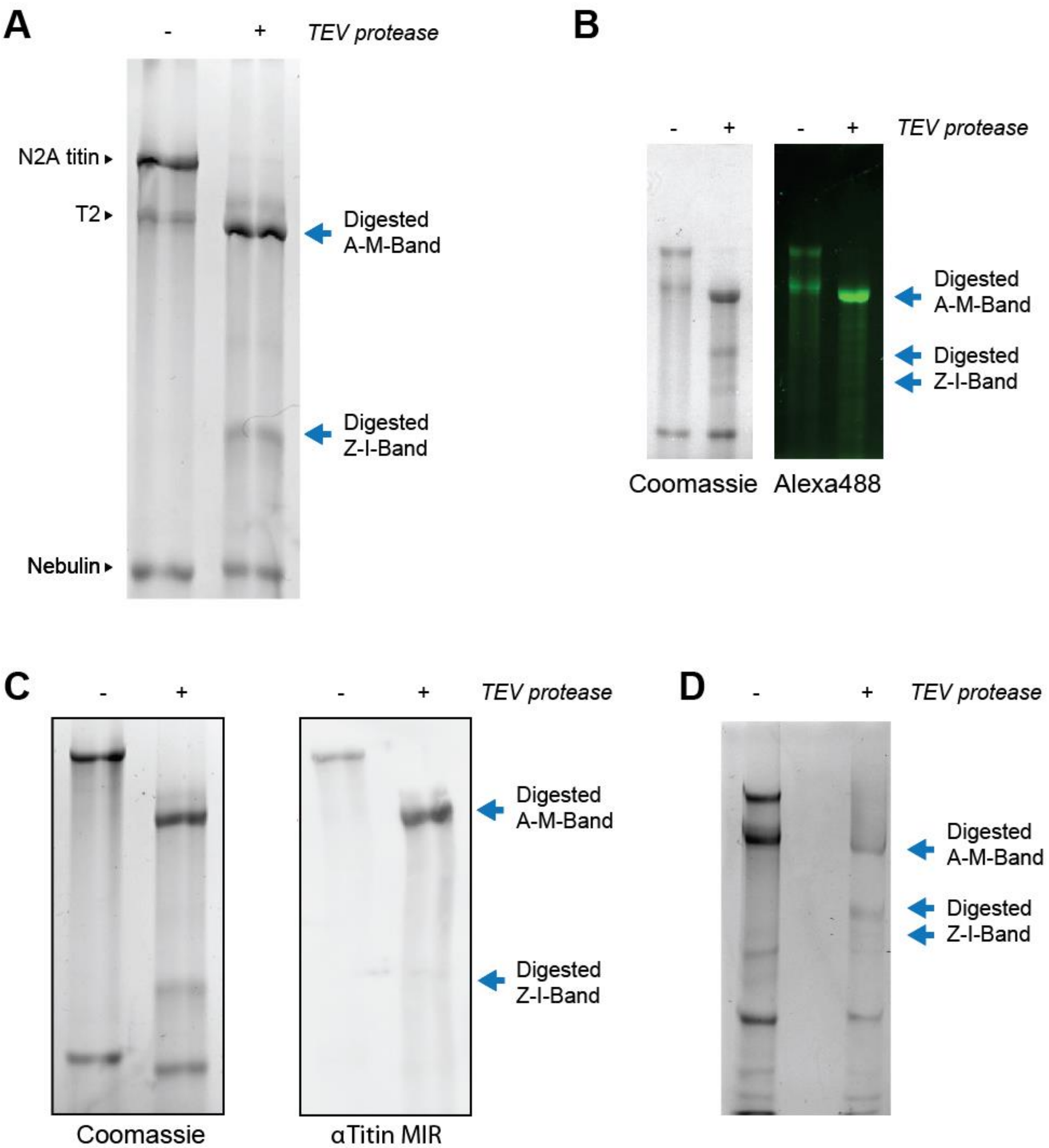
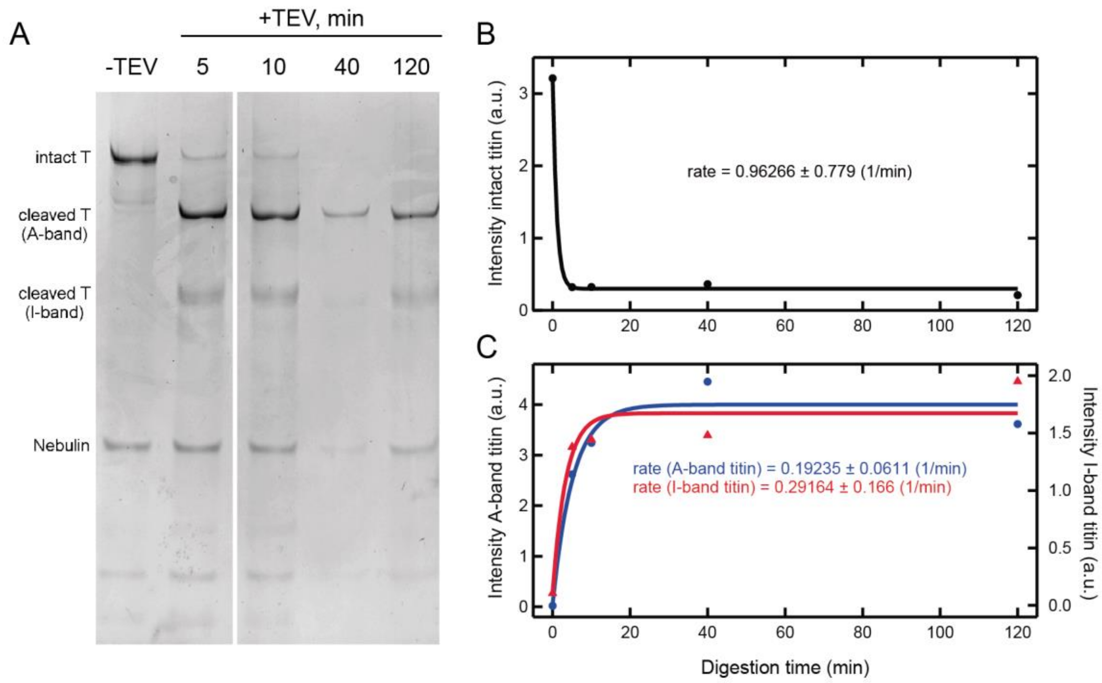


