

Mechanically knocking out titin reveals protein tension loss as a trigger of muscle disease

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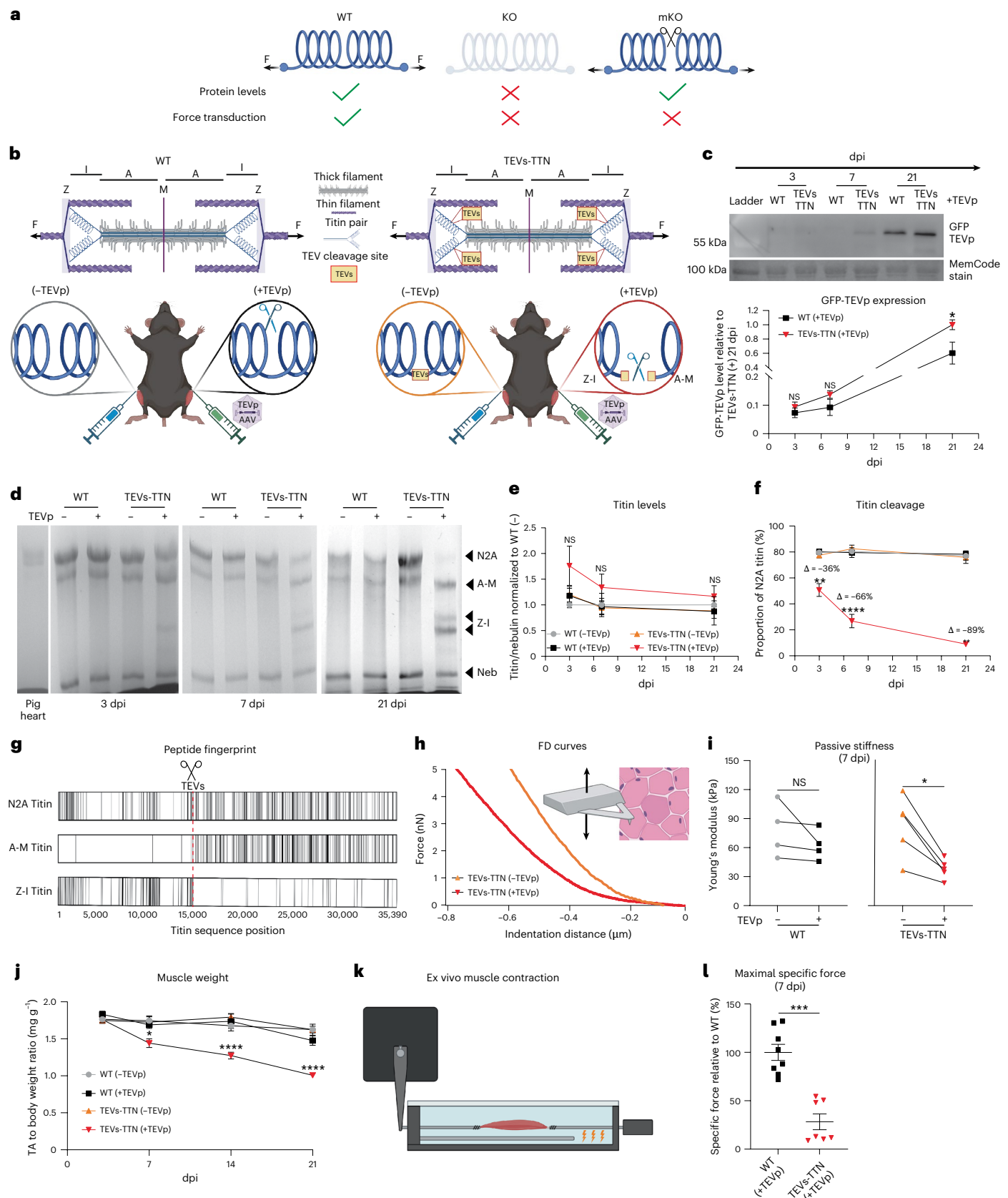
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Titin, the elastic protein scaffold of muscle sarcomeres, has multifunctional roles in mechanosignalling and is implicated in muscle disease. However, the consequences of disrupting titin's mechanical function in vivo remain incompletely understood. Here, by leveraging site-directed polypeptide severing as a 'mechanical knock-out' method for abolishing force transmission across titin, we show that the loss of titin tension in homozygous mechanically knocked-out muscles reduces force generation and induces severe atrophy and widespread transcriptional dysregulation. Although mechanically knocked-out myofibres persist, they shrink and undergo progressive sarcomere depletion, which correlates with the rapid upregulation of muscle-specific RING finger protein 1 (MuRF1) and with altered levels of other titin-associated atrophy regulators. The affected fibres also exhibit mitochondrial aggregation and myonuclear internalization, preceded by desmin mislocalization. Heterozygous mechanically knocked-out muscles show milder phenotypes that closely resemble titin-related human myopathy. Our findings suggest that slack titin molecules drive muscle disease, potentially through mechanisms shared with other mechanical proteins.

In cells, many multifunctional proteins must work under mechanical tension, including talin and α -catenin at the basis of mechanosensing and mechanotransduction in adhesion sites^{1,2}; lamin, a guardian of nuclear integrity³; and dystrophin, which ensures efficient force transmission in striated muscle and is mutated in Duchenne muscular dystrophy⁴. Despite progress using in vitro systems, current knowledge on the integrative mechanical functions of proteins in vivo remains incomplete. A limitation in this regard is that loss of protein levels in

conventional knock-out (KO) or knock-down approaches results in the suppression of both the mechanical and non-mechanical functions of the targeted protein, complicating interpretation of results^{5–11} (Fig. 1a). In this work, we focus on titin, another example of a protein with coexistent mechanical and non-mechanical functions¹². With a giant size of up to 3.8 MDa and connecting the Z- and M-lines of sarcomeres (Fig. 1b), titin is the major contributor to the passive stiffness of muscle tissue at physiological levels of stretch^{13,14}. In addition to these structural

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and mechanical roles, titin domains also serve as binding hubs for protein partners that regulate muscle hypertrophy/atrophy^{10,11,14,15}. Of note, the longitudinal location of titin in sarcomeres makes it a prime candidate to integrate muscle cell responses to mechanical forces, which have been shown in vitro to change the affinity of titin for its

interactors^{16,17}, modulate mechanosignalling pathways at the M-line region of the protein^{18–21} and enable post-translational modifications that modulate titin mechanics²².

Consistent with the fundamental functions of the protein, truncating variants in the titin gene (*TTN*) are responsible for many

Fig. 1 | Titin mKO induces muscle atrophy and reduced force generation.

a, Conceptual comparison of traditional KO and mKO strategies to study a mechanical protein. An mKO achieves loss of force transmission across the targeted protein without affecting its expression levels. **b**, Scheme of experimental set-up. WT and TEVs-TTN legs are injected either with vehicle saline buffer (–TEVp, blue) or AAV6-GFP-TEVp (+TEVp, green). Representations of sarcomeres are shown. Titin cleavage can only occur in AAV6-GFP-TEVp-injected TEVs-TTN muscles. Insets: the region of I-band titin where the TEV recognition site (yellow box) is inserted in TEVs-TTN mice. Titin fragments resulting from TEVp activity are indicated. **c**, GFP-TEVp expression measured by WB using anti-GFP antibody. MemCode staining was used as the loading control (n = muscles per group, 3 dpi, WT (+) n = 6, TEVs-TTN (+) n = 6; 7 dpi, WT (+) n = 5, TEVs-TTN (+) n = 6; 21 dpi, WT (+) n = 6, TEVs-TTN (+) n = 6). Two-tailed Student's t -test WT (+) versus TEVs-TTN (+): 3 dpi P = 0.4111, 7 dpi P = 0.1647 and 21 dpi P = 0.0412. $^*P < 0.05$. **d**, Titin integrity measured on Coomassie-stained 2% acrylamide SDS–PAGE gels. Position of detected titin and nebulin bands are indicated. **e**, Total titin levels (sum of all titin bands). Nebulin was used for normalization (n = muscles per group, 3 dpi, WT (–) n = 2, WT (+) n = 2, TEVs-TTN (–) n = 3, TEVs-TTN (+) n = 3; 7 dpi, WT (–) n = 4, WT (+) n = 4, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 21 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 4, TEVs-TTN (+) n = 4). Two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+): 3 dpi P = 0.249, 7 dpi P = 0.2083 and 21 dpi P = 0.4402. **f**, Quantification of proportion of N2A with respect to all titin bands (n = muscles per group, 3 dpi, WT (–) n = 4, WT (+) n = 3, TEVs-TTN (–) n = 4, TEVs-TTN (+) n = 4; 7 dpi, WT (–) n = 4, WT (+) n = 4, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 21 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6).

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severe diseases, including dilated cardiomyopathy²³ and congenital myopathies^{24–26}. However, the underlying molecular mechanisms of this pathology remain largely unknown²⁷. Recently, truncated titin molecules have been observed to be incorporated into cardiac sarcomeres, although in a less stable manner than wild-type (WT) counterparts potentially compromising force transmission^{28,29}. Considering also that sarcomere stability *ex vivo* requires that the mechanical integrity of titin is preserved^{30,31}, it is plausible that loss of mechanical transduction across the protein could contribute *per se* to the pathogenic load of truncating *TTN* variants, a hypothesis that remains untested.

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To dissect the functional role of titin mechanics, here we exploit site-directed cleavage of the protein by heterologous expression of tobacco etch virus protease (TEVp) in living mice (Fig. 1a,b). This strategy, which we name titin mechanical KO (mKO), results in loss of force transduction across titin's polypeptide backbone while preserving the total levels of the protein and therefore its non-mechanical functions. For implementation, we used TEVs-TTN mice carrying the TEVp recognition site in the I-band of titin³², a model used before for *ex vivo* studies confirming the major contribution of titin to muscle cell stiffness^{31–33} and uncovering the role of titin-based force on myofilament structure, regulation and stability^{31,34–36}, as well as for examination of sarcomere turnover in cultured cardiomyocytes and *in vivo* via HaloTag chemistry³⁷. Here we demonstrate that TEVp-directed severing can be applied to study the mechanical contribution of proteins to organ physiology *in vivo*. Our results reveal that titin mKO muscles develop a severe

TEVs-TTN (+) n = 6). Two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+): 3 dpi P = 0.0023, 7 dpi P < 0.0001 and 21 dpi P = 0.0022. $^{**}P < 0.01$, $^{***}P < 0.0001$. **g**, Position of identified peptides in mass-spectrometry experiments (at 1% false discovery rate) using the indicated titin bands (N2A band coming from a contralateral-vehicle-injected TEVs-TTN sample). The position of the TEV recognition site is indicated by a dashed line. **h**, Cartoon representing mechanical characterization of TA muscle cryosections using atomic force microscopy, and representative indentation traces from n = muscles per group TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5). **i**, Young's moduli of TA myofibres obtained using AFM at 7 dpi. (–TEVp) and (+TEVp) data points coming from the same animal are paired (n = muscles per group, WT (–) n = 4, WT (+) n = 4, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5). Two-tailed Student's t -test WT (–) versus WT (+) P = 0.2575 and two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+) P = 0.0113. $^{*}P < 0.05$. **j**, TA muscle weight to body weight ratio (n = muscles per group, 3 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 7 dpi, WT (–) n = 7, WT (+) n = 8, TEVs-TTN (–) n = 7, TEVs-TTN (+) n = 9; 14 dpi, WT (–) n = 5, WT (+) n = 4, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5); 21 dpi, WT (–) n = 8, WT (+) n = 9, TEVs-TTN (–) n = 8, TEVs-TTN (+) n = 9). Two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+): 3 dpi P = 0.4318, 7 dpi P = 0.0115, 14 dpi P < 0.0001 and 21 dpi P < 0.0001. $^{*}P < 0.05$, $^{***}P < 0.0001$. **k**, Scheme of *ex vivo* muscle mechanical characterization. **l**, Maximal specific active force produced by AAV6-injected EDL muscles at 7 dpi (n = muscles per group, WT (+) n = 8, TEVs-TTN (+) n = 7). Two-tailed Student's t -test WT (+) versus TEVs-TTN (+) P = 0.0003. $^{***}P < 0.001$. Data are represented as mean of the biological replicates $\pm$ s.e.m. Panels **a**, **b**, **h** and **k** were created with BioRender.com.

form of myopathy recapitulating a number of structural, histological and functional phenotypes in human patients who carry pathogenic *TTN* variants. Unexpectedly, we also find that titin mechanical integrity is required for stable positioning of myonuclei, and demonstrate the viability and long-term persistence of sarcomere-free, titin mKO myofibres.

Results

Titin mKO induces loss of muscle mass and strength

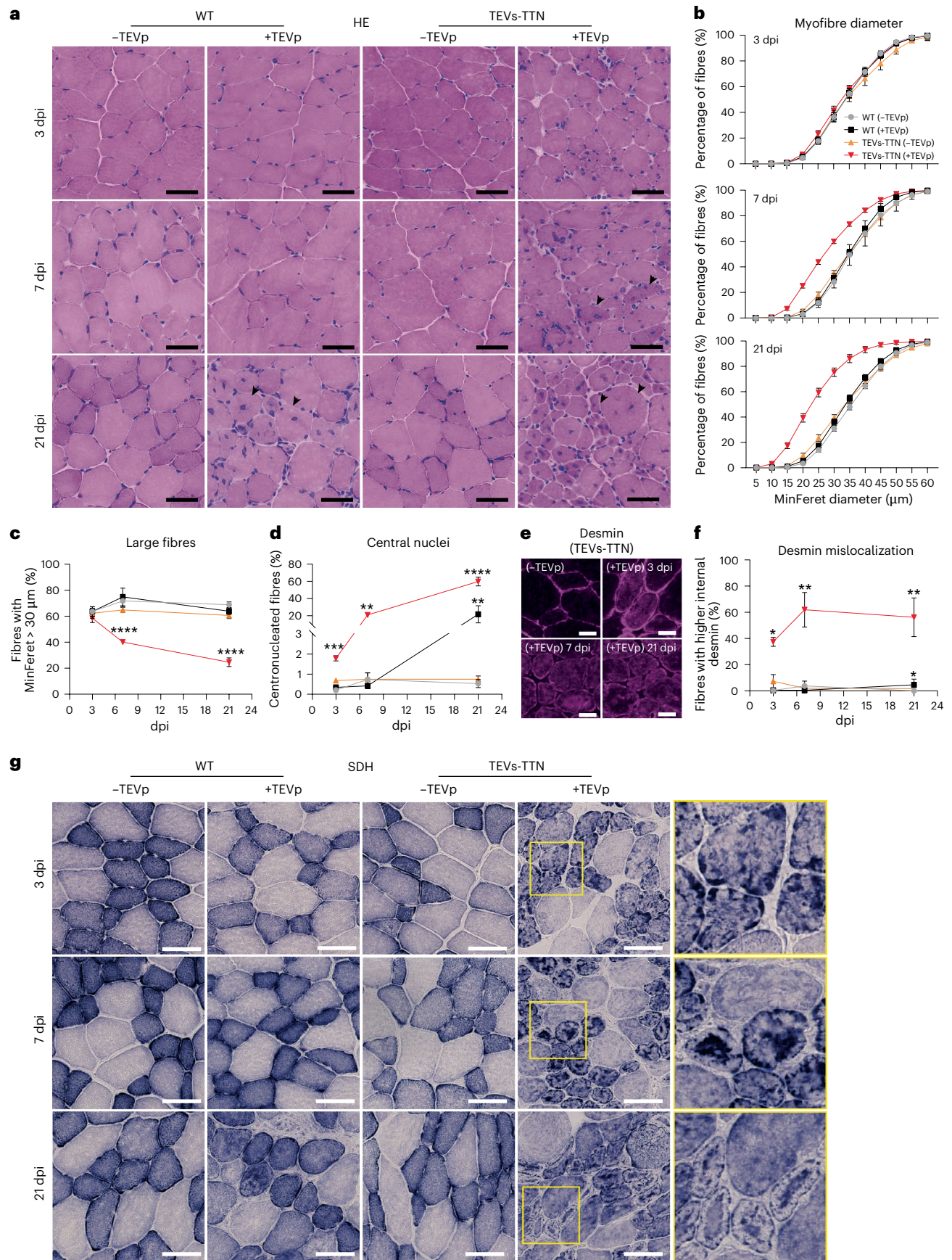
To implement titin mKO, we injected AAV6 viral vectors encoding a GFP-tagged version of a TEV protease with optimized catalytic activity (GFP-TEVp)³⁸ into the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles of homozygous TEVs-TTN mice³². Vehicle saline buffer was injected in contralateral legs, which served as internal controls (Fig. 1b). To ensure specificity of phenotypes resulting from titin mKO, we also treated WT mice (Fig. 1b). Using western blot (WB) and immunofluorescence, we verified time-dependent accumulation of GFP-TEVp in AAV6-injected legs from both WT and TEVs-TTN mice (Fig. 1c and Extended Data Fig. 1a–c). We noted that GFP-TEVp levels are higher in TEVs-TTN muscles, probably reflecting substrate-mediated stabilization of the protease since mRNA levels of the transgene do not follow the same trend (Supplementary Table 1).