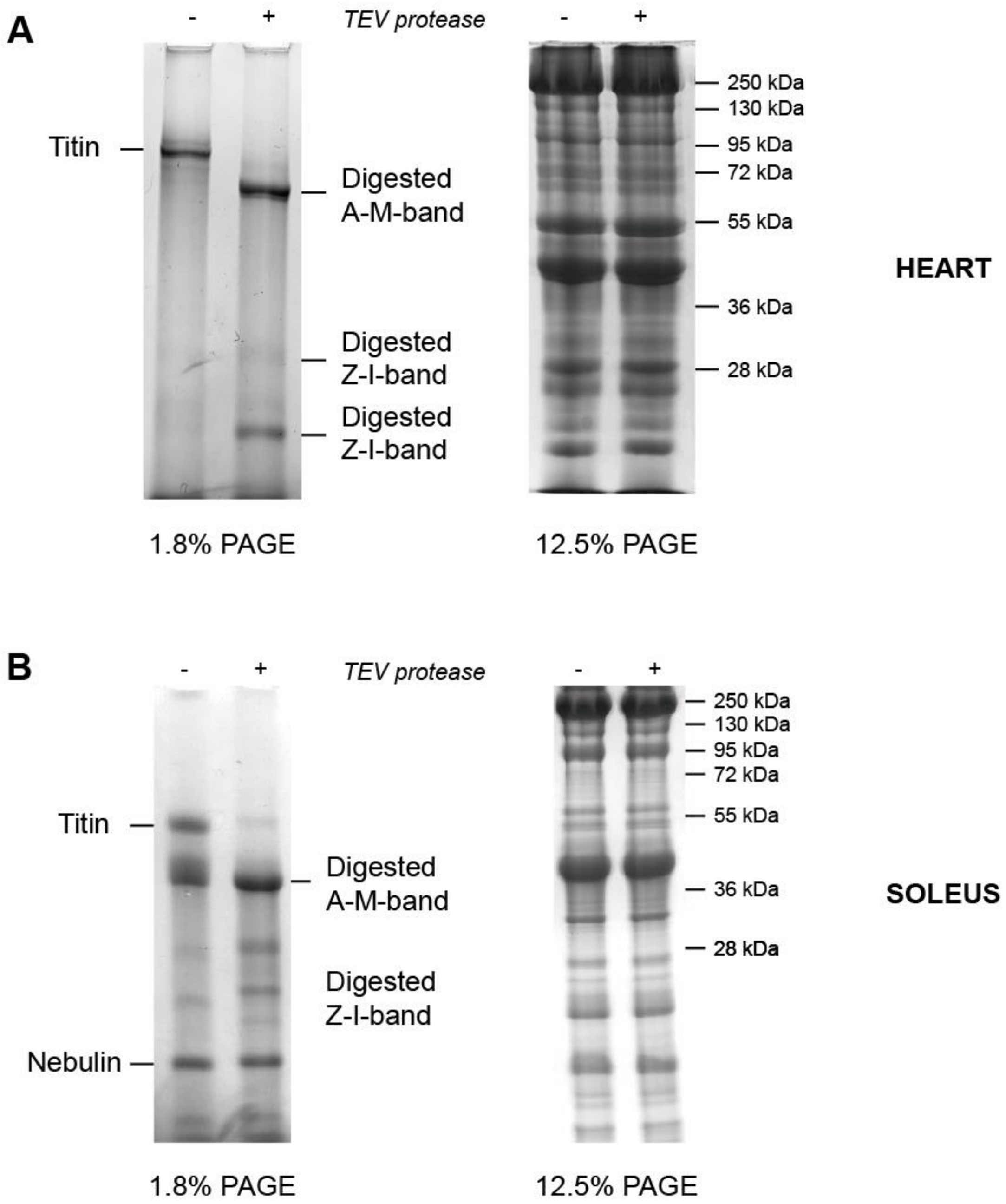
Supplementary Figure 6. Digestion of recombinant HaloTag-TEV titin fragment. (A) Scheme of I86-HaloTag-TEV-I87 (60 kDa) showing the size of the fragments that result from TEV digestion. **(B)** I86-HaloTag-TEV-I87 was treated or not with TEV protease (28 kDa) at 34ºC for 1 hour, and results were analyzed by 17% SDS-PAGE. Digestion resulted in the appearance of two new bands at the expected mobility (*left*, Coomassie staining). Specificity of TEV-cleavage is demonstrated by labeling with HaloTag Alexa488 ligand, which only reacts with HaloTag-containing bands (*right*, Alexa488 fluorescence). n = 1 experiment; a replicate using a different batch of TEV protease showed the same result.