Next, we assessed titin levels and cleavage using low-percentage SDS–PAGE gels and titin peptide fingerprinting by mass spectrometry (Fig. 1d–g). The results reveal progressive loss of full-length ~3.8 MDa

Fig. 2 | Histopathology of titin mKO muscles.

a, HE stainings of TA muscle. Black arrowheads indicate myofibres with centralized myonuclei. Scale bars, 50 μ m. **b**, Distribution of the MinFerret diameter of myofibres measured using WGA stainings in Extended Data Fig. 1a. n = muscles per group, 3 dpi, WT (–) n = 5, WT (+) n = 5, TEVs-TTN (–) n = 4, TEVs-TTN (+) n = 4; 7 dpi, WT (–) n = 3, WT (+) n = 4, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 21 dpi, WT (–) n = 4, WT (+) n = 5, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6. **c,d**, Time evolution of the fraction of myofibres with MinFerret diameter larger than 30 μ m (**c**) or centralized myonuclei (**d**) (n = muscles per group, 3 dpi, WT (–) n = 5, WT (+) n = 4, TEVs-TTN (–) n = 4, TEVs-TTN (+) n = 4; 7 dpi, WT (–) n = 3, WT (+) n = 4, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 21 dpi, WT (–) n = 4, WT (+) n = 5, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6). Large fibres two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+): 3 dpi P = 0.4318, 7 dpi P < 0.0001 and 21 dpi P < 0.0001. Centronucleated fibres two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+): 3 dpi P = 0.0001

and 21 dpi P < 0.0001. Centronucleated fibres two-tailed Mann–Whitney's t -test TEVs-TTN (–) versus TEVs-TTN (+): 7 dpi P = 0.0022. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. **e**, Desmin staining in transverse TA muscle sections. Scale bars, 20 μ m. **f**, Fraction of myofibres with increased sarcoplasmic desmin staining (n = muscles per group, 3 dpi, WT (–) n = 5, WT (+) n = 5, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5; 7 dpi, WT (–) n = 4, WT (+) n = 4, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5; 21 dpi, WT (–) n = 4, WT (+) n = 5, TEVs-TTN (–) n = 4, TEVs-TTN (+) n = 3). Two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+): 7 dpi P = 0.0019 and 21 dpi P = 0.007. Two-tailed Mann–Whitney's t -test TEVs-TTN (–) versus TEVs-TTN (+): 3 dpi P = 0.0159. $^{*}P < 0.05$ and $^{**}P < 0.01$. **g**, SDH mitochondrial activity staining of TA muscles (n = 5 muscles). Colour legends are indicated in insert in **b**. Scale bars, 50 μ m. Data are represented as mean of the biological replicates $\pm$ s.e.m.



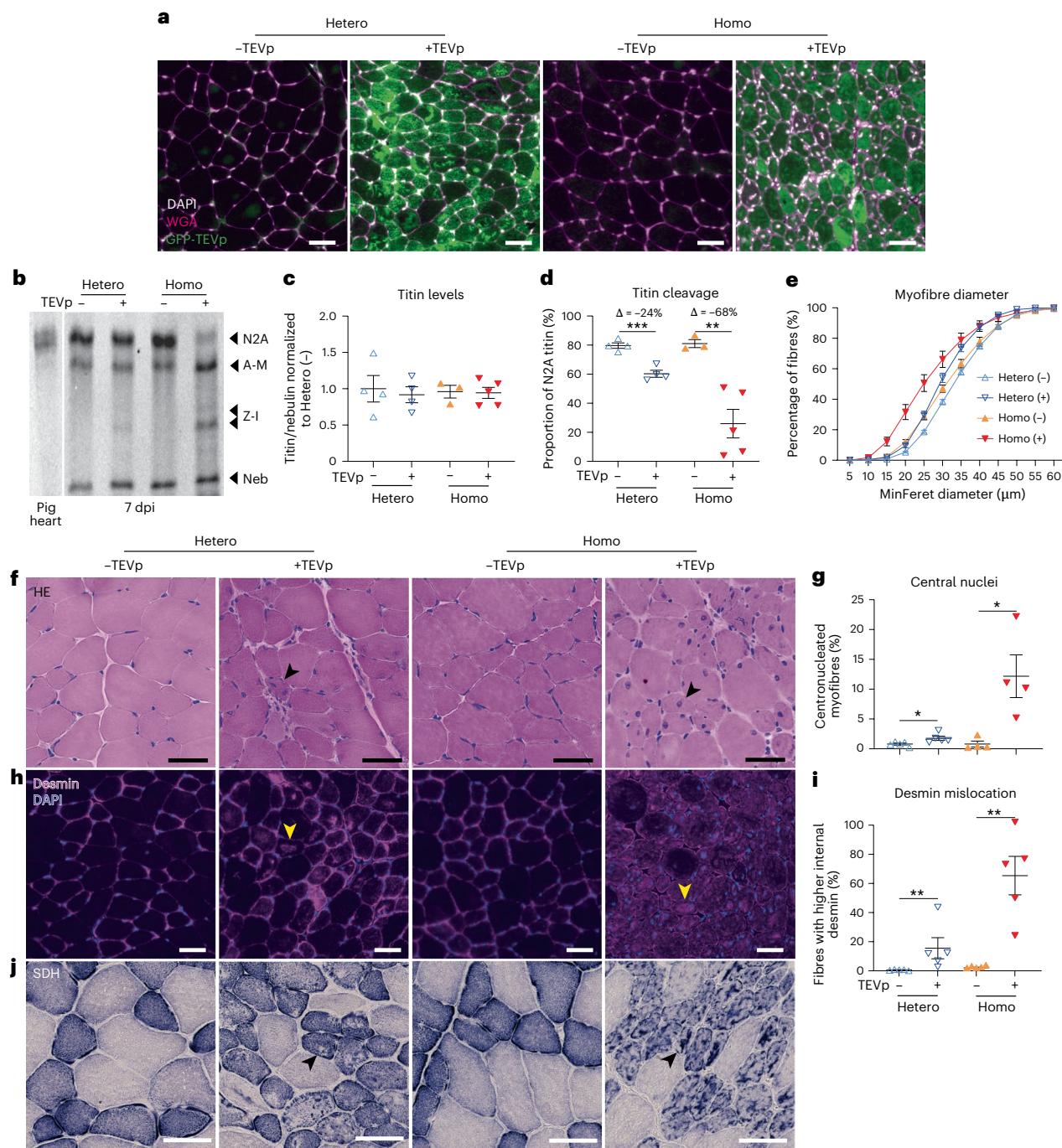


Fig. 3 | Intermediate phenotypes in heterozygous titin mKO muscles.

a, Immunofluorescence using WGA and anti-GFP antibodies of homozygous and heterozygous TEVs-TTN muscles at 7 dpi. Scale bars, 50 μ m. **b**, Titin integrity measured on Coomassie-stained 2% acrylamide SDS-PAGE gels. Position of detected titin bands are indicated. **c**, Total titin levels (sum of all titin bands). Nebulin was used for normalization ($n =$ muscles per group, Hetero (-) $n = 4$, Hetero (+) $n = 4$, Homo (-) $n = 3$, Homo (+) $n = 5$). Two-tailed Student's *t*-test Hetero (-) versus Hetero (+) $P = 0.7093$ and two-tailed Student's *t*-test Homo (-) versus Homo (+) $P = 0.8944$. **d**, Quantification of proportion of N2A with respect to all titin bands ($n =$ muscles per group, Hetero (-) $n = 4$, Hetero (+) $n = 4$, Homo (-) $n = 3$, Homo (+) $n = 5$). Two-tailed Student's *t*-test Hetero (-) versus Hetero (+) $P = 0.0007$ and two-tailed Student's *t*-test Homo (-) versus Homo (+) $P = 0.006$. $**P < 0.01$ and $****P < 0.0001$. **e**, Distribution of myofiber MinFerret diameter measured in WGA immunofluorescence ($n =$ muscles per group, Hetero (-) $n = 11$, Hetero (+) $n = 10$, Homo (-) $n = 9$, Homo (+) $n = 9$). **f**, HE staining of homozygous and heterozygous TEVs-TTN muscles at 7 dpi. Arrowheads mark myofibers with central myonuclei. Scale bars, 50 μ m. **g**, Fraction of myofibers with central

myonuclei measured in HE staining ($n =$ muscles per group, Hetero (-) $n = 5$, Hetero (+) $n = 5$, Homo (-) $n = 4$, Homo (+) $n = 4$). Two-tailed Mann-Whitney's *U*-test Hetero (-) versus Hetero (+) $P = 0.0238$ and two-tailed Mann-Whitney's *U*-test Homo (-) versus Homo (+) $P = 0.0286$. $*P < 0.05$. **h**, Immunofluorescence to visualize desmin in homozygous and heterozygous TEVs-TTN muscles at 7 dpi. Arrowheads mark selected myofibers showing desmin mislocalization. Scale bars, 50 μ m. **i**, Fraction of myofibers with mislocalized desmin ($n =$ muscles per group, Hetero (-) $n = 5$, Hetero (+) $n = 5$, Homo (-) $n = 5$, Homo (+) $n = 5$). Two-tailed Mann-Whitney's *U*-test Hetero (-) versus Hetero (+) $P = 0.0079$ and two-tailed Student's *t*-test Homo (-) versus Homo (+) $P = 0.0019$. $**P < 0.01$. **j**, SDH staining on transverse TA sections of heterozygous and homozygous TEVs-TTN muscles (representative images from $n =$ muscles per group from Hetero (-) $n = 3$, Hetero (+) $n = 3$, Homo (-) $n = 5$ and Homo (+) $n = 5$). Arrowheads mark myofibers with cores in heterozygous muscle and mitochondrial aggregation in homozygous muscle. Scale bars, 50 μ m. Data are represented as mean of biological replicates $\pm$ s.e.m.

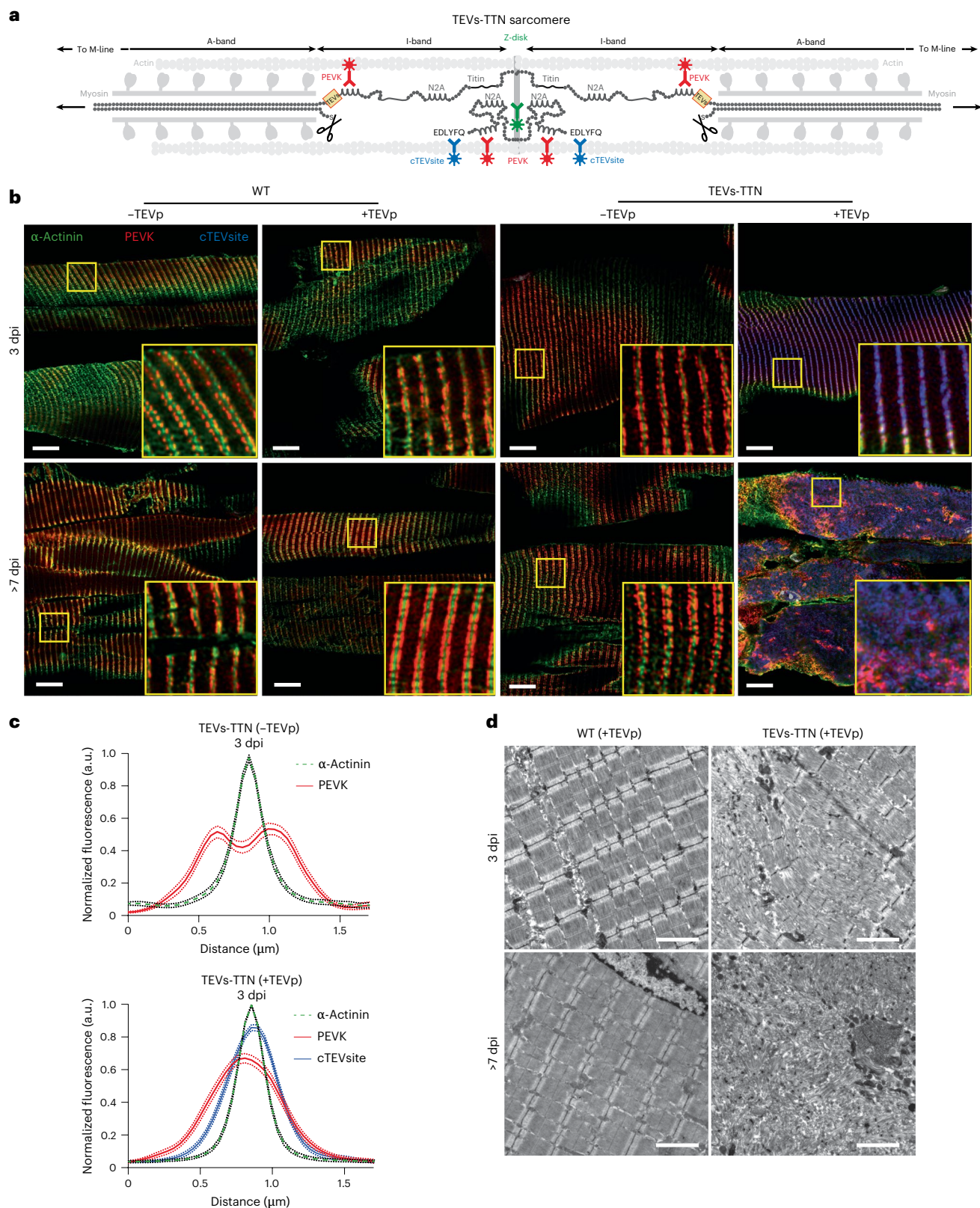


Fig. 4 | Progressive sarcomeric disassembly in titin mKO muscles. a, Scheme of two half sarcomeres in TEVs-TTN muscles. TEV sites are represented by yellow boxes. The C-terminal sequence resulting from TEV cleavage is indicated. This epitope is recognized by anti-cTEVsite antibody only in cleaved titin. The scheme shows recoiling of cleaved titin. **b**, Triple α -actinin/PEVK-titin/cTEVsite immunofluorescence on EDL longitudinal sections at different time points. Scale bars, 10 μ m (representative images from 3 muscles per group from WT (-), WT (+), Homo (-) and Homo (+)). **c**, Relative distribution of α -actinin, PEVK-titin and

cTEVsite fluorescent signals in vehicle and AAV6-injected conditions at 3 dpi (TEVs-TTN (-), 76 sarcomeres analysed from 17 myofibres from 3 muscles; TEVs-TTN (+), 102 sarcomeres analysed from 24 myofibres from 5 muscles). Data are represented as mean of the normalized signal $\pm$ s.e.m. For AAV6-injected TEVs-TTN muscles, only cTEVsite-positive myofibres were assessed. **d**, Transmission electron microscopy longitudinal sections of EDL myofibres at different time points (representative images from n = muscles per group, WT (+) n = 2, TEVs-TTN (+) n = 4). Scale bars, 2 μ m.

N2A titin only in AAV6-injected TEVs-TTN muscles (Fig. 1f). Concomitantly, we observed the appearance of an ~2.2 MDa A-M titin fragment with slightly higher mobility than naturally present T2/Cronos titin, and ~1.6 MDa Z-I fragments as expected from the existence of alternatively spliced forms of titin in the I-band of the protein³² (Fig. 1d,g). Importantly, AAV6-injected TEVs-TTN muscles show preserved titin protein levels (Fig. 1e). As expected from the loss of titin mechanical integrity, AAV6-injected TEVs-TTN myofibres show a 35–65% drop in passive stiffness at 7 days post-infection (dpi), an effect that is not observed in WT samples³¹ (Fig. 1h,i).

At the tissue level, titin mKO muscles show noticeable atrophy from 7 dpi, an effect that reaches 38% mass loss at 21 dpi (Fig. 1f). In addition, AAV6-injected TEVs-TTN muscles generate less active force than WT counterparts at 7 dpi even when data are normalized by the muscle cross-sectional area, a result that cannot be explained by a tendency to slightly lower force generation of TEVs-TTN muscles in basal conditions (Fig. 1k,l and Extended Data Fig. 1d,e). By contrast, titin mKO does not result in detectable alterations of contraction kinetics (Extended Data Fig. 1f).

Altogether, our results indicate that titin mKO leads to atrophy and reduced force generation of targeted muscles. These effects are not observed in controls where GFP-TEVp is expressed in WT animals.

Histopathological features of titin mKO muscles

The phenotypes that we observe in titin mKO muscles appear to happen in the absence of remarkable myofibre death. Indeed, we only have limited evidence of titin-mKO-induced necrosis at 3 dpi, when 6.5% of myofibres show IgG infiltration³⁹ (Extended Data Fig. 2a). To further characterize the myopathic response in titin mKO muscles, we did histological staining routinely used to classify congenital myopathies. We first stained sections with haematoxylin and eosin (HE) (Fig. 2a) or wheat germ agglutinin (WGA) (Extended Data Fig. 1a). The results demonstrate a shift towards smaller myofibre size at 7 and 21 dpi only in titin mKO muscles (Fig. 2b), which is evidenced by 40–60% less myofibres with minimal Feret diameter (MinFeret) larger than 30 μm (Fig. 2c). Notably, our results reveal a marked increase of myofibres with internalized myonuclei in titin mKO muscles, which is already evident at 3 dpi (Fig. 2a,d). In some AAV6-injected WT muscles at 21 dpi, we also detect internal myonuclei. We hypothesize that these internal myonuclei probably belong to new, immature myofibres resulting from muscle regeneration induced by non-specific GFP-TEVp immunogenicity as observed in other settings⁴⁰. Consistent with this interpretation, we find that most central myonuclei in AAV6-injected WT are found in GFP-TEVp-negative myofibres, a population expected to be enriched in regenerative myofibres where non-replicative AAV6 particles have

been diluted during progenitor cell divisions (Extended Data Fig. 2b). By contrast, central myonuclei are found both in GFP-negative and GFP-positive cells in titin mKO muscles (Extended Data Fig. 2b). These results support that many myofibres with internal myonuclei in the AAV6-injected TEVs-TTN group do not come from regeneration and that myonuclei internalization in these myofibres is a direct consequence of titin cleavage. Accordingly, mislocalization of desmin intermediate filaments is present in ~40% of AAV6-injected TEVs-TTN myofibres already at 3 dpi (Fig. 2e,f and Extended Data Fig. 2c) and therefore precedes nuclear centralization in many cases (Fig. 2d) as observed in centronuclear myopathy, a muscle disorder where the presence of central myonuclei is unrelated to myofibre regeneration⁴¹. Importantly, no overt desmin mislocalization is detected in AAV6-injected WT muscles (Fig. 2f and Extended Data Fig. 2c), providing additional support that myonuclei internalization induced by GFP-TEVp expression in WT animals is related to immunogenicity-related muscle regeneration.

We extended histological characterization using succinate dehydrogenase (SDH) staining, which shows mitochondrial aggregates in titin mKO samples from 3 dpi. These aggregates become centralized from 7 dpi eventually resulting in a halo-ring appearance of many myofibres at 21 dpi (Fig. 2g). Complementary nicotinamide adenine dinucleotide dehydrogenase (NADH) staining confirms mitochondrial alterations in titin mKO specimens (Extended Data Fig. 3a). In addition, Gomori staining detects dye-free, irregular regions induced by titin mKO (Extended Data Fig. 3b). Although this result could be compatible with the presence of intracellular vacuoles, we do not observe typical signs of vacuolization in HE staining (Fig. 2a). Gomori also reveals increased interfibre spacing in titin mKO samples (Extended Data Fig. 3b). In agreement with this observation, we find that titin mKO sections show increased picrosirius staining from 7 dpi, indicative of interstitial fibrosis (Extended Data Fig. 3c).

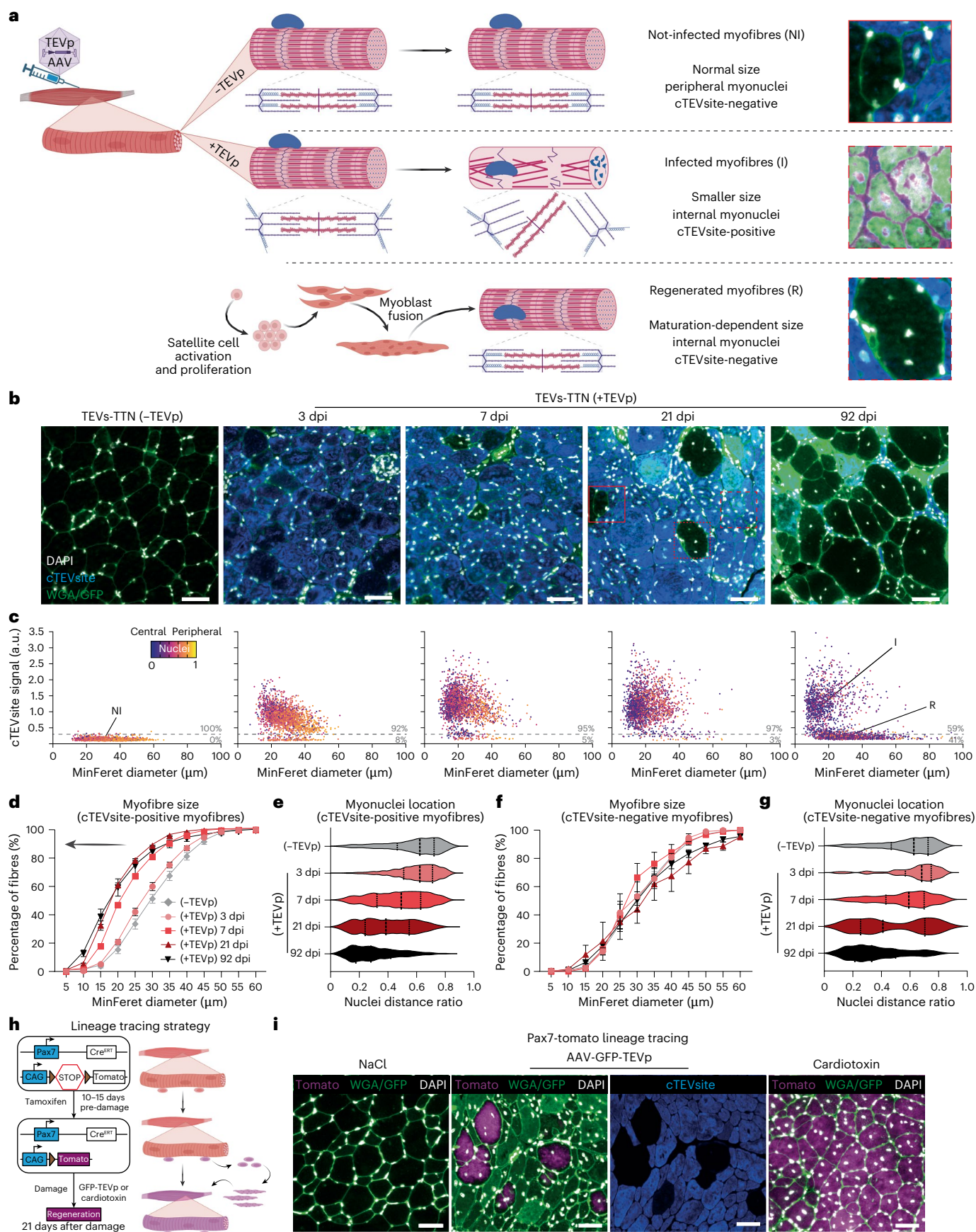
In combination, our histological characterization demonstrates that titin mKO muscles develop common histopathological features of muscle disease, including myofibre atrophy, myonuclei internalization, mitochondrial mislocalization and fibrosis^{24–26}.

Heterozygous titin mKO muscles show milder phenotypes

To study consequences of titin mKO in heterozygosity, we injected independent cohorts of homozygous and heterozygous TEVs-TTN mice with GFP-TEVp-expressing AAV6 vectors. Both groups show equivalent expression of GFP-TEVp in injected muscles at 7 dpi (Fig. 3a and Extended Data Fig. 4a,b), which results in preserved titin levels but 68% and 24% titin cleavage in homozygous and heterozygous samples, respectively (Fig. 3b–d). Consistent with our results in Fig. 1j, homozygous titin mKO muscles weigh 16% less than control counterparts at 7

Fig. 5 | Coexistence of regenerative and titin mKO myofibres. **a**, Scheme of the different types of TEVs-TTN myofibres expected after AAV6-GFP-TEVp infection. Non-infected myofibres have normal size and peripheral myonuclei and are negative for cTEVsite staining. Infected myofibres are small, show internal myonuclei and are stained by anti-cTEVsite antibody. Regenerative myofibres originating from satellite cell proliferation and fusion have variable size and internal myonuclei and are expected to be less cTEVsite positive as a result of dilution of GFP-TEVp-expressing, non-replicative AAV6 viral particles during satellite cell proliferation. **b**, DAPI/WGA-GFP/cTEVsite triple immunofluorescence allows visualization of myofibre size, myonuclei position and cTEVsite signal for every myofibre. Scale bars, 50 μm . **c**, Quantification of cTEVsite signal, myofibre diameter and myonuclei-to-myofibre-centroid distance for every myofibre at different time points ($n = 2,056$ randomly chosen myofibres per group, cTEVsite-positive threshold marked as dashed grey line). **d,e**, Distribution of the MinFeret myofibre diameter (represented as mean $\pm$ s.e.m.) (**d**) and violin plot of myonuclei distance ratio (only myofibres where nuclei was detected were counted, violin plot with quartiles and median as dashed lines) (**e**) in cTEVsite-positive myofibres ($n =$ myofibres per group, TEVs-TTN (–TEVp) $n = 2,059$ (all myofibres), 3 dpi TEVs-TTN (+TEVp) $n = 1,710$,

7 dpi TEVs-TTN (+TEVp) $n = 3,663$, 21 dpi TEVs-TTN (+TEVp) $n = 2,229$, 92 dpi TEVs-TTN (+TEVp) $n = 2,175$). **f,g**, Distribution of the myofibre MinFeret diameter (represented as mean $\pm$ s.e.m.) (**f**) and violin plot of myonuclei distance ratio (only myofibres where nuclei was detected were counted, violin plot with quartiles and median as dashed lines) (**g**) in cTEVsite-negative myofibres ($n =$ myofibres per group, TEVs-TTN (–TEVp) $n = 2059$ (all myofibres), 3 dpi TEVs-TTN (+TEVp) $n = 155$, 7 dpi TEVs-TTN (+TEVp) $n = 216$, 21 dpi TEVs-TTN (+TEVp) $n = 69$, 92 dpi TEVs-TTN (+TEVp) $n = 1,566$). **h**, Scheme of the lineage tracing experiment based on the elimination of STOP codons upon tamoxifen-induced Cre recombination in Pax7-expressing cells. In this strategy, regenerative myofibres originating from satellite cells are fluorescently labelled by a Tomato reporter. **i**, Immunofluorescence identifies Tomato-positive regenerative myofibres in AAV6-GFP-TEVp-injected TEVs-TTN muscles surrounded by many Tomato-negative, cTEVsite-positive counterparts. A positive control of regeneration using cardiotoxin is also shown. Representative images from $n =$ muscles per group, TEVs-TTN (–TEVp) $n = 5$, 3 dpi TEVs-TTN (+TEVp) $n = 4$, 7 dpi TEVs-TTN (+TEVp) $n = 5$, 21 dpi TEVs-TTN (+TEVp) $n = 4$, 92 dpi TEVs-TTN (+TEVp) $n = 5$. Scale bars, 50 μm . Panels **a** and **h** were created with [BioRender.com](#).



dpi (Extended Data Fig. 4c). By contrast, AAV6-injected heterozygous TEVs-TTN muscles do not show any evidence of weight loss (Extended Data Fig. 4c), although the fraction of myofibres with MinFerret larger than 30 μm is reduced by 25% (Fig. 3e and Extended Data Fig. 4d). Remarkably, myofibres in heterozygous titin mKO muscles have a higher fraction of central myonuclei than controls, an effect that correlates with the extent of desmin mislocalization (Fig. 3f–i). Both effects are not as pronounced as in titin mKO homozygous animals. At the histological level, histological staining with SDH (Fig. 3j) and NADH (Extended Data Fig. 4e) reveals cores in heterozygous titin mKO samples. We also observe dye-free regions in Gomori (Extended Data Fig. 4f). Overall, our results indicate that induction of titin mKO in heterozygosis results in a milder form of myopathy compared with homozygous conditions.

Titin mKO leads to sarcomere disassembly

Having verified the effects of titin mKO at the tissue and cellular levels, we next examined the underlying sarcomere alterations. Mechanically, titin can be divided into two main regions (Fig. 4a). While A-band titin is an integral part of the thick filament participating in fundamental structural interactions^{42,43}, the I-band of the protein has a prominent mechanical role, thanks to the ability of I-band domains to adapt their extension to the fluctuating force experienced or generated by sarcomeres⁴⁴. TEVs-TTN muscle contains the TEV protease cleavage site at the end of the I-band part of titin (Fig. 4a); hence, we hypothesized that releasing force upon titin cleavage by TEV protease would primarily affect titin's I-band. We did fluorescent immunohistochemistry on longitudinal muscle sections using antibodies against alpha-actinin (labelling the Z-disk of sarcomeres), the PEVK region within I-band titin and the cleaved TEV site (cTEVsite) (Fig. 4a; validation of specificity of anti-cTEVsite shown in Extended Data Fig. 5a,b). At 3 dpi, muscles from the three control groups show normal PEVK immunostaining resulting in two maxima flanking the Z-disk (Fig. 4b,c). By contrast, the PEVK epitope shows a single, broader maximum co-localizing with the Z-disk in titin mKO myofibres that are positive for cTEVsite (Fig. 4b,c). In these myofibres, the cTEVsite signal also locates to the Z-disk (Fig. 4b,c). These results indicate that the cleaved I-band of titin retracts towards the Z-disk of targeted sarcomeres upon tension release, as observed also *in vitro*³¹. Interestingly, this recoiling occurs in a context where the overall sarcomeric structure is well preserved according to Z-disk staining (Fig. 4b). At 7 and 21 dpi, sarcomere organization is completely lost in most myofibres of the homozygous titin mKO group (Fig. 4b).

To characterize sarcomere disassembly induced by titin mKO at higher resolution, we assessed muscle ultrastructure by transmission electron microscopy. Transmission electron microscopy micrographs show that homozygous titin mKO muscles show focal disarray of the sarcomeric structure already at 3 dpi (Fig. 4d). In agreement with the immunofluorescence results in Fig. 4b, no evidence of the organized sarcomere structure is observed at 7 and 21 dpi in most myofibres of the homozygous titin mKO group (Fig. 4d). We also find limited sarcomere disassembly in heterozygous titin mKO samples at 7 dpi (Extended Data Fig. 5c). Electron microscopy images also reveal mitochondrial aggregation in affected homozygous titin mKO myofibres (Extended Data Fig. 5d), consistent with histological determinations (Fig. 2g and Extended Data Fig. 3a). Membrane whorls or multilamellar bodies are also found in micrographs of homozygous titin mKO samples (Extended Data Fig. 5d).

Altogether, immunofluorescence and ultrastructural characterization confirm that titin cleavage by TEV protease results in mechanical unloading of the protein preceding sarcomere disassembly. As a result, many myofibres in titin mKO muscles are fully devoid of sarcomeres from 7 dpi.

Coexistence of regenerative and titin mKO myofibres

Our data so far suggest that sarcomere-free, titin mKO myofibres remain viable at 21 dpi, although they shrink and show central myonuclei. We

examined the long-term persistence of these myofibres in the context of muscle regeneration (Fig. 5a). With this aim, we analysed by immunohistochemistry 21,720 individual TEVs-TTN myofibres and quantified their size, myonuclei position and cTEVsite signal, including also samples obtained at 92 dpi (Fig. 5b). Myofibres in non-injected controls are cTEVsite negative and show peripheral myonuclei (Fig. 5b,c). By contrast, a population of infected myofibres showing high cTEVsite signal marking titin cleavage is evident from 3 dpi onwards (Fig. 5b,c). These cTEVsite-positive myofibres show progressively reduced size and more centralized myonuclei as time passes (Fig. 5c–e). Remarkably, a population of cTEVsite-negative myofibres with central myonuclei and preserved size becomes apparent at 92 dpi. We hypothesize that this new population corresponds to myofibres originating from muscle regeneration (Fig. 5c,f,g). Indeed, using a Pax7 lineage tracing strategy based on the inducible expression of a Tomato reporter that therefore labels myofibres originating from myofibre regeneration^{45,46} (Fig. 5h), we verified the existence of 4.4% regenerative myofibres in the AAV6-injected TEVs-TTN group already at 21 dpi (Fig. 5i). Remarkably, this level of muscle regeneration agrees well with the proportion of cTEVsite-negative myofibres showing central myonuclei at 21 dpi in Fig. 5c.

Taken together, our results confirm that titin mKO is a direct trigger of myofibre atrophy and myonuclei internalization, and that affected cells are only slowly taken over by intact-titin-containing myofibres (Extended Data Fig. 6a) that originate from muscle regeneration. This replenishment of myofibres correlates with a slight recovery of muscle mass at 92 dpi (Extended Data Fig. 6b).

Altered titin interactors in titin mKO muscles

Considering that titin is a signalling hub that interacts with many proteins relevant for muscle function⁴⁴ (Fig. 6a), and that the levels of some of these interactors are altered in muscles with stiffer titins^{7–9}, we hypothesized that titin unloading could perturb titin interactors contributing to downstream myopathic responses. Using WB, we assessed the levels of five titin interactors that are involved in muscle atrophy/hypertrophy and response to stress (Fig. 6b). At 21 dpi in homozygous titin mKO muscles, we detect increased levels of ANKRD1 (Fig. 6c), a transcriptional coregulator that has been observed to link titin to the thin filament, and is also upregulated in several stress conditions such as intensive care unit-acquired weakness, denervation and dystrophy^{47–49}. Interestingly, from 7 dpi in titin mKO muscles we detect increased levels of autoprocessed and active calpain-3, a titin-interacting protease involved in protein turnover and known to cleave ANKRD1⁵⁰ (Fig. 6d). Total levels of calpain-3 remain unaltered (Extended Data Fig. 7a). In agreement with higher calpain-3 activity, the presence of cleaved ANKRD1 is also evident from 7 dpi (Fig. 6e). The levels of chaperones HSP27 and $\alpha\beta$ -crystallin and the ubiquitin ligase MuRF1, all of them involved in protein homeostasis^{14,16,51,52}, are increased in titin mKO muscles at 21 dpi (Fig. 6f–h). Remarkably, increased expression of MuRF1 in titin mKO samples is detected as soon as 3 dpi (Fig. 6h). At this time point, the protein remains at the M-line of sarcomeres (Extended Data Fig. 7b). From 7 dpi, MuRF1 staining in titin mKO cells becomes disorganized as expected from the absence of noticeable sarcomere structure (Extended Data Fig. 7b). We also measured the levels of FHL1, a transcriptional co-activator regulating skeletal muscle mass⁵³, and found the protein to be upregulated by 21 dpi in titin mKO muscles (Extended Data Fig. 7c). Intriguingly, basal levels of HSP27, $\alpha\beta$ -crystallin and FHL1 levels are increased in TEVs-TTN mice compared with WT counterparts, indicating that the 35 kDa insert in TEVs-TTN may lead to some baseline stress. The mechanisms underlying this phenotype in TEVs-TTN muscles remain unknown, although we speculate that they may result from softening of the titin filament from the extra length of the TEVs-containing insert and/or alteration of protein–protein interactions involving the insertion site.