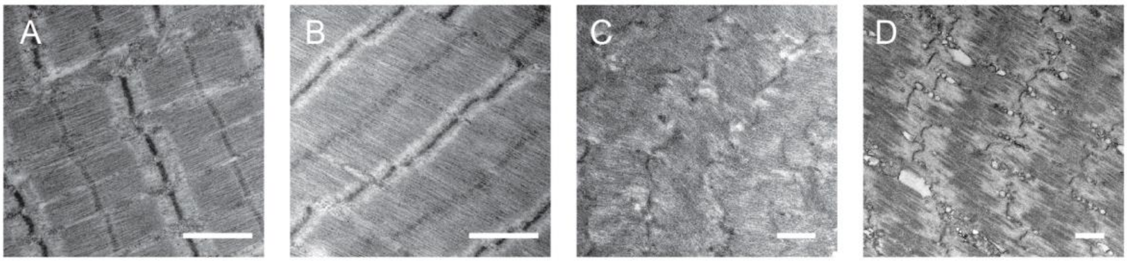
Supplementary Figure 7. TEV digestion of skeletal muscles from homozygous HaloTag-TEV titin mice, as analyzed using 1.8% acrylamide SDS-PAGE gels. (A) Results of TEV-digestion of isolated soleus myofibrils (Coomassie staining, representative image, n = 9). **(B)** Analysis of the digestion of psoas myofibrils with TEV. Both Coomassie staining and HaloTag Alexa488 ligand are used to visualize proteins. The HaloTag-specific Alexa488 ligand only labels the digested A-M-band fragment (representative image, n = 14). **(C)** Equivalent results are obtained in TEV digestions of soleus myofibrils, as analyzed by western blot using the MIR antibody, which recognizes the A-band segment of titin (representative image, n = 6) **(D)** The HaloTag-TEV-titin fibers used to collect the mechanical data in Figure 2C were analyzed by 1.8% SDS-PAGE to verify full digestion of titin (+ lane). A control sample with no TEV was used for reference (- lane). Representative image, n =12.



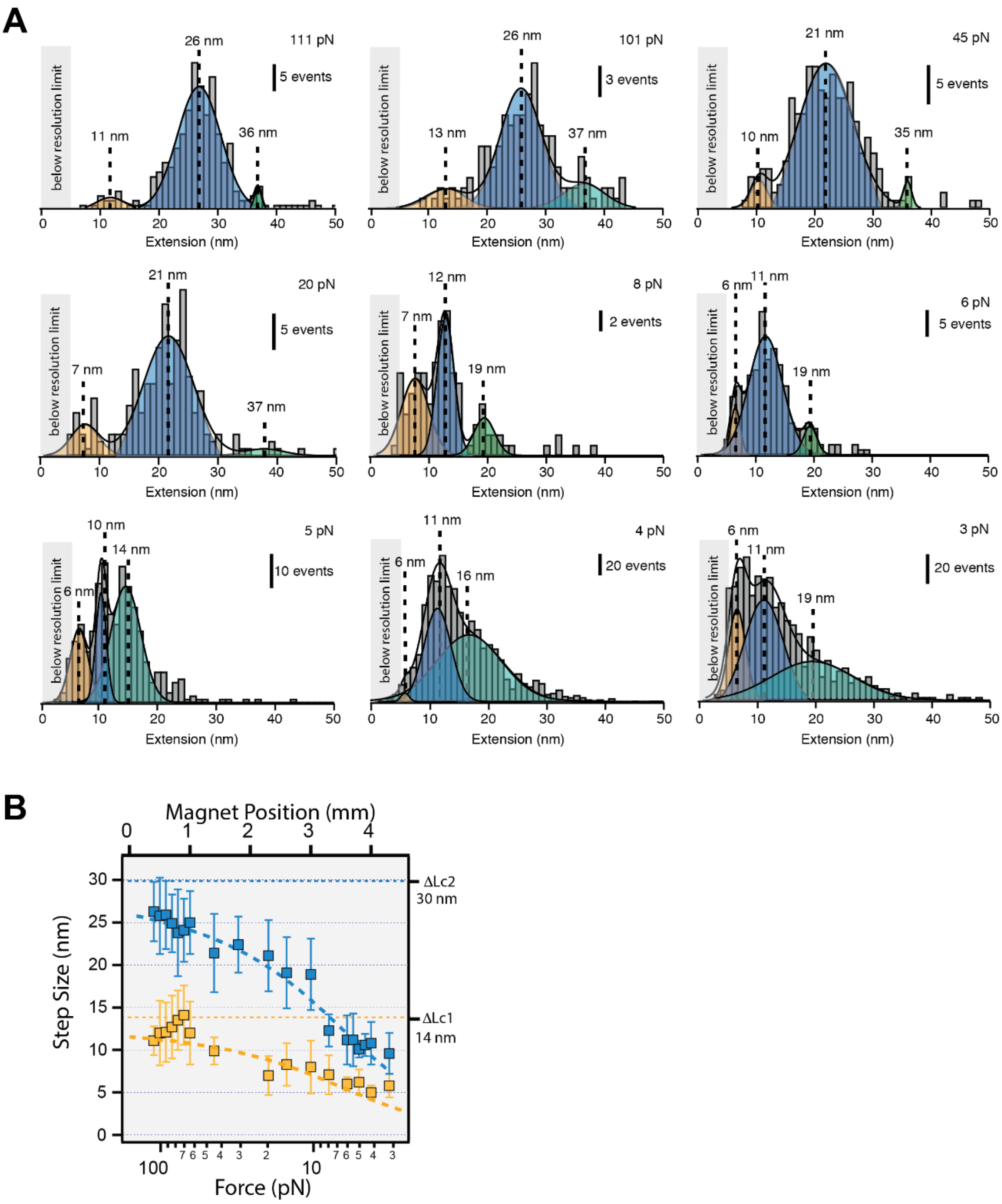
Supplementary Figure 8. Representative kinetics of HaloTag-TEV-titin digestion by TEV protease. (A) Psoas fibers were digested with TEV protease. Digestion was stopped at different time points by boiling aliquots in Laemmli buffer, and results were analyzed by 1.8% SDS-PAGE and Coomassie staining. Bands corresponding to nebulin, and intact and cleaved titin molecules are indicated. **(B,C)** Quantification of titin bands by densitometry. Nebulin intensity was used for normalization (black, intact titin; blue circles, A-band titin fragment; red triangles, I-band titin fragment). Solid lines are exponential fits, rate constants are indicated. These data show that at 30 min reaction time, the digestion of HaloTag-TEV titin is >99.9 % complete in this particular experiment (n =1 experiment analyzing one sample per digestion time, similar results were obtained in 6 experiments).