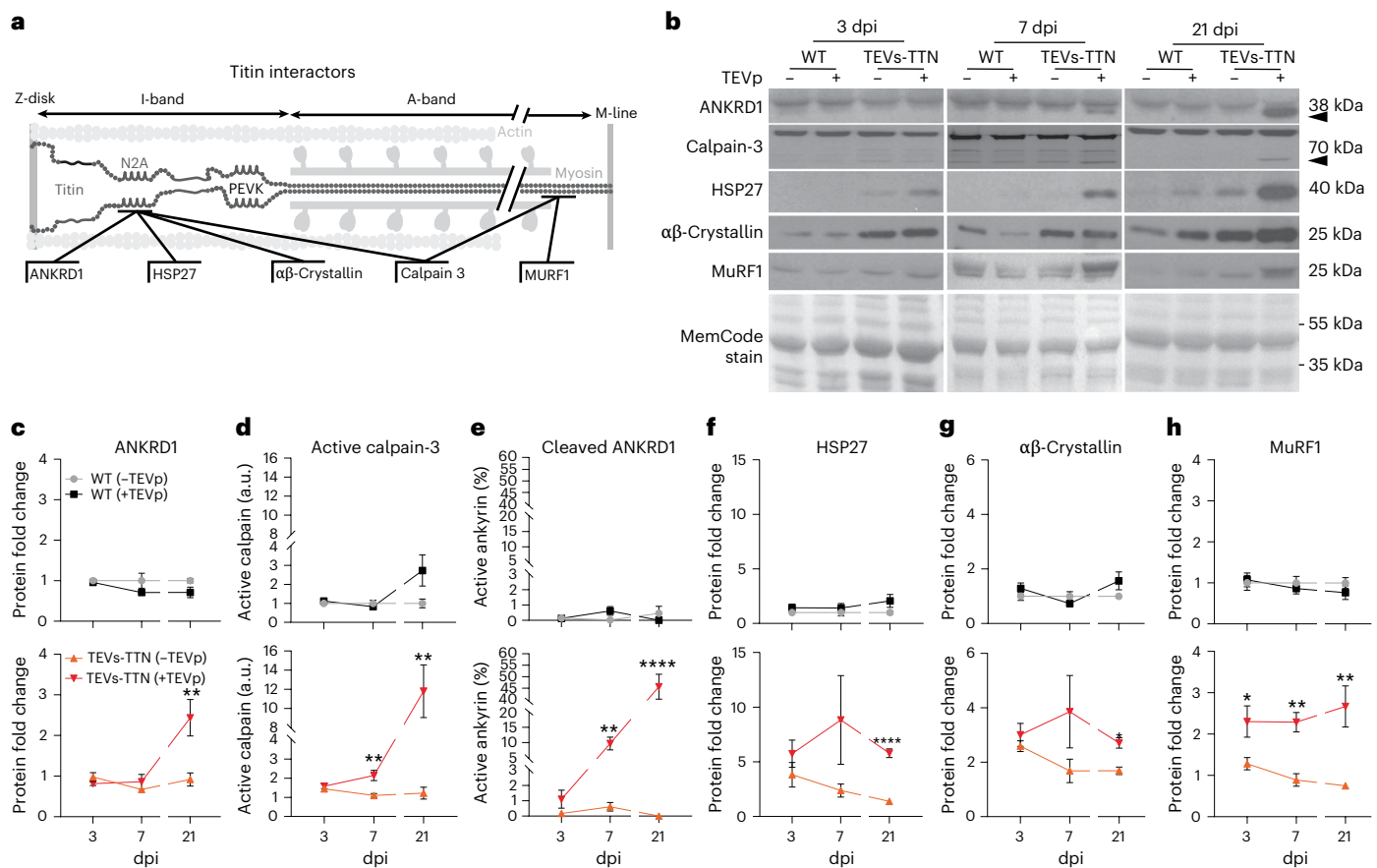


Fig. 6 | Levels of titin interactors in titin mKO muscles. **a**, Scheme representing the binding sites of several titin interactors. **b**, Protein expression of the different titin interactors evaluated by WB. The arrowheads mark active calpain-3 and cleaved ANKRD1. MemCode membrane staining was used for normalization. **c–h**, Quantification of WB results: ANKRD1 (**c**) (n = muscles per group, 3 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 7 dpi, WT (–) n = 4, WT (+) n = 5, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5; 21 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6). Two-tailed Student's t -test WT (–) versus WT (+), 3 dpi P = 0.623, 7 dpi P = 0.1163 and 21 dpi P = 0.0644; two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+), 3 dpi P = 0.1911, 7 dpi P = 0.3289 and 21 dpi P = 0.0097. $^{**}P < 0.01$. Active calpain-3 (**d**) (n = muscles per group, 3 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 7 dpi, WT (–) n = 3, WT (+) n = 4, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5; 21 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6). Two-tailed Student's t -test WT (–) versus WT (+), 3 dpi P = 0.132, 7 dpi P = 0.4072 and 21 dpi P = 0.0692; two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+), 3 dpi P = 0.4678, 7 dpi P = 0.0019 and 21 dpi P = 0.0034. $^{**}P < 0.01$. Cleaved ANKRD1 (**e**) (n = muscles per group, 3 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 7 dpi, WT (–) n = 4, WT (+) n = 5, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5; 21 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6). Two-tailed Student's t -test WT (–) versus WT (+), 3 dpi P = 0.879, 7 dpi P = 0.132 and 21 dpi P = 0.3409; two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+), 3 dpi P = 0.1597, 7 dpi P = 0.0024 and 21 dpi P < 0.0001. $^{**}P < 0.01$ and $^{****}P < 0.0001$. HSP27 (**f**) (n = muscles per group, 3 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN

(–) n = 6, TEVs-TTN (+) n = 6; 7 dpi, WT (–) n = 3, WT (+) n = 4, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5; 21 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6). Two-tailed Student's t -test WT (–) versus WT (+), 3 dpi P = 0.315, 7 dpi P = 0.4965 and 21 dpi P = 0.1108; two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+), 3 dpi P = 0.2767 and 21 dpi P < 0.0001; two-tailed Mann-Whitney's U -test TEVs-TTN (–) versus TEVs-TTN (+), 7 dpi P = 0.0952. $^{****}P < 0.0001$. αβ-Crystallin (**g**) (n = muscles per group, 3 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 7 dpi, WT (–) n = 4, WT (+) n = 5, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5; 21 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6). Two-tailed Student's t -test WT (–) versus WT (+), 3 dpi P = 0.2755, 7 dpi P = 0.229 and 21 dpi P = 0.1276; two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+), 3 dpi P = 0.3932 and 21 dpi P = 0.0016; two-tailed Mann-Whitney's U -test TEVs-TTN (–) versus TEVs-TTN (+), 7 dpi P = 0.1775. $^{*}P < 0.05$. MuRF1 (**h**) (n = muscles per group, 3 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6; 7 dpi, WT (–) n = 4, WT (+) n = 5, TEVs-TTN (–) n = 5, TEVs-TTN (+) n = 5; 21 dpi, WT (–) n = 6, WT (+) n = 6, TEVs-TTN (–) n = 6, TEVs-TTN (+) n = 6). Two-tailed Student's t -test WT (–) versus WT (+), 3 dpi P = 0.7722, 7 dpi P = 0.5191 and 21 dpi P = 0.2882; two-tailed Student's t -test TEVs-TTN (–) versus TEVs-TTN (+), 3 dpi P = 0.0309, 7 dpi P = 0.0013 and 21 dpi P = 0.0033. $^{*}P < 0.05$ and $^{*}P < 0.01$. Cleaved ANKRD1 was calculated as the fraction of cleaved ANKRD1 relative to total ANKRD1. Active calpain-3 was similarly calculated and the result was normalized to WT NaCl-injected samples. Protein fold changes were calculated relative to WT NaCl-injected samples. Data are represented as mean of the biological replicates $\pm$ s.e.m.

Considering the early alteration of MuRF1 in titin mKO muscles, we assessed whether MuRF1 inhibition could block the myopathic response induced by titin cleavage (Extended Data Fig. 8). However, we found that oral treatment with the MuRF1 inhibitor MyoMed205, a compound that limits atrophy in heart failure with preserved ejection fraction (HFpEF)⁵⁴, muscle disuse⁵⁵ and tumour cachexia associated myopathy⁵⁶, was not able to normalize levels of MuRF1 (Extended Data Fig. 8b), global protein ubiquitination (Extended Data Fig. 8c,d) and myofibre size (Extended Data Fig. 8e) in titin mKO samples.

Pathological transcriptional signatures of titin mKO muscles

To examine transcriptional changes induced by titin mKO, we did RNA-seq analysis of the 4 experimental groups at 3, 7 and 21 dpi, including also heterozygous samples at 7 dpi. Principal component analysis (PCA) reveals a progressive change in the transcriptional state of homozygous titin mKO muscles starting at 3 dpi, which reaches 5,821 differentially expressed genes (DEGs) at 21 dpi (Fig. 7a and Extended Data Fig. 9a). In addition, PCA also detects transcriptional alterations in heterozygous titin mKO muscles (Fig. 7a). Consistent with our histology

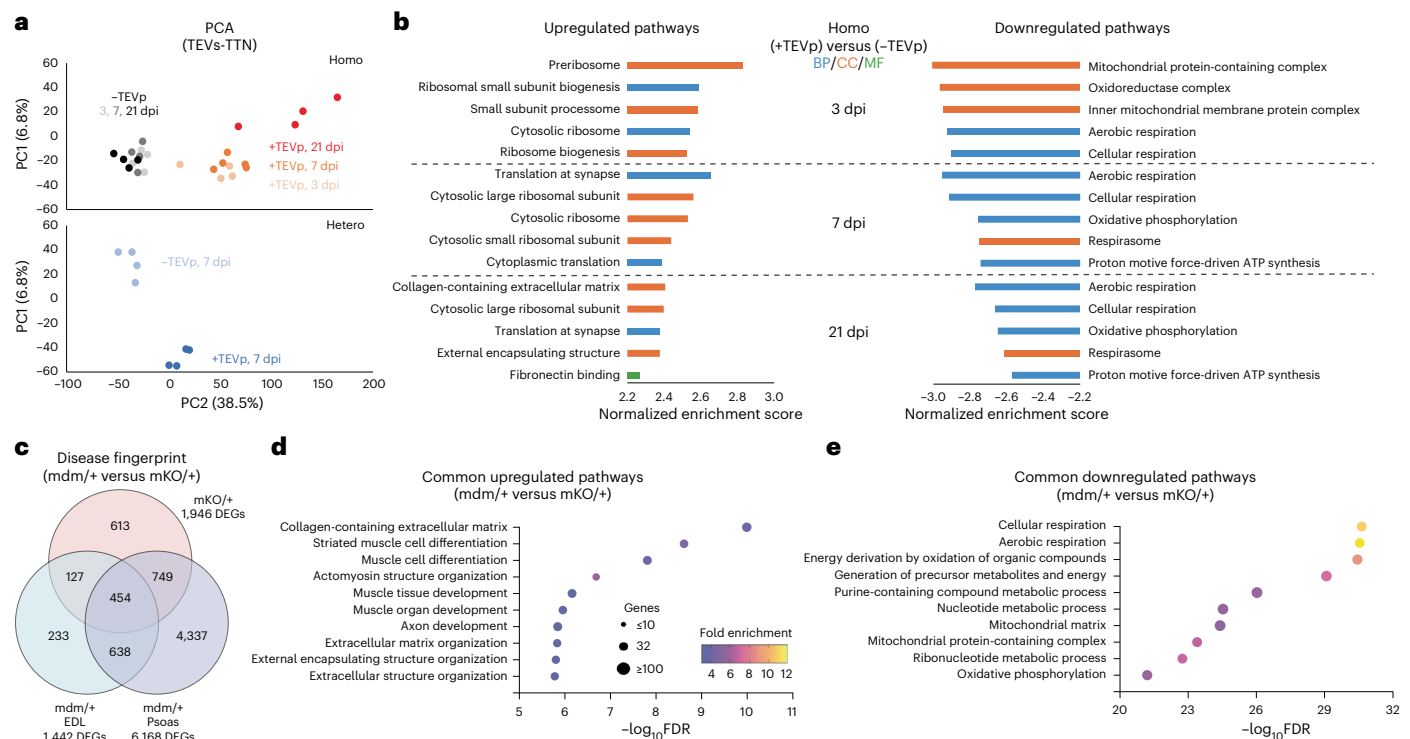


Fig. 7 | Myopathic transcriptional state of titin mKO muscles. a, PCA of gene expression in homozygous and heterozygous TEVs-TTN TA muscles ($n = 4$ muscles per group). **b**, Top 5 most enriched upregulated and downregulated Gene Ontology terms in AAV6-injected homozygous TEVs-TTN muscles relative to contralateral control legs (BP, biological process; CC, cellular component;

MF, molecular function). **c**, Shared DEGs in heterozygous titin mKO muscles, and heterozygous *mdm* EDL and psoas muscles⁵⁸. **d, e**, Top 10 most upregulated (**d**) and downregulated (**e**) Gene Ontology terms of the 1,330 shared DEGs between heterozygous titin mKO at 7 dpi and heterozygous EDL and psoas *mdm* muscles.

results, expression of TEV protease in WT animals leads to measurable changes in gene expression by PCA, but only at 21 dpi (Extended Data Fig. 9b). To capture specific transcriptional responses induced by titin mKO, for all downstream analyses, we removed DEGs shared with the WT group, which mostly belong to Gene Ontology terms related to immune function as expected (Extended Data Fig. 9a). Gene set enrichment analysis⁵⁷ shows upregulation of terms related to protein biogenesis in titin mKO muscles at all time points, both in homozygous and heterozygous conditions (Fig. 7b and Extended Data Fig. 9c). In agreement with histology results, we also find that titin mKO leads to upregulation of terms related to fibrotic processes and a consistent downregulation of terms related to mitochondria (Fig. 7b and Extended Data Fig. 9c). Remarkably, Gene Ontology analyses detect common overexpression of genes involved in fibrosis and cytoskeletal remodeling and downregulation of genes related to mitochondrial activity in both titin mKO muscles and in samples from the classical titinopathy *mdm* mouse model⁵⁸ (Fig. 7c–e and Extended Data Fig. 9d–f). Indeed, 47–68% of DEGs in titin mKO muscles show also altered expression in the *mdm* mice, indicative of similar transcriptional states in these two models (Fig. 7c and Extended Data Fig. 9d–f).

Discussion

Titin, the largest protein in vertebrates, plays multiple fundamental roles in cardiac and skeletal muscle. These range from providing structural support and passive stiffness to sarcomeres, to facilitating assembly of signalling hubs that control muscle physiology^{10,11,14,15}. In addition, titin can be considered as a homeostatic integrator of mechanical signals, as indicated, for example, by the remarkable capacity of muscle to balance sarcomere length and longitudinal hypertrophy to keep force across titin constant⁵. However, understanding how these mechanical mechanisms involving titin operate in vivo has proven challenging, a situation that is shared with other proteins with mechanical function.

Here we demonstrate that cessation of force transmission across titin results in severe muscle atrophy and dysfunction correlating with gradual sarcomere depletion (Figs. 1 and 4). A corollary of this result is that myofibres do not have redundant mechanisms that can effectively compensate for the loss of titin's mechanical function, implying that every single peptide bond out of the several thousands in titin can be regarded as an Achilles heel of muscle tissue. Remarkably, phenotypes stemming from titin mKO rapidly propagate to compartments other than the sarcomere, including the mitochondria, the nucleus and the extracellular matrix (Fig. 2 and Extended Data Fig. 3). An unexpected finding in this regard is that the mechanical integrity of titin is required to stabilize peripheral location of myonuclei, a function that complements the described role of active sarcomeres driving myonuclei positioning during myofibre formation⁵⁹. Another unexpected result is the notable persistence of titin mKO myofibres, which are only partially taken over by regenerating counterparts in a timescale of months (Fig. 5). Considering that targeted EDL and TA muscles contribute little to the physiological state of animals, the local delivery of TEV protease that we have followed ensures that the phenotypes that we capture do not result from global perturbation of organismal homeostasis, in agreement with the lack of noticeable effects in control contralateral legs in AAV6-injected TEVs-TTN mice compared with WT counterparts.

In recent years, gene variants in *TTN* have been linked to a growing number of human muscle dystrophies, including tibial muscle dystrophy^{60–62}, limb girdle muscle dystrophy (R10 titin-related)⁶³, Emery-Dreifuss-like dystrophy⁶⁴, hereditary myopathy with early respiratory failure^{65,66}, and congenital myopathies such as centronuclear myopathy⁶⁷ and autosomal recessive multi-minicore disease with heart disease⁶⁸. Indeed, the term titinopathies is now used to refer to all the above forms of muscle disease when they are caused by titin mutations²⁴. Together with the different types of heart disease triggered by

titin variants⁶⁹, the existence of titinopathies makes *TTN* an important human disease gene. In this context, the histological similarities of titin mKO muscles with human counterparts affected by titinopathy are remarkable. For instance, sarcomere disorganization, myofibre size variability, central myonuclei, altered mitochondrial organization and fibrosis are typical features of most titinopathies^{24–26}; we find all of them in titin mKO muscles (Figs. 2 and 4). This resemblance is particularly notable in the case of heterozygous titin mKO samples, which also show cores in SDH staining and electron microscopy^{24,67} (Fig. 3 and Extended Data Figs. 4e and 5c). Furthermore, gene expression profiles induced by titin mKO and in titinopathy muscles overlap to a considerable extent (Fig. 7c–e and Extended Data Fig. 9d–f). Considering these similarities and the fact that truncated titins recoil towards the Z-disk under force²⁹, a phenotype also observed in mKO titins (Fig. 4b,c), we speculate that loss of mechanical transduction across titin molecules could contribute to disease induced by pathogenic *TTN* truncating variants. Similarly, it is tempting to hypothesize that mutations inducing cleavage of titin (for instance, through the generation of aberrant cleavage sites or by overactivation of endogenous proteases) could be a trigger of muscle disease. In this regard, titin has been shown to be the substrate of calpain-3 and MMP-2 proteases¹⁴. Similar to transgenic mice overexpressing full-length calpain-3 (ref. 70), we find that AAV6-mediated MMP-2 expression in skeletal muscle is not enough to produce myopathy neither at 7 nor at 21 dpi (Extended Data Fig. 10a,b). Accordingly, there is no evidence of titin cleavage in these experiments (Extended Data Fig. 10a). Our observations suggest that MMP-2 requires additional activation (for example, through genetic mutations targeting autoinhibitory regions) to unleash its catalytic activity as reported in the cardiac setting^{71–73}. Considering the activation of calpain-3 that we have detected in titin mKO muscles (Fig. 6d), it is possible that titin cleavage by this protease can amplify the effects of TEVp on titin in TEVs-*TTN* muscles from 7 dpi. Q17

In the past, a few titin KO mouse lines have been generated to model muscle disease. While systemic loss of titin is embryonically lethal, peri- or postnatal muscle-specific counterparts show phenotypes resembling those in our titin mKO samples, including sarcopenia and reduced muscle mass, internal myonuclei, sarcomeric disassembly and increased expression of titin interactors^{10,11}. Hence, cessation of force transduction across titin appears to be sufficient to recapitulate structural and molecular anomalies in titin KO muscles. It is possible that disruption of sarcomere structure is a common contributor to the detected phenotypes. However, some alterations in homozygous titin mKO muscles at 3 dpi precede sarcomere disassembly, which is apparent only at ≥ 7 dpi. These early-onset phenotypes, which also occur in heterozygous titin mKO muscles despite the fact that sarcomere disruption is limited in this case, include mislocalization of desmin, myonuclei and mitochondria (Figs. 2–4 and Extended Data Figs. 4 and 5). We speculate that alteration of force-dependent interactions in slack titin, as proposed for calpain-3 (ref. 74), may sustain these primordial effects even when sarcomere structure is preserved. Also, the fact that titin shows multimeric organization in sarcomeres^{42,43} implies that tension on intact titin molecules may transiently increase as partner titins are cleaved. Hence, we cannot rule out the possibility that some extent of high-force titin mechanosensing is also triggered in mKO muscles. This effect could be particularly relevant in conditions where less than 50% titin molecules are cleaved, such as in heterozygous titin mKO muscles and in homozygous counterparts at 3 dpi. Indeed, the proportion of dysregulated genes in titin mKO muscles that are also dysregulated in *mdm* muscles is higher for heterozygous and 3 dpi homozygous samples (68% and 67%, respectively) than for 7 and 21 dpi counterparts (47% and 44%, respectively), which would agree with the expected high-force mechanosensing in deletion-bearing *mdm* titins⁷⁵ (Fig. 7c–e and Extended Data Fig. 9d–f).

Consistent with perturbed titin-based mechanosensing, in titin mKO samples, we detect altered levels and activity of several titin

interactors that modulate transcription or participate in protein quality control pathways (Fig. 6 and Extended Data Fig. 7b). Remarkably, higher levels of M-line-associated MuRF1 is the only change that we detect already at 3 dpi in homozygous titin mKO muscles, therefore preceding overt sarcomere and muscle mass loss. This result suggests that this titin-interacting ubiquitin ligase is a mediator of the myopathic response in targeted tissues. Supporting this scenario, overexpression of MuRF1 has been shown to cause muscle atrophy⁷⁶. Future research is needed to understand how loss of force transmission across titin results in altered levels of titin interactors and the mechanistic links with the phenotypes present in titin mKO muscles. In this regard, further examination of whether MuRF1 inhibition can prevent the myopathic response induced by titin unloading will be highly informative. Our results in Extended Data Fig. 8 indicate that current strategies to block MuRF1 that limit muscle atrophy in several settings^{54–56} are not enough to prevent the MuRF1 upregulation nor the associated protein ubiquitination in homozygous titin mKO muscles, suggesting that more aggressive treatments may be required in this highly severe setting. Also, the fact that the worst of the mKO phenotypes are avoided at ~25% cleavage in TEVs-*TTN* heterozygous muscles at 7 dpi suggests that the effects of any given fraction of cleaved titin may be dependent on the zygosity of the targeted muscles, with potentially relevant mechanistic implications. Exploring this scenario will require titration of GFP-TEVp expression to ensure equivalent kinetics and extent of titin cleavage in both hetero- and homozygous titin mKO muscles. Q18

Outlook

The development of gene KO technology in mice revolutionized functional genetics in mammals as it allowed to assess biological fitness of animals deprived of a single protein, in many instances uncovering the biological function of the targeted gene and its causative role in familial conditions⁷⁷. However, KO approaches cannot be applied to scrutinize the mechanical roles of multifunctional proteins that must operate under tension. Our work demonstrates the use of TEVp to achieve protein mKOs in vivo, that is, ceased force transduction across the targeted protein with no alteration of protein levels. Taking into account enticing observations using optogenetics to mechanically knock out proteins in vitro^{78,79}, the protease-based strategy that we describe here could be exploited to dissect the complex functions of other mechanical proteins, including pathomechanisms of mutant proteins relevant for human disease such as dystrophin⁴, nebulin⁸⁰, integrins⁸¹, lamins⁸² and cadherins⁸³. Protease-based methods could also be extended to directly compare phenotypes of KO and mKO animals by tuning cleavage sites to couple proteolysis with degran activation⁸⁴. Q19

Methods

Animal experimentation

Mice were housed in ventilated cages with free access to food and water and 12 h day/night cycles. Heterozygous TEVs-*TTN* animals were crossed to obtain WT, heterozygous and homozygous animals. We have described production and basal characterization of TEVs-*TTN* animals before³². 5'-TGCAGTACTGGAGTGTGAAGTATCC-3' (exon 226), 5'-GAAACGTGTGAAGTATCAGGTTAGG-3' (WT) and 5'-GAGCGATGCATCTATGTGTAGG-3' (TEVs-*TTN*) primers were used for genotyping. For Pax7 lineage tracing experiments, heterozygous Pax7^{CreERT} (JAX line #017763) and Rosa26^{tdTomato} (JAX line #007914) were crossed with TEVs-*TTN* animals to generate homozygous TEVs-*TTN* animals heterozygous for Pax7^{CreERT} and Rosa26^{tdTomato}. Treatment with MyoMed205-spiked food followed published protocols⁸⁵. Only female mice were used for the experiments. All experimental procedures were done in accordance with Spanish and European legislation and approved by Comunidad de Madrid ethics committee (protocols PROEX 042/18 and PROEX 107.8/23).

AAV6 production

AAV6 particles were produced by double transfection of HEK293T cells (ATCC) with plasmids containing adenovirus helper sequences together with rep (AAV2) and cap (AAV6) genes (PF0406, Plasmid-Factory), and the pAAV plasmid encoding GFP-tagged uTEV3 protease under the cytomegalovirus promoter (Addgene 234320)³⁸ or N-terminal-HA-tagged MMP-2 under the MHCK7 promoter (Vector Builder). Cell lysates were treated with 150 U ml⁻¹ Benzonase (Millipore) for 30 min at 37 °C and clarified by centrifugation for 5 min at 3,000 × *g* at room temperature. Viral particles were purified by iodixanol gradient ultracentrifugation for 3 h at 183,000 × *g* at 16 °C using Polypropylene Optiseal tubes (361625, Beckman Coulter), further concentrated using Amicon Ultra-15 tubes (100 K MWCO, Millipore) and resuspended in PBS buffer supplemented with 0.001% Pluronic F-68. Particle quantity was determined by real-time PCR targeting cytomegalovirus promoter: ACCATGGTGATGCGGTTTGT (forward) and ATGGGGTGGAGACTTGGAAATC (reverse) primers. Titres are expressed as viral genomes per ml (vg ml⁻¹).^{Q20}

Intramuscular injection of AAV6

Female mice (10–12 weeks old) were anaesthetized using ketamine/xylazine (100 mg kg⁻¹/10 mg kg⁻¹) and injected with 1.5 × 10¹⁰ viral genomes per leg (resulting in infection of both EDL and TA muscles), or 25 µl of NaCl 0.9% as the control. Mice were placed in a recovery machine until full recovery from anaesthesia and placed back in their breeding cages until dissection. Mice were euthanized by CO₂ inhalation or by cervical dislocation. For lineage tracing experiments, AAV6 injection procedure was preceded by 5 consecutive days of intraperitoneal injections of 75 mg kg⁻¹ tamoxifen diluted in corn oil, followed by a resting period of 10 days.^{Q21}

Protein expression analyses

A small piece of frozen muscle was weighed and placed in a TaKaRa BioMasher Standard tube (9791A, Takara) with 40 µl of protein extraction buffer (50 mM Tris buffer, pH 6.8, 10 mM EGTA, 3% SDS, 50 mM *N*-ethylmaleimide) per mg of tissue. Muscles were homogenized using a TaKaRa BioMasher Standard microtube homogenizer (9790B, Takara) and heated at 60 °C for 10 min, and the soluble fraction was obtained by centrifugation at 18,000 × *g* for 5 min at room temperature. To detect titin cleavage, the protein extract was diluted with a 4X bromophenol blue mix (0.3% bromophenol blue/50% glycerol) and loaded in 2% polyacrylamide/0.5% agarose gels⁸⁶. Gels were stained using Coomassie Blue. For WB, protein extracts in the 4X bromophenol blue mix were loaded in 12% polyacrylamide gels, transferred to nitrocellulose membranes and stained with primary and subsequent horseradish peroxidase-coupled secondary antibodies (antibodies are reported in Supplementary Table 1). Chemiluminescence staining was performed using Amersham ECL Western Blotting Reagent (RPN2106, Cytiva). Membrane MemCode Reversible Protein Stain (24580, Thermo Scientific) was performed and imaged before antibody incubations and results were used for load normalization. For fingerprinting mass spectrometry, proteins were digested in-gel with trypsin (V5111)⁸⁷ and analysed using an Evosep One chromatographic system (Evosep Biosystems) coupled with a Thermo Orbitrap Eclipse (Thermo Fisher) mass spectrometer, using a 21 min gradient separation and data-dependent MS/MS spectra. Protein identification was conducted using Sequest HT Proteome Discoverer (Thermo Fisher). Identifications were validated based on probability thresholds and grouped according to the principles of parsimony by Scaffold Software (Proteome Software).^{Q22}

Histology

Upon dissection, TA was frozen in liquid-nitrogen-cooled isopentane, whereas EDL was fixed using 4% paraformaldehyde, cryopreserved in 30% sucrose, embedded in Tissue-Tek (4583, Sakura) and frozen.^{Q23}

For MuRF1, ANKRD1 and MMP-2 (HA tag) immunofluorescence, EDL was fixed using 40% methanol/40% acetic acid/20% ultrapure water before sucrose treatment and embedding. Cryosections (8 µm) were permeabilized using 0.3% Triton X-100 (X100, Sigma), blocked with bovine serum albumin (A7906, Sigma) and stained with Alexa-coupled WGA or with primary and secondary antibodies in the case of immunofluorescence (Supplementary Table 2). Preparations were imaged on Nikon Eclipse 90i Timelapse (transverse TA sections) or Leica SP8 (longitudinal EDL sections) confocal microscopes. Full muscle transverse section images were recorded in a Zeiss AxioScan 7 Scanner Microscope (Zeiss) and images processed with ZEN 2.6.

Atomic force microscopy

Measurements were performed using a commercial JPK NanoWizard 4XP instrument (Bruker-JPK, Berlin) coupled to an inverted Axio Observer D1 optical microscope (Carl Zeiss) and processed using JPK Data Analysis Software. TA muscle cryosections with a thickness of 8 µm were probed at room temperature in PBS using CP-qp-CONT-SiO-A-5 cantilevers (NanoAndMore GmbH) with 2-µm-diameter colloidal spherical probes and pre-calibrated spring constant of 0.6 N m⁻¹. Force–distance (FD) curves were recorded in contact mode on the tissue surface to determine Young's moduli. Approach–retract cycles were performed at 5 µm s⁻¹, a ramp size of 2 µm and a setpoint force of 5 nN, limiting the indentation to 0.8 µm. FD measurements were spread across at least 3 different areas of 100 × 100 µm² and several millimetres apart on each tissue sample. We analysed between 150 and 500 FD curves per tissue sample. Young's modulus values (*E*) were calculated using the JPK Data Analysis Software, by fitting the approach section of the curve to the Hertz⁸⁸ model for spherical indenters using equation (1):

$$F = \frac{4}{3} \frac{E}{1 - \nu^2} \sqrt{R\delta^3} \quad (1)$$

In equation (1), *F* is the applied force, *R* is the probe radius, *ν* is Poisson's ratio, and *δ* is the indentation. Tissues mostly consist of water and are considered incompressible; hence, a Poisson ratio of 0.5 was chosen.^{Q24}

Ex vivo muscle force production

Mice were euthanized and EDL was isolated, tied using 5-0 silk suture thread (Fine Science Tools) and attached to a force transducer (1200A Isolated Muscle Test System using 610A Dynamic Muscle Control Lab-Book, Aurora Scientific). EDL muscle was placed between platinum electrodes in a chamber continuously perfused with Ringer solution (137 mM NaCl, 5 mM KCl, 1.2 mM NaH₂PO₄, 1 mM MgSO₄, 2 mM CaCl₂, 10 mM glucose, 24 mM NaHCO₃). Optimal muscle length was determined using 1 Hz twitches and finally adjusted using 100 Hz stimulations spaced by 1 min. A force–frequency protocol consisting on stimulation trains of 0.5 s (1 Hz, 10 Hz, 20 Hz, 50 Hz, 80 Hz, 100 Hz, 120 Hz, 150 Hz and 200 Hz) spaced by 1 min was applied. Specific force was calculated with 611A Dynamic Muscle Analysis v.501 by dividing the maximal force (mN) with the EDL cross-sectional area calculated as wet muscle weight (mg)/optimal muscle length (mm) × mammalian muscle density (1.06 mg mm⁻³).

Image analysis

WGA or HaloTMR-stained myofibres in histological sections were segmented using Cellpose 2.0 (ref. 89). Thresholds for GFP/necrosis/eMHC/desmin mislocalization were calculated within every experimental time point as these were stained, imaged and analysed separately. For the analysis of myonuclei localization, additional segmentation of DAPI signal was performed using Fiji plugin StarDist⁹⁰; assignment of their corresponding myofibre was calculated from myofibre ROI. Myonuclei distance to myofibre centroid was obtained. The ratio of distances of myonuclei and myofibre membrane to myofibre centroid was calculated according to

$$\text{Myonuclei distance ratio} = \frac{\text{Myonuclei to myofibre centre distance}}{\text{Myofibre centre to periphery distance}} \quad (2)$$

For the co-localization test of α -actinin, PEVK-Titin and cTEVsite, images of longitudinal EDL sections were first deconvolved using Huygens deconvolution software (Science Volume Imaging). Deconvolved images from 17–24 myofibres imaged in at least 3 different mice were used to obtain fluorescence signals across 73–106 sarcomeres, which were normalized and averaged at the α -actinin peak for every sarcomere. Counting of internal myonuclei in HE sections was performed manually using a CellCounter ImageJ tool. Quantification of the degree of fibrosis was performed using a custom script written in ImageJ macro language and normalized with the contralateral leg in the same slide.

Q25

In vitro validation of cTEVsite antibody

TEVp and a recombinant fragment encompassing Ig86-TEVs-HaloTag-Ig87 were produced, and proteolytic digestion was performed as previously described³². We transferred the fragments to a nitrocellulose membrane and stained with a primary antibody targeting the TEV recognition site (NBP2-37831, Novus Biologicals) and peroxidase-coupled secondary targeting rabbit IgG (A0545, Sigma-Aldrich). In parallel, the products of digestion were also loaded in a dot blot transfer apparatus, where native proteins were air-vacuum-transferred into the nitrocellulose membrane and stained with primary and secondary antibodies as above. Total protein staining with the Membrane MemCode Reversible Protein Stain (24580, Thermo Scientific) was performed and imaged before antibody incubations.

Q26

Electron microscopy

For electron microscopy, EDL muscle was fixed in 2.5% glutaraldehyde and 2.5% paraformaldehyde in 50 mM cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide for 60 min and dehydrated through a series of ethanol solutions (30%, 50%, 70%, 95% and 100%) and acetone. After the last dehydration step, samples were incubated consecutively in 1:3, 1:1 and 3:1 DURCUPAN resin/acetone and cured at 60 °C for 48 h. Ultrathin sections (50–60 nm) were obtained using a diamond knife (Diatome) in a Leica Reichert Ultracut S Ultramicrotome (Leica) and collected in microscope nickel grids (200 mesh). The sections were counterstained with 2% uranyl acetate in water for 20 min, followed by a lead citrate solution for 10 min. They were examined with a JEM1010 electron microscope (JEOL) equipped with an Orius SC200 digital camera (Gatan).

Q27

Gene expression analysis

Muscles were lysed using Precellys homogenizing CK28 beads and Minilys homogenizer (Bertin Technologies) using TRI Reagent (Sigma) following the manufacturer's instructions. RNA quality was quantified using RNA Integrity Number (RIN) in a 2100 Bioanalyzer (Agilent Technologies) and next-generation sequencing was performed with samples showing RIN > 7. Total RNA (150 ng) was used to generate barcoded RNA-seq libraries using the NEBNext Ultra II Directional RNA Library preparation kit (New England Biolabs) according to the manufacturer's instructions. First, poly A+ RNA was purified using oligo-dT-attached magnetic beads, followed by fragmentation and first and second cDNA strand synthesis. Next, cDNA ends were repaired and adenylated. The NEBNext adaptor was then ligated, followed by second strand removal, uracil excision from the adaptor and PCR amplification. The size of the libraries was checked using the Agilent 2100 Bioanalyzer, and the concentration was determined using the Qubit fluorometer (ThermoFisher). Libraries were sequenced at 650 pM on a NextSeq 2000 (Illumina) using a P3 flow cell to generate 50 bases paired-end reads. FastQ files for each sample were obtained using bcl2fastq v2.2.20 software (Illumina). RNA-seq experiments were performed in the Genomics Unit of the CNIC. Sequencing reads were pre-processed by a pipeline that used FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) to assess read quality, and Cutadapt⁹⁷ to remove

adaptor remains from Illumina and to discard reads that were shorter than 30 bp. The resulting reads were mapped against reference transcriptome GRCm38.99 and quantified with RSEM⁹², using Bowtie as the aligner. The expected expression counts calculated with RSEM were processed with a pipeline that used Limma package⁹³, from Bioconductor, for normalization and differential expression testing, taking into account those genes expressed with at least 1 count per million (CPM) in at least three samples. Changes in gene expression were considered significant if associated to Benjamini and Hochberg adjusted $P < 0.05$ and absolute log2 fold change higher than 0.5 and lower than -0.5. Functional enrichment analyses were performed with gene set enrichment analysis of pathways comprising more than 15 and less than 500 genes (GSEA)⁹⁴. Graphical representations were generated with R. For comparison of the genes differentially expressed in titin mKO and mdm⁵⁸ muscles, data were reanalysed using Galaxy web platform tools⁹⁵, including alignment (STAR), expression counts (featureCounts) and differences in gene expression (DESeq2). The Gene Ontology enrichment of commonly dysregulated genes in the two lines was calculated using PANTHER⁹⁶. Accession codes are GSE271916 for the RNA-seq data of TEVs-TTN mice and GSE158283 and GSE210263 for mdm mice⁵⁸.

Statistical analysis and preparation of figures

All experiments were performed and analysed in a blinded manner, and the investigators were unaware of the genotype of the mice. Data are presented as mean $\pm$ standard error of the mean (s.e.m.). Normal distribution of data was assessed using the Shapiro–Wilk test. For normally distributed data, the significance of changes was examined by two-tailed Student's *t*-test for comparison of two groups or by one-way ANOVA, followed by Tukey's post hoc test for comparison of more than two groups. In the case of not-normally distributed data, the Mann–Whitney test was used to compare two groups. Significant differences are illustrated in the figures as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. Statistical analyses were performed using GraphPad Prism and tests were two-sided. Figures have been generated using GraphPad Prism 10, Adobe Illustrator and BioRender.com for Figs. 1a,b,h,k and 5a,h and Extended Data Figs. 8a and 10a.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The main data supporting the results in this study are available within the paper and its Supplementary Information. Non-commercial materials are available from the corresponding authors. Transcriptomic data from bulk RNA-seq referenced GRCm38.99 transcriptome are available from the Gene Expression Omnibus (GEO) with GSE271916 accession code and transcriptomic data of EDL and psoas muscles were obtained from GSE158283 ref. 58 and GSE210263 ref. 58. Source data are provided with this paper.

Code availability

The custom code for the measurement of fibrosis extent on picrosirius red-stained muscles is available in the Supplementary Information of this article.

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Author contributions

R.S.-R., N.V., D.V.-C., E.H.-G., M.A.L.-U., M.R.P. and J.A.-C. conceived and designed the experiments. R.S.-R., N.V., M.G.-H., A.C.D., M.G.-V. and J.A.L. executed and/or analysed the experiments. V.L.-C., J.S., O.G.-S., M.I.S. and J.V. contributed to the materials/analysis tools. R.S.-R. and J.A.-C. drafted the paper with input from all authors.

Competing interests

The authors declare no competing interests.