Supplementary Figure 9. Effect of TEV treatment on the protein composition of striated muscle from homozygous HaloTag-TEV titin mice. (A) *Left*: 1.8% SDS-PAGE shows TEV-induced specific digestion of cardiac titin. *Right*: the pattern of protein bands in 12.5% SDS-PAGE gels remains unaffected by TEV treatment. (Representative gel, n = 8). **(B)** Equivalent results are obtained with soleus samples (Representative experiment, n = 6). In this particular experiment, specific assignment of Z-I-band fragments is hindered by some non-specific degradation of titin already present in the –TEV sample.



Supplementary Figure 10. Ultrastructure of TEV-treated psoas fibers from homozygous HaloTag-TEV titin mice. **(A)** Sample in which no TEV is addedd, fixation for EM after a stretch-release protocol (Representative image, n = 7). **(B)** Sample in which TEV is added, fixation in the absence of mechanical perturbation. (Representative image, n = 18). **(C)** Sample in which TEV is added, fixation following a stretch-release cycle. (Representative image, n = 20)**. (D)** Sample in which TEV is added, fixation after holding the sample at long sarcomere length (Representative image, n = 16). All scale bars are 1 μm.



Supplementary Figure 11. (Un)folding step sizes. (A) Single titin molecules extracted from gastrocnemius muscles were pulled at different forces between 3 and 111 pN, and the size of the unfolding and refolding events was measured. We found three main populations of step sizes (solid lines are Gaussian fits to the data). Data is originated from n = 19 molecules. The number of events at each force is: 285 (111 pN), 146 (101 pN), 247 (45 pN), 213 (20 pN), 114 (8 pN), 202 (6 pN), 405 (5 pN), 1458 (4 pN), 1264 (3 pN). **(B)** Force-dependency of the step sizes for the two populations corresponding to single-domain unfolding events, and fits to the worm like chain model of polymer elasticity (dashed lines). We obtain $\Delta L_C = 30 \pm 1$ nm (blue) and $\Delta L_C = 14 \pm 1$ nm (yellow). Data are presented as mean values ± SD of the corresponding Gaussian distributions. The number of events for the distributions not shown in panel A is: 174 (93 pN), 685 (85 pN), 206 (77 pN), 1092 (71 pN), 467 (64 pN), 231 (15 pN), 91 (10 pN), 31 (5.5 pN), 578 (4.6 pN), 322 (3.8 pN).

Supplementary Note 1. Sequence of the targeting vector to introduce HaloTag-TEV in titin. Exons and Introns are shown in grey and white background, respectively.