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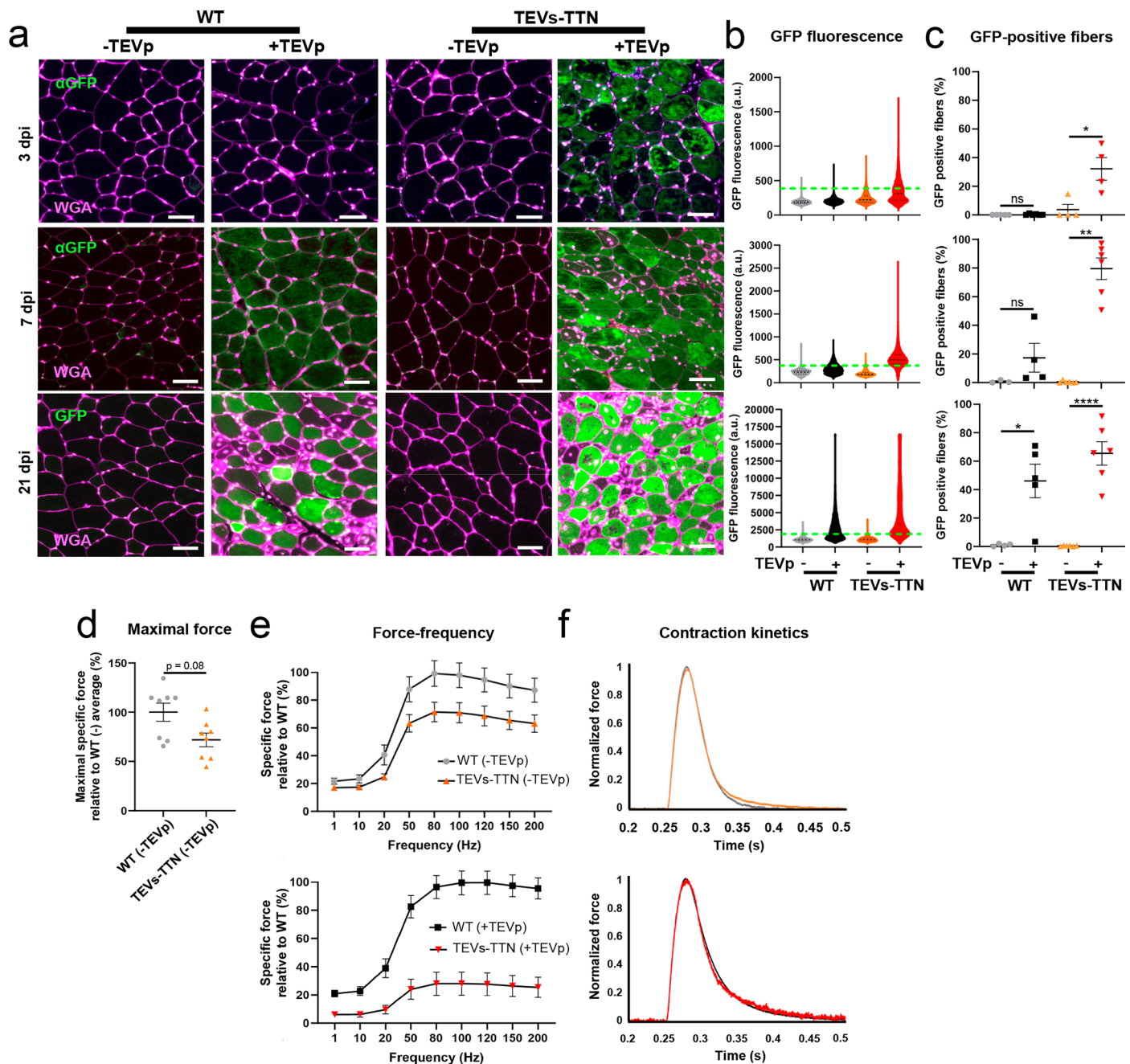
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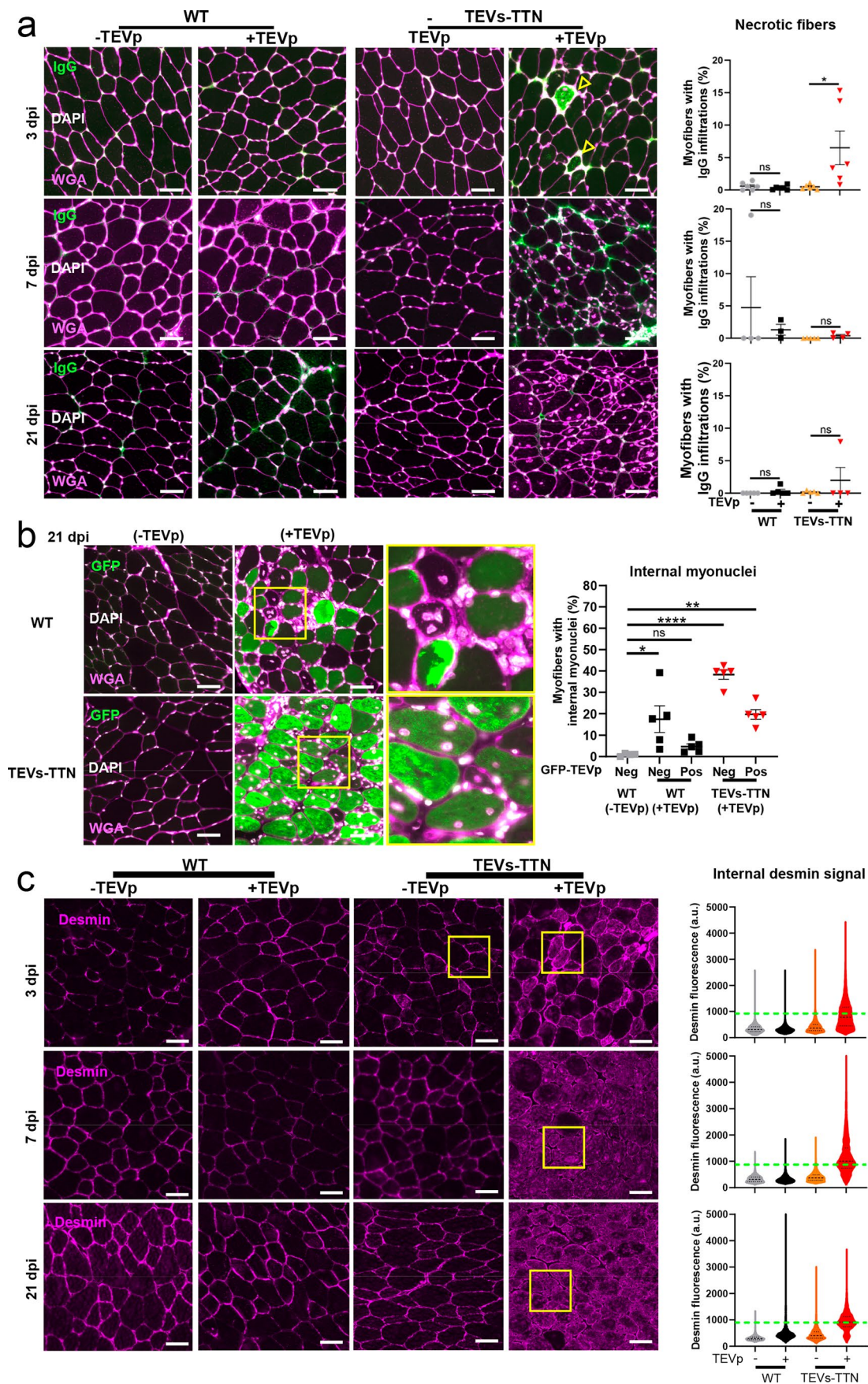
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Extended Data Fig. 1 | Phenotyping of titin mKO muscles. **a**, GFP-TEVp immunofluorescence on transverse TA sections. Scale bars represent 50 μm. **b**, Violin plot of GFP levels per individual myofiber (n=myofibers/group, 3 dpi: WT (-) n=8351, WT (+) n=9820, TEVs-TTN (-) n=7529, TEVs-TTN (+) n=5680; 7 dpi: WT (-) n=2949, WT (+) n=5612, TEVs-TTN (-) n=7026, TEVs-TTN (+) n=8474; 21 dpi: WT (-) n=6150, WT (+) n=8537, TEVs-TTN (-) n=8783, TEVs-TTN (+) n=8440), threshold marked with a dashed line. Experiments at different time points were done independently so the resulting GFP fluorescence intensities cannot be compared directly. **c**, fraction of GFP-TEVp-positive myofibers (n=muscles/group, 3 dpi: WT (-) n=4, WT (+) n=5, TEVs-TTN (-) n=3, TEVs-TTN (+) n=4; 7 dpi: WT (-) n=3, WT (+) n=4, TEVs-TTN (-) n=6, TEVs-TTN (+) n=6; 21 dpi: WT (-) n=4, WT (+) n=5, TEVs-TTN (-) n=6, TEVs-TTN (+) n=6). Data are represented as mean of the biological replicates ± SEM. Two-tailed Student's t-test WT (-) vs WT (+): 3 dpi p=0.2234, and 21 dpi p=0.0121. Two-tailed Student's t-test TEVs-TTN (-) vs TEVs-TTN (+): 21 dpi p<0.0001. Two-tailed Mann-Whitney's t-test WT (-) vs WT (+): 3

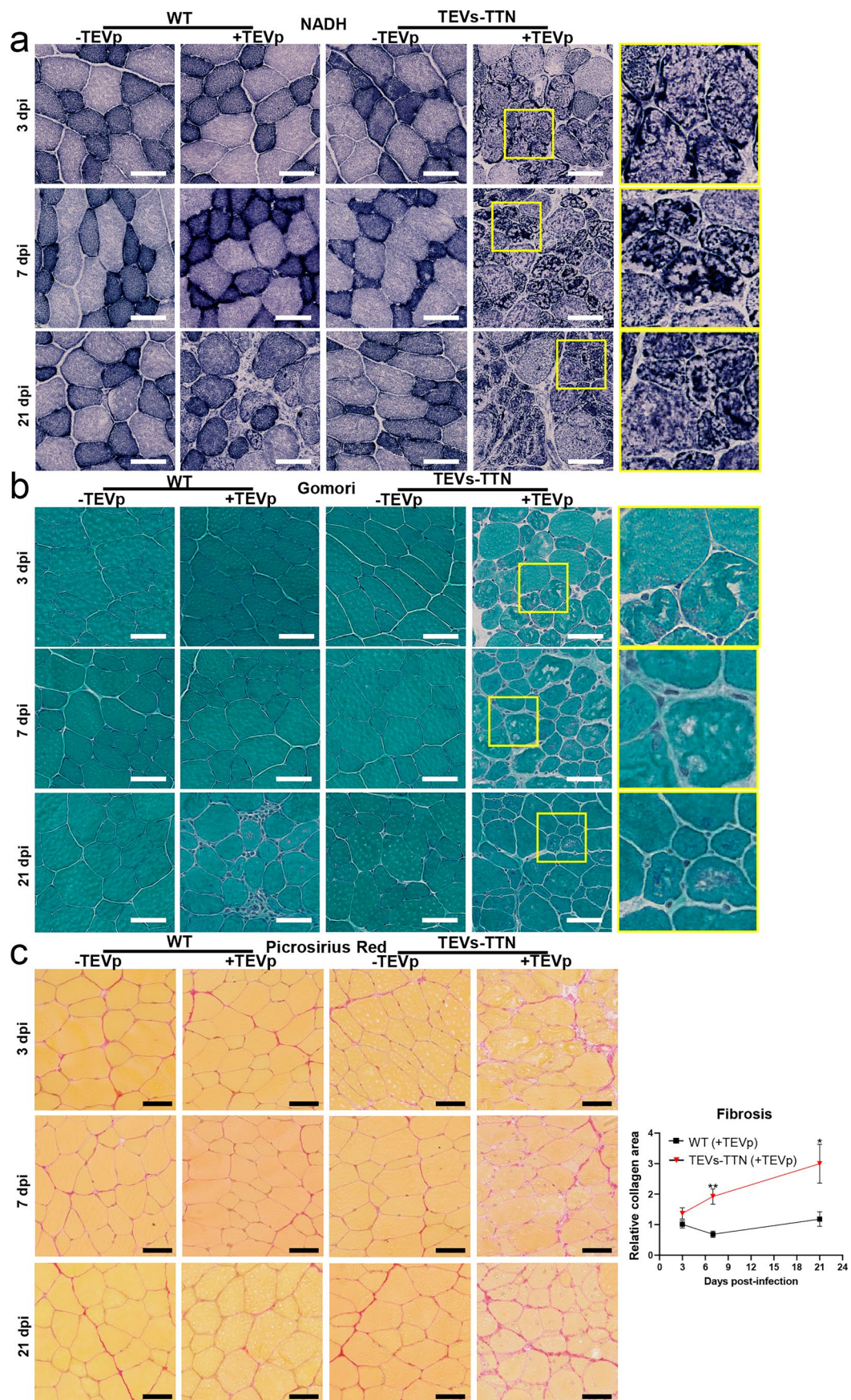
dpi p=0.1342. Two-tailed Mann-Whitney's t-test TEVs-TTN (-) vs TEVs-TTN (+): 3 dpi p=0.0286, and 7 dpi p=0.0022. **d**, Maximal specific active force produced by control EDL muscles (n=muscles/group, WT (-) n=8, TEVs-TTN (-) n=8). Two-tailed Student's t-test WT (-) vs TEVs-TTN (-) p=0.08. Data are represented as mean of the biological replicates ± SEM. **e**, Specific force produced by EDL of the different groups at different stimulation frequencies at 7 dpi. Data are represented as mean ± SEM. (n=muscles/group, WT (-) n=8, WT (+) n=8, TEVs-TTN (-) n=8, TEVs-TTN (+) n=7). Two-tailed Student's t-test WT (-) vs WT (+): 3 dpi p=0.2234, and 21 dpi p=0.0121. Two-tailed Student's t-test TEVs-TTN (-) vs TEVs-TTN (+): 21 dpi p<0.0001. Two-tailed Mann-Whitney's t-test WT (-) vs WT (+): 3 dpi p=0.1342. Two-tailed Mann-Whitney's t-test TEVs-TTN (-) vs TEVs-TTN (+): 3 dpi p=0.0286, and 7 dpi p=0.0022. **f**, Average normalized force kinetics upon single stimulation of muscles from the different groups. Data are represented as mean. (n=muscles/group, WT (-) n=8, WT (+) n=8, TEVs-TTN (-) n=8, TEVs-TTN (+) n=7). Source data.



Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Quantification of myofiber necrosis and myonuclei location. a, Immunofluorescence using anti mouse IgG to study the presence of immunoglobulins in necrotic myofibers and quantification (n=muscles/group, 3 dpi: WT (-) n=6, WT (+) n=6, TEVs-TTN (-) n=6, TEVs-TTN (+) n=6; 7 dpi: WT (-) n=4, WT (+) n=3, TEVs-TTN (-) n=5, TEVs-TTN (+) n=5; 21 dpi: WT (-) n=5, WT (+) n=5, TEVs-TTN (-) n=5, TEVs-TTN (+) n=4). Data are represented as mean of the biological replicates $\pm$ SEM. Scale bars represent 50 μ m. Two-tailed Student's t-test WT (-) vs WT (+): 3 dpi p= 0.3492 and two-tailed Student's t-test TEVs-TTN (-) vs TEVs-TTN (+): 3 dpi p= 0.0427. Two-tailed Mann-Whitney's t-test WT (-) vs WT (+): 7 dpi p= 0.6571, and 21 dpi p= 0.444; and two-tailed Mann-Whitney's t-test TEVs-TTN (-) vs TEVs-TTN (+): 7 dpi p= 0.0873 and 21 p= 0.6825. **b,** GFP-TEVp immunofluorescence on transverse TA sections (regions zoomed on the left are indicated). Fraction of myofibers with internalized myonuclei depending on GFP-TEVp positivity (n=muscles/group, WT (-TEVp) n=4, WT (+TEVp) negative

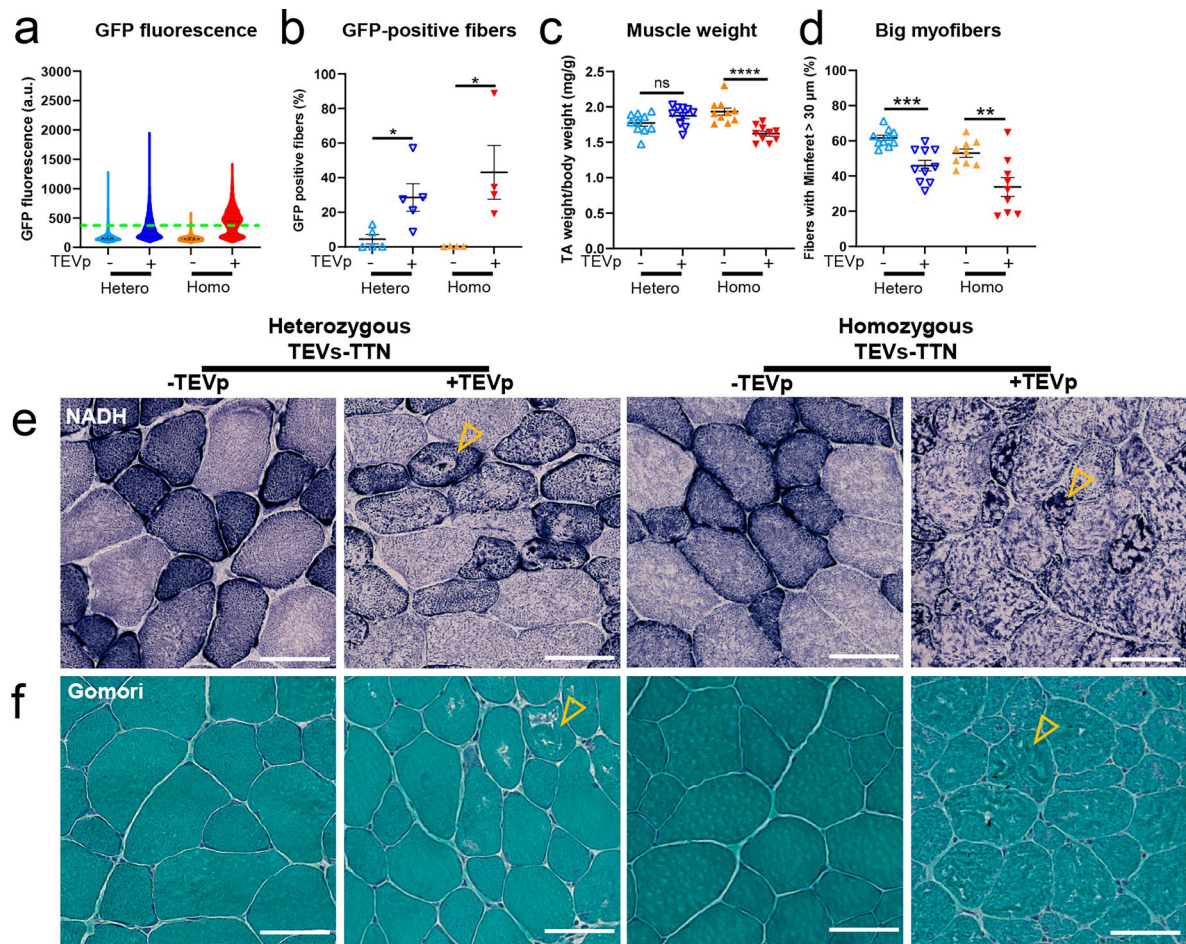
n=5), WT (+TEVp) positive n=5), TEVs-TTN (+TEVp) negative n=5), TEVs-TTN (+TEVp) positive n=5). Data are represented as mean $\pm$ SEM. Scale bars represent 50 μ m. One-way ANOVA with multiple comparisons corrected with Dunnett's test. WT-TEVp GFP-negative vs: WT+TEVp GFP-negative (p= 0.0212), WT+TEVp GFP-positive (p= 0.9278), TEVs-+TEVp GFP-negative (p< 0.0001), and TEVs-+TEVp GFP-positive (p=0.0081). **c,** Desmin immunofluorescence in transverse TA sections and violin plots of its sarcoplasmic intensity in individual myofibers (n=myofibers/group, 3 dpi: WT (-) n=10515, WT (+) n=10028, TEVs-TTN (-) n=8070, TEVs-TTN (+) n=7445; 7 dpi: WT (-) n=7543, WT (+) n=8100, TEVs-TTN (-) n=10258, TEVs-TTN (+) n=7630; 21 dpi: WT (-) n=5293, WT (+) n=8384, TEVs-TTN (-) n=5571, TEVs-TTN (+) n=3641). Boxes indicate highlighted regions in Fig. 2e. The threshold used for quantification in Fig. 2f is marked with a dashed line. Scale bars represent 50 μ m.



Extended Data Fig. 3 | See next page for caption.

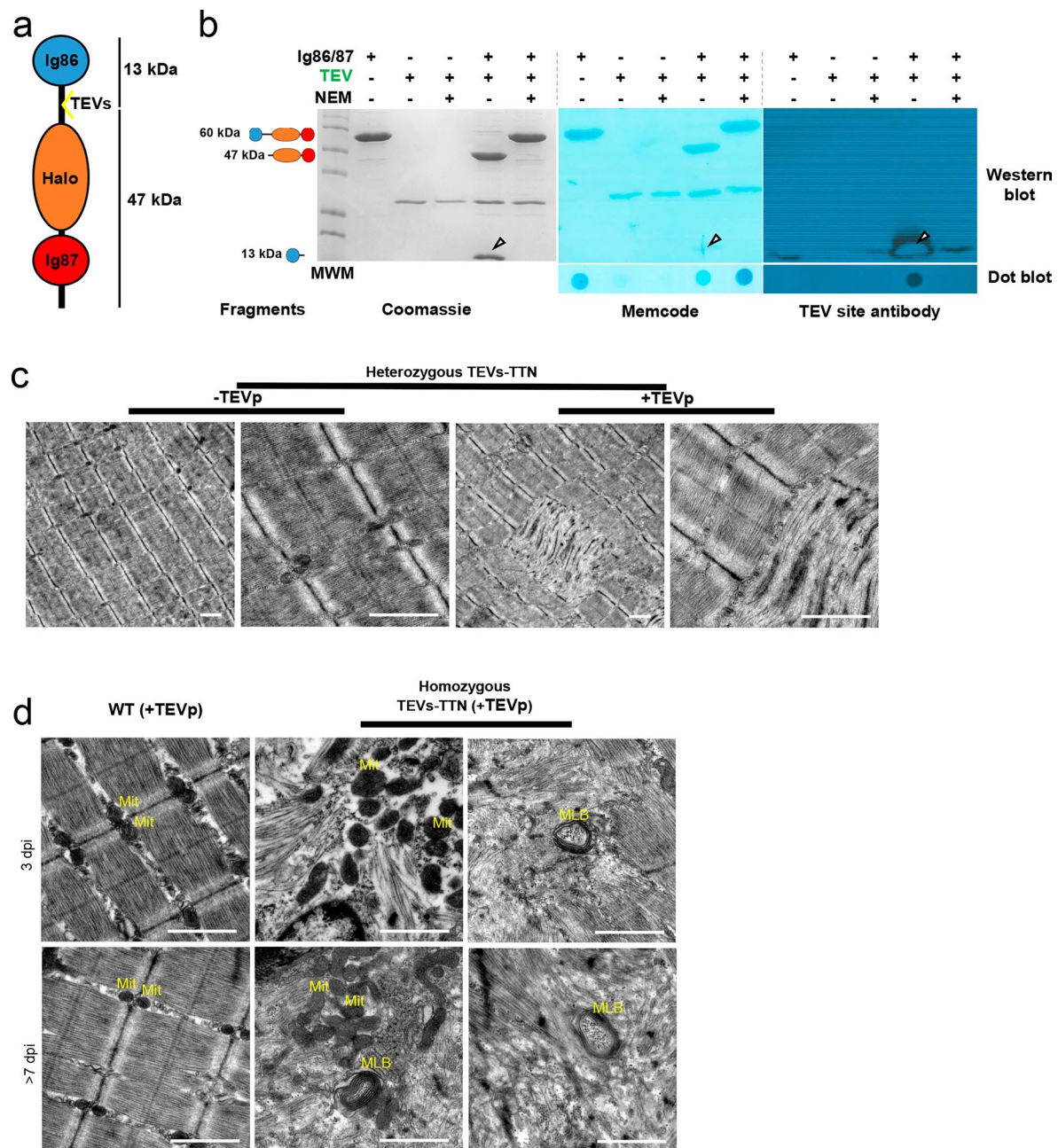
Extended Data Fig. 3 | Mitochondrial mislocalization and aggregation and fibrosis induced by titin mKO. **a**, NADH activity staining of muscle TA cryosections (n=5 muscles/group). Scale bars represent 50 μm . **b**, Modified Gomori staining (n=5 muscles/group). Scale bars represent 50 μm . **c**, Picrosirius staining marking mature collagen deposition and quantification of the fibrosis

extent in AAV6-injected TA muscles normalized with their contralateral leg (n=5 muscles/group except for WT samples at 7 dpi where n=4). Scale bars represent 50 μm . Data are represented as mean of the biological replicates $\pm$ SEM. Two-tailed Student's t-test WT (+) vs TEVs-TTN (+): 3 dpi p= 0.1344, 7 dpi p= 0.0023, and 21 dpi p= 0.0235.



Extended Data Fig. 4 | Comparative effects of GFP-TEVp expression in heterozygous and homozygous TEVs-TTN mice at 7 dpi. **a**, Violin plot of GFP levels in the immunofluorescence shown in Fig. 3a (n=myofibers/group, Hetero (-) n=7477, Hetero (+) n=6118, TEVs-TTN (-) n=7400, TEVs-TTN (+) n=4834). **b**, Fraction of GFP-TEVp-positive myofibers (n=muscles/group, Hetero (-) n=5, Hetero (+) n=5, TEVs-TTN (-) n=4, TEVs-TTN (+) n=4). Data are represented as mean of the biological replicates $\pm$ SEM. Two-tailed Student's t-test Hetero (-) vs Hetero (+) $p=0.0211$ and two-tailed Student's t-test Homo (-) vs Homo (+) $p=0.0331$. **c**, TA muscle weight to body weight ratio (n=10 muscles/group). Data are represented as mean of the biological replicates $\pm$ SEM. Two-tailed Student's t-test Hetero (-) vs Hetero (+) $p=0.1197$ and two-tailed Student's t-test Homo (-)

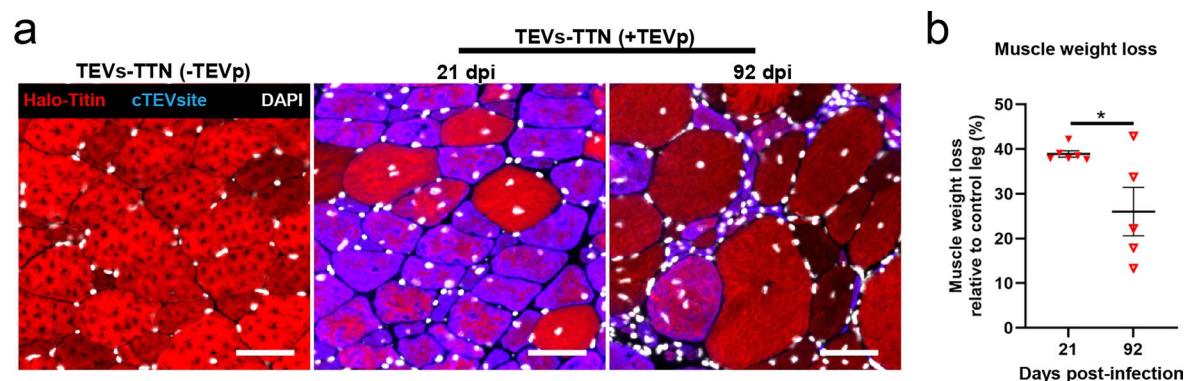
vs Homo (+) $p<0.0001$. **d**, Fraction of myofibers with MinFerret diameter larger than 30 μm (n=muscles/group, Hetero (-) n=10, Hetero (+) n=10, TEVs-TTN (-) n=9, TEVs-TTN (+) n=9). Data are represented as mean of the biological replicates $\pm$ SEM. Two-tailed Student's t-test Hetero (-) vs Hetero (+) $p=0.0002$ and two-tailed Student's t-test Homo (-) vs Homo (+) $p<0.0046$. **e**, NADH staining in heterozygous and homozygous mKO muscles. Arrowheads mark cores in heterozygous samples and mitochondrial mislocalization in homozygous samples (n=5 muscles/group). Scale bars represent 50 μm . **f**, Gomori staining in heterozygous and homozygous mKO muscles. Arrowheads mark stain-free zones both in heterozygous and homozygous mKO muscles. (n=5 muscles/group). Scale bars represent 50 μm .



Extended Data Fig. 5 | Sarcomere disassembly in titin mKO muscles.

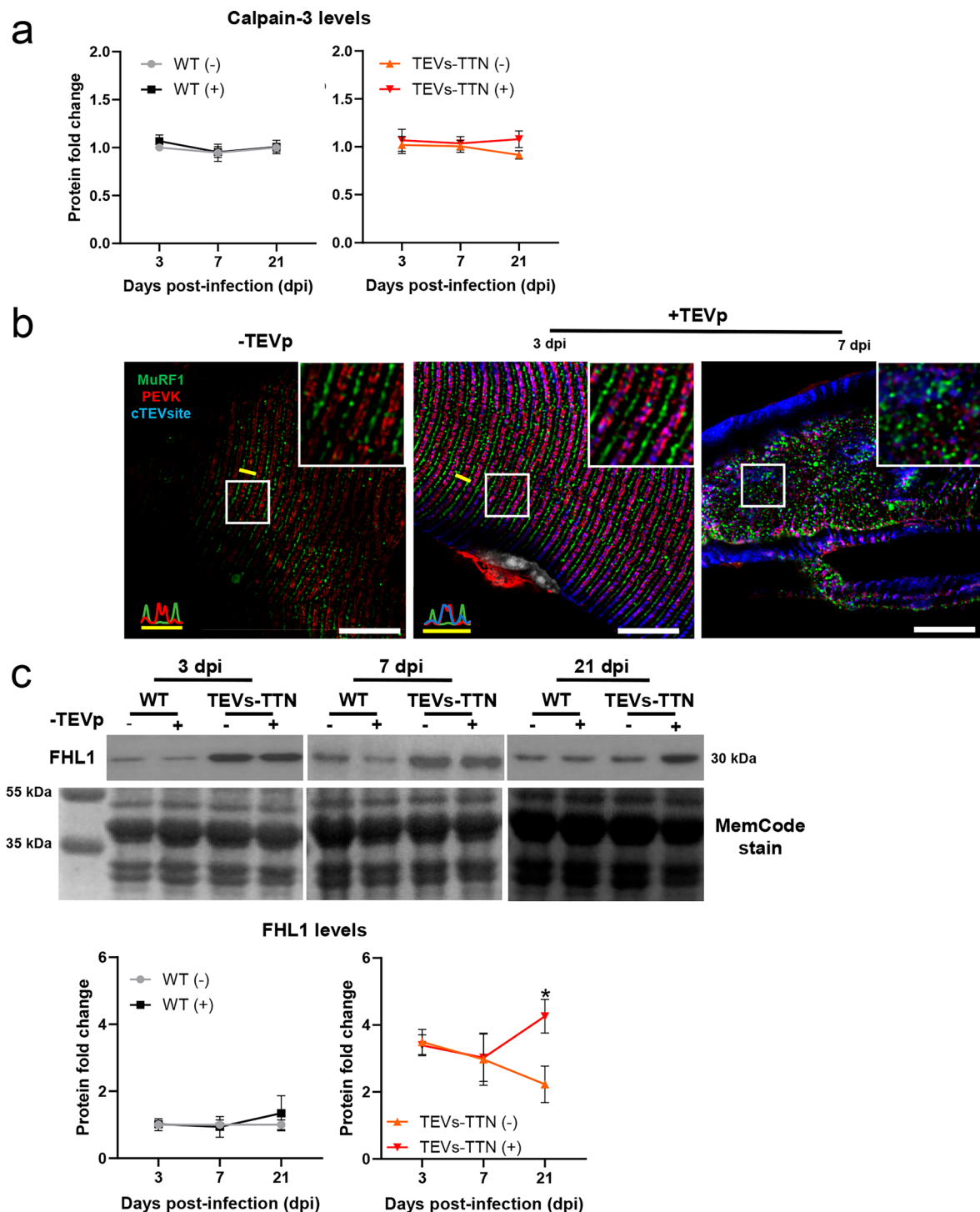
a, Scheme of the recombinant Ig86-TEVs-Halo-Ig87 titin fragment that, upon TEV protease treatment, results in 13 kDa and 47 kDa fragments. **b**, Validation of the specificity of anti-cTEVsite antibody by SDS-PAGE and WB (denaturing conditions) and dot-blot (native conditions). Ig86-TEVs-Halo-Ig87 is incubated with TEV protease to induce cleavage. A control sample where TEV protease activity is blocked by treatment with N-ethylmaleimide (NEM) is also included. Anti cTEVsite signal only occurs in samples where the 13-KDa fragment is present,

both in native and denaturing conditions. Molecular weight marker (MWM) used in Coomassie was Precision Plus Protein Dual Color Standards (1610374, BioRad). **c**, Transmission electron microscopy on longitudinal EDL sections of heterozygous muscles at different magnifications (representative images from $n=3$ muscles/group). Scale bars represent 1 μm . **d**, Transmission electron microscopy on longitudinal homozygous EDL sections of AAV6-injected WT and TEVs-TTN muscles (representative images from $n=3$ muscles/group, Mit: Mitochondria, MLB: multilamellar body). Scale bars represent 1 μm .



Extended Data Fig. 6 | Titin mKO muscles at long time points. a, Halo-Titin and cTEVsite immunofluorescence in AAV6-injected TEVs-TTN muscles at 21 and 92 dpi showing titin expression in myofibers that are cTEVsite-negative and have centrally-located myonuclei (representative images from $n =$ muscles/group, TEVs-TTN (-) $n = 5$, 3 dpi TEVs-TTN (+) $n = 4$, 7 dpi TEVs-TTN (+) $n = 4$, 21 dpi TEVs-TTN (+) $n = 4$, 92 dpi TEVs-TTN (+) $n = 5$). Scale bars represent 50 μm . **b**, Muscle weight

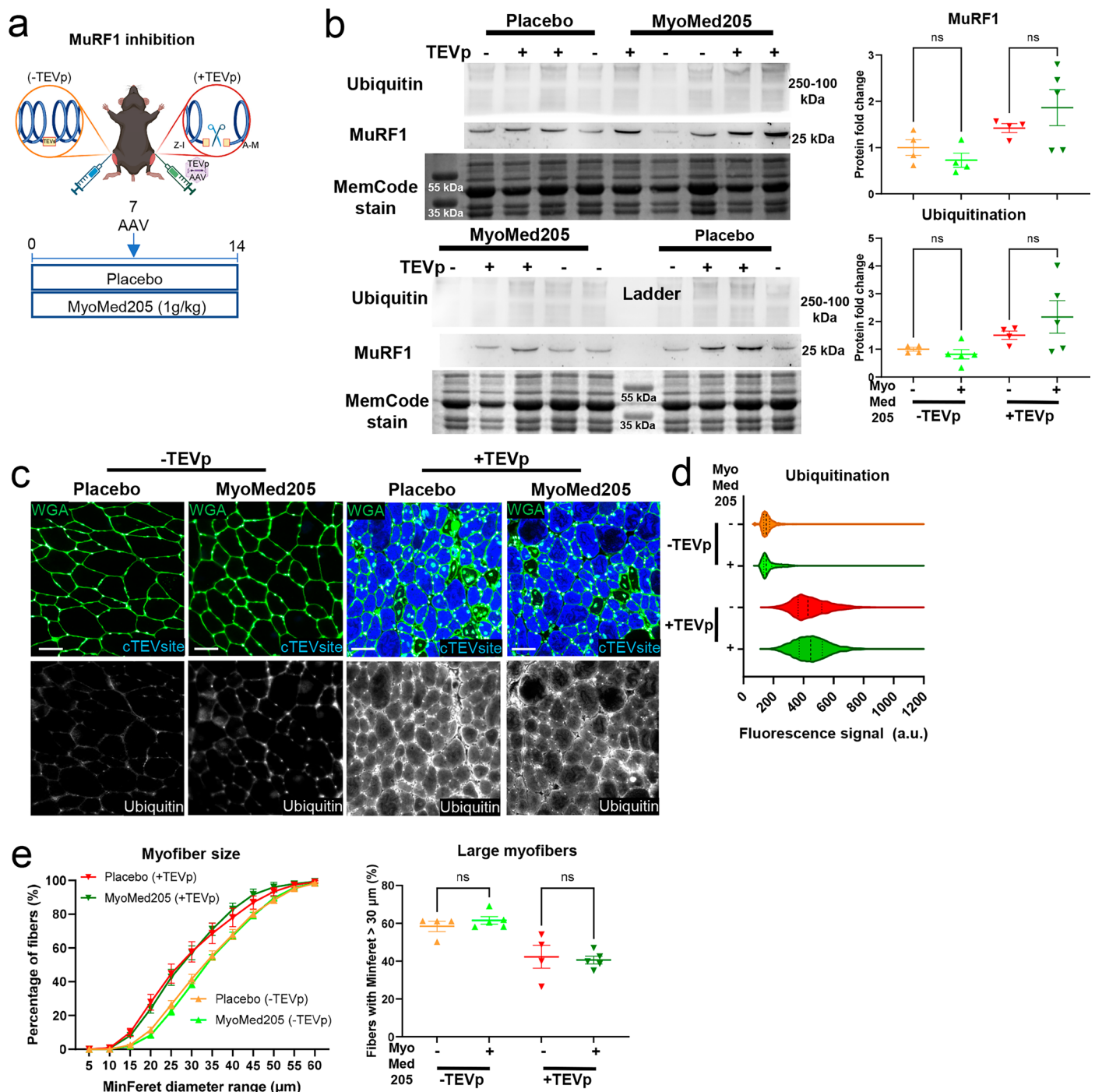
loss in AAV6-injected TEVs-TTN muscles relative to their contralateral control legs at 21 and 92 dpi ($n =$ muscles/group, 21 dpi TEVs-TTN (+) $n = 6$, 92 dpi TEVs-TTN (+) $n = 5$). Part of these data are presented also in Fig. 11. Data are represented as mean of the biological replicates $\pm$ SEM. Two-tailed Student's t -test 21 dpi vs 92 dpi $p = 0.0288$.