Exon224—Exon225-**TEV**-HaloTag-**EK**—NeoFRT—Exon226—Exon227—Exon228—
Exon229—Exon230—Exon231—Exon232—Exon233—Exon234

Exon224

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Exon225

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HaloTag

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Exon226

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Exon227

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tgcctaatactcatgctacaagctaactaatcattcaatctcttctttag
Exon232
ATACAAGTTTGAGAAGGATGGGAGCATCCACAGGCTCATCATAAAAGACTGCAGGCTGGA
GGATGAGTGTGAATACGCTTGTGGTGTAGAGGACCGCAAGTCCCGAGCTAGACTTTTTGT
AGAAG
gttagtattggcttcaaggataattctagctgaagtgacaatcttttacaatgctaata
aaaaatacaaacacatatctgttttattttacattttctctccag
Exon233
AAATTCCAGTTGAGATTATCAGGCCTCCTCAAGACATTCTTGAAGCCCCTGGTGCAGACG
TTATCTTCTTGGCTGAGCTCAACAAAGATAAAGTGGAGGTCCAATGGCTTAGAAATAACA
TGATCGTCGTCCAGGGTGACAAGCACCAGATGATGAGTGAAGGAAAGATACACAGGCTAC
AGATTTGTGATATTAAGCCACGTGACCAGGGCGAATACAGATTCATTGCCAAAGATAAAG
AAGCCAGGGCTAAACTTGAATTAGCAG
gtaaattgctttcttctgcttcctggtgtcctccccatatcacaaacctctgtagatttg
agattttacaattaaggcaaaacactccttgtgaaagcatatggctcgaacctgtgctca
cgtgttagctattctactttgtctacctacaaagtggaagtggtgagcagcatctttaca
tgtcaaaccactgttattcctggcatgggcagctgctcatgacttcctagaagagttcct
cactcaactgataggcaactcctgtggctcttttaaatatgaaacctgcataggtttccc
cccaaagccccattgtacatgagagaaagctgtctgacttatcctgaactaagtatgcag
gactttatcaaaaacatattttataaaataatttcaacaagaaaagcaaaacttgtttca
ccatattctaggtcctctgcaattatttttaactaagaccagatgtgaatgatggtatgc
catcttcaccaaaccactgtttccaaacatgaatagttaggagtaaaacaataacaacaa
caacaagctattataactctccaacagaatgcactgctatgaaattactgtggttatgat
aatactgtggtttatcaaatgtatagaaaaaaaaacctatgtcatagtatgactatagta
tataactcaaacatgtttgtgtgttctctttgtcactag
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Exon234

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CTGCACCTAAAATCAAGACAGCTGATCAAGATCTCGTCGTTGATGCTGGCCAGCCTCTGA
CAATGGTGGTACCCTATGATGCCTACCCCAAAGCAGAAGCTGAATGGTTTAAAGAGAACG
AACCTCTATCTACAAAAACCGTTGACACTACGGCTGAGCAGACTTCTTTCAGAATCTCAG
AAGCCAAGAAGGACGACAAGGGGAGGTATAAAATCGTGCTTCAGAACAAGCATGGGAAAG
CAGAGGGCTTCATCAATTTACAAGTTATTG
```

Supplementary Note 2. Sequence of the I86-HaloTag-TEV-I87 recombinant construct. The QS peptide bond cleaved by TEV is indicated. Please note that DNA sequence do not match the murine DNA because it was codon-optimized for optimal protein expression in *E.coli*. The first alanine residue of I87 in the HaloTag-TEV-titin construct is actually a proline in wild-type I87.

I86-TEV site-Halotag-EK site-I87

cDNA:

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ATGAGAGGATCGCATCACCATCACCATCACGGATCCCCTCCGGTTGAATTTACCAAACCGCTGG
AAGATCAGACCGTTGAAGAAGAAGCAACCGCAGTTCTGGAATGTGAAGTTAGCCGTGAAAATGC
CAAAGTGAAATGGTTTAAAAACGGCACCGAAATCCTGAAAAGCAAGAAATATGAAATTGTGGCC
GATGGTCGTGTGCGCAAACTGATTATTCATGGTTGTACACCGGAAGATATCAAGACCTATACCT
GTGATGCCAAAGATTTCAAAACCAGCTGCAATCTGAATGTTGTTCTGGCAAGCGATAATACCAC
TCCGGAAGAGGATCTGTATTTTCAGAGTGATAATACAACCCCTGAAGCAGAAATCGGTACTGGC
TTTCCATTCGACCCCCATTATGTGGAAGTCCTGGGCGAGCGCATGCACTACGTCGATGTTGGTC
CGCGCGATGGCACCCCTGTGCTGTTCCTGCACGGTAACCCGACCTCCTCCTACGTGTGGCGCAA
CATCATCCCGCATGTTGCACCGACCCATCGCTGCATTGCTCCAGACCTGATCGGTATGGGCAAA
TCCGACAAACCAGACCTGGGTTATTTCTTCGACGACCACGTCCGCTTCATGGATGCCTTCATCG
AAGCCCTGGGTCTGGAAGAGGTCGTCCTGGTCATTCACGACTGGGGCTCCGCTCTGGGTTTCCA
CTGGGCCAAGCGCAATCCAGAGCGCGTCAAAGGTATTGCATTTATGGAGTTCATCCGCCCTATC
CCGACCTGGGACGAATGGCCAGAATTTGCCCGCGAGACCTTCCAGGCCTTCCGCACCACCGACG
TCGGCCGCAAGCTGATCATCGATCAGAACGTTTTTATCGAGGGTACGCTGCCGATGGGTGTCGT
CCGCCCGCTGACTGAAGTCGAGATGGACCATTACCGCGAGCCGTTCCTGAATCCTGTTGACCGC
GAGCCACTGTGGCGCTTCCCAAACGAGCTGCCAATCGCCGGTGAGCCAGCGAACATCGTCGCGC
TGGTCGAAGAATACATGGACTGGCTGCACCAGTCCCCTGTCCCGAAGCTGCTGTTCTGGGGCAC
CCCAGGCGTTCTGATCCCACCGGCCGAAGCCGCTCGCCTGGCCAAAAGCCTGCCTAACTGCAAG
GCTGTGGACATCGGCCCGGGTCTGAATCTGCTGCAAGAAGACAACCCGGACCTGATCGGCAGCG
AGATCGCGCGCTGGCTGTCGACGCTCGAGATTTCCGGCGATAACACGACACCTGAAGATGATGA
TGATAAAGACAATACGACACCGGAAACACGTGCACCGCATGTGGAATTTCTGCGTCCGCTGACC
GATCTGCAGGTTAAAGAAAAAGAAACCGCACGTTTTGAATGCGAGATCAGCAAAGAAAATGAAA
AGGTGCAGTGGTTTAAAGATGGTGCCGAAATCAAAAAAGGCAAAAAATACGACATCATCTCCAA
AGGTGCCGTTCGTATTCTGGTTATTAACAAATGTCTGCTGAACGATGAAGCCGAATATAGCTGT
GAAGTTCGTACCGCACGTACCAGCGGTATGCTGACCAGATCTTAA
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Protein:

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MRGSHHHHHHGSPPVEFTKPLEDQTVEEEATAVLECEVSRENAKVKWFKNGTEILKSKKYEIVA
DGRVRKLIIHGCTPEDIKTYTCDAKDFKTSCNLNVVLASDNTTPEEDLYFQ'SDNTTPEAEIGT
GFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAPDLIGMG
KSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAFMEFIRP
IPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREPFLNPVD
REPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAKSLPNC
KAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISGDNTTPEDDDDKDNTTPETRAPHVEFLRPL
TDLQVKEKETARFECEISKENEKVQWFKDGAEIKKGKKYDIISKGAVRILVINKCLLNDEAEYS
CEVRTARTSGMLTRS
```

Supplementary Table 1. Sequence of primers used to produce and genotype HaloTag-TEV-titin mice.

Primer	**Sequence**
P1	5'-ACCCTGAGGGATGTGAAGC-3'
PS1	5'-TGCAGTACTGGAGTGTGAAGTATCC-3'
PSR2	5'-GAAACGTGTGAAGTATCAGGTTAGG-3'
PR2	5'-CTGGCACTCTGTCGATACCC-3'
P2	5'-GGGTTTGCTCGACATTGG-3'
PR3	5'-GTAAATTGATGAAGCCCTCTGC-3'
Pmin	5'-CGTGGTGGCTTATCTTCTAGC-3'
PRmin	5'-CTGTTGGTTCATGCATCTCC-3'