Extended Data Fig. 7 | Localization and levels of titin-related proteins.

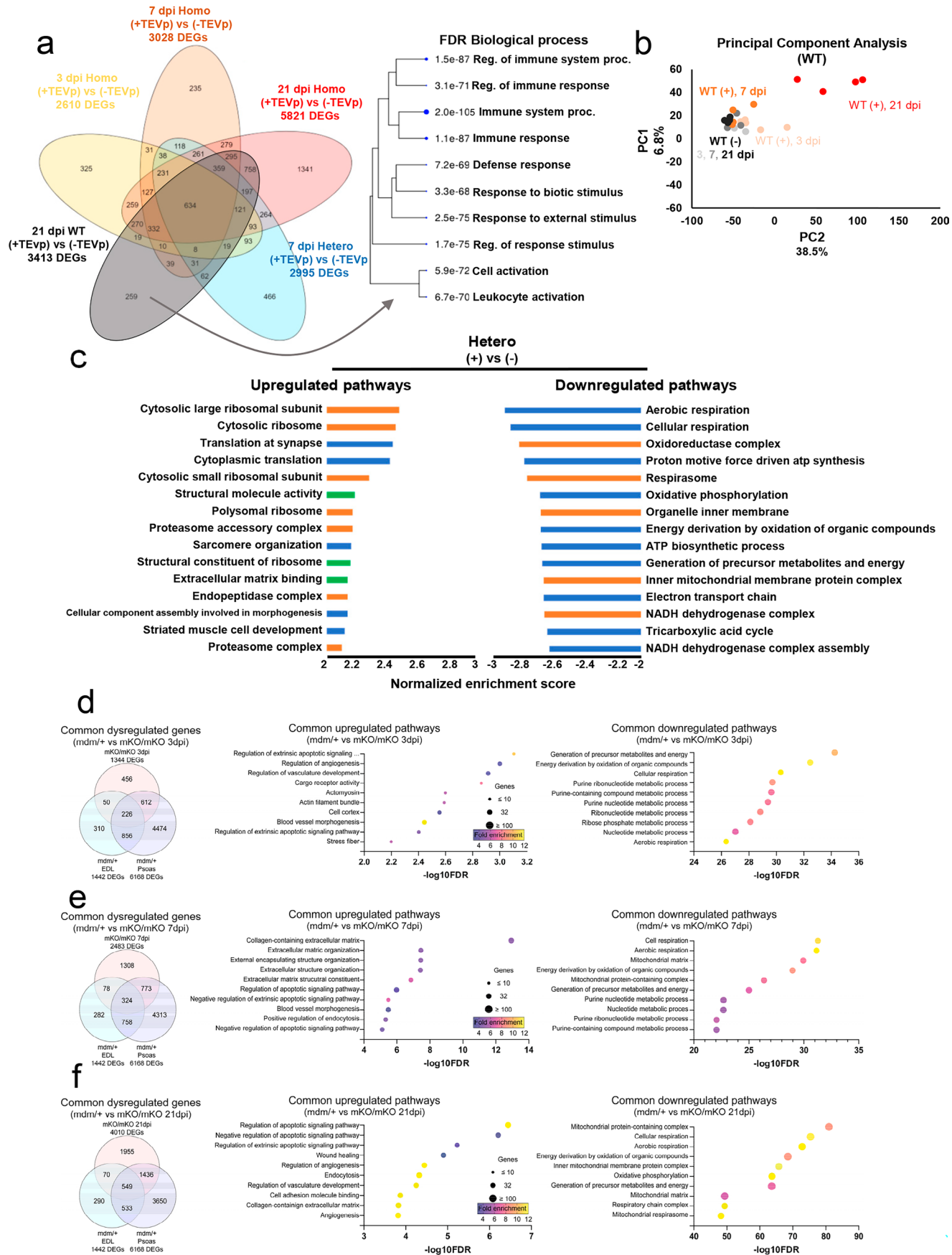
a, Total calpain 3 levels including cleaved and intact calpain 3 at the different timepoints as measured in western blot (n =muscles/group, 3 dpi: WT (-) n =6, WT (+) n =6, TEVs-TTN (-) n =6, TEVs-TTN (+) n =6; 7 dpi: WT (-) n =3, WT (+) n =4, TEVs-TTN (-) n =5, TEVs-TTN (+) n =5; 21 dpi: WT (-) n =6, WT (+) n =6, TEVs-TTN (-) n =6, TEVs-TTN (+) n =6). Two-tailed Student's t -test WT (-) vs WT (+): 3 dpi p =0.333, 7 dpi p =0.5545, and 21 dpi p =0.9497. Two-tailed Student's t -test TEVs-TTN (-) vs TEVs-TTN (+): 3 dpi p =0.749 and 7 dpi p =0.0.8825. Two-tailed Mann-Whitney's U -test TEVs-TTN (-) vs TEVs-TTN (+): 21 dpi p =0.1223. **b**, ANKRD1 immunofluorescence in control and AAV6-injected TEVs-TTN muscles at 3 showing sarcomeric localization of the protein in titin-intact and titin-cleaved conditions (representative images from n =3 muscles/group). Normalized fluorescence intensity profile along the lines indicated in yellow are shown (for simplicity, the cTEVsite profile is not shown in the -TEVp sample). **c**, MuRF1 immunofluorescence in control and AAV6-injected TEVs-TTN muscles at 3 and

7 dpi showing sarcomeric localization of the protein at 3 dpi and dotted staining in the absence of sarcomeres (as marked by antibodies against titin PEVK and cTEV site) at 7 dpi (representative images from n =3 muscles/group). Normalized fluorescence intensity profile along the lines indicated in yellow are shown (for simplicity, the cTEVsite profile is not shown in the -TEVp sample). **d**, FHL1 expression of the different titin interactors evaluated by WB (n =muscles/group, 3 dpi: WT (-) n =6, WT (+) n =6, TEVs-TTN (-) n =6, TEVs-TTN (+) n =6; 7 dpi: WT (-) n =4, WT (+) n =5, TEVs-TTN (-) n =5, TEVs-TTN (+) n =5; 21 dpi: WT (-) n =6, WT (+) n =6, TEVs-TTN (-) n =6, TEVs-TTN (+) n =6). MemCode protein staining was used for normalization. Protein fold changes were calculated relative to WT NaCl-injected samples. Scale bars represent 10 μ m. Data are represented as mean of the biological replicates $\pm$ SEM. Two-tailed Student's t -test WT (-) vs WT (+): 3 dpi p =0.9398, 7 dpi p =0.8661, and 21 dpi p =0.5461. Two-tailed Student's t -test TEVs-TTN (-) vs TEVs-TTN (+): 3 dpi p =0.8418, 7 dpi p =0.9649, and 21 dpi p =0.0211.



Extended Data Fig. 8 | MyoMed205 treatment. **a**, Homozygous TEVp-TTN mice were treated with MyoMed205 (1 g/kg) spiked food or control placebo diet for two weeks. Mice were intramuscularly injected with either saline solution or AAV6-GFP-TEVp at the first week of treatment and muscles were harvested 7 dpi. **b**, MuRF1 and protein ubiquitination levels measured by WB (MuRF1, n=muscles/group Placebo (-TEVp) n=4, MyoMed205 (-TEVp) n=4, Placebo (+TEVp) n=4, MyoMed205 (+TEVp) n=5) (ubiquitination, n=muscles/group, Placebo (-TEVp) n=4, MyoMed205 (-TEVp) n=4, Placebo (+TEVp) n=5, MyoMed205 (+TEVp) n=5). MemCode total protein staining was used for normalization. Protein fold changes were calculated relative to placebo-treated, NaCl-injected samples. MuRF1, two-tailed Student's t-test: -TEVp (-MyoMed205) vs (+MyoMed205) p=0.2752; two-tailed Student's t-test: +TEVp (-MyoMed205) vs (+MyoMed205) p=

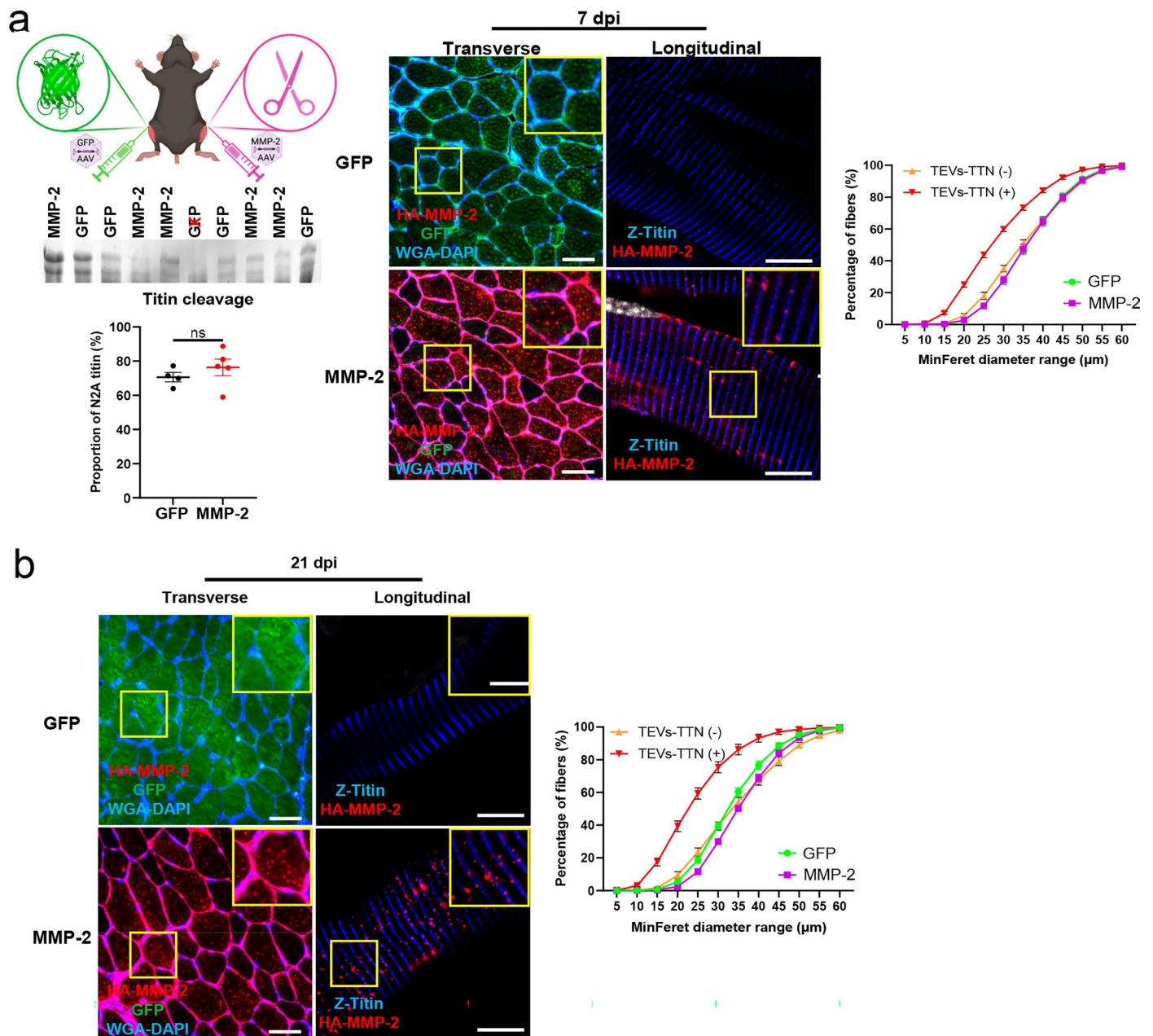
0.3564. Protein ubiquitination: two-tailed Student's t-test: -TEVp (-MyoMed205) vs (+MyoMed205) p=0.4044; two-tailed Student's t-test: +TEVp (-MyoMed205) vs (+MyoMed205) p=0.364. **c**, Representative cTEVsite and ubiquitin immunofluorescences. Scale bars represent 50 μm. **d**, Violin plot quantification of intramyofiber ubiquitin fluorescence intensity (n=myofibers/group, Placebo (-TEVp) n=4784, MyoMed205 (-TEVp) n=7103, Placebo (+TEVp) n=4103, MyoMed205 (+TEVp) n=5872). **e**, Analysis of myofiber size (n=muscles/group Placebo (-TEVp) n=4, MyoMed205 (-TEVp) n=5, Placebo (+TEVp) n=4, MyoMed205 (+TEVp) n=5). Large myofibers: two-tailed Student's t-test: -TEVp (-MyoMed205) vs (+MyoMed205) p=0.8571; two-tailed Student's t-test: +TEVp (-MyoMed205) vs (+MyoMed205) p=0.7731. Data are represented as mean of the biological replicates ± SEM. Panel **a** was created with [Biorender.com](https://www.biorender.com).



Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Gene expression in titin mKO muscles. **a**, Shared DEGs between AAV6-injected and saline-injected legs for WT muscles at 21 dpi, heterozygous TEVs-TTN muscles at 7 dpi and homozygous TEVs-TTN muscles at 3, 7 and 21 dpi (n=4 muscles/group). To the right, hierarchical clustering tree of the shared 3,413 DEGs between WT and the rest of the groups (n=4 muscles). **b**, Principal component analysis of gene expression in WT TA

muscles. **c**, Dysregulated pathways in heterozygous titin mKO muscles. **d-f**, Left, shared DEGs in heterozygous *mdm* EDL and psoas muscles⁴⁴, and homozygous titin mKO TA muscles at 3 (**d**), 7 (**e**), 21 (**f**) dpi. Middle and right, top 10 most dysregulated gene ontology terms of the corresponding shared DEGs at the different experimental times.



Extended Data Fig. 10 | MMP-2 overexpression does not result in titin cleavage nor myopathic response. a, Mice were intramuscularly injected with AAV6 vectors encoding either GFP or HA-tagged MMP-2. SDS-PAGE gel to stain titin did not show any effect on full length N2A titin at 7 dpi (n=muscles/group, GFP n=4, MMP-2 n=5) (crossed sample was not analyzed due to technical titin degradation). Two-tailed Student's t-test: MMP-2 vs GFP p=0.3729. Transverse TA sections evidence major HA-MMP-2 location in the interfiber space, but also intracellular dots. Longitudinal EDL sections evidence a location of HA-MMP-2 near the Z line of the sarcomeres without any sign of sarcomeric disarrays produced at 7 dpi. No effect was observed on myofiber size by the overexpression

of MMP-2 (n=muscles/group, 7 dpi TEVs-TTN (-TEVp) from Fig. 2b n=6, 7 dpi TEVs-TTN (+TEVp) from Fig. 2b n=6, 7 dpi GFP n=5, 7 dpi MMP-2 n=5). Scale bars represent 50 μm for transverse sections and 10 for longitudinal sections. **b,** the effect of MMP-2 overexpression was tested after 21dpi obtaining similar results in location and effects on myofiber size (n=muscles/group, 7 dpi TEVs-TTN (-TEVp) from Fig. 2b n=6, 7 dpi TEVs-TTN (+TEVp) from Fig. 2b n=6, 7 dpi GFP n=5, 7 dpi MMP-2 n=6). Scale bars represent 50 μm for transverse sections and 10 for longitudinal sections. Data are represented as mean $\pm$ SEM. For reference, TEVs-TTN data from Fig. 2b are also plotted in both panels. Panel a was created with [BioRender.com](#).

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Q5:	You cannot alter accepted Supplementary Information files except for critical changes to scientific content. If you do resupply any files, please also provide a brief (but complete) list of changes. If these are not considered scientific changes, any altered Supplementary files will not be used, only the originally accepted version will be published.
Q6:	Please check Figures for accuracy as they have been relabelled. Please markup minor changes in the eProof. For major changes, please provide revised figures. (Please note that in the eProof the figure resolution will appear at lower resolution than in the pdf and html versions of your paper.)
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<i>Query No.</i>	<i>Nature of Query</i>
Q9:	Reference [34] is a duplicate of [32] and hence the repeated version has been deleted. Please check.
Q10:	In Fig. 1 caption, please write 'AFM' in full.
Q11:	Per style, genetic material is set in italics and gene products (including the mature forms of non-coding RNAs) are set upright. Please confirm that the italics/upright is appropriate or mark any instances that need to be changed.
Q12:	In the sentence beginning 'Next, we assessed titin levels...', please define 'SDS-PAGE'.
Q13:	In the sentences beginning 'Indeed, we only have limited...' and 'We transferred the fragments to...', please write 'IgG' in full.
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Q17:	In the sentence beginning 'In this regard, titin has...', please define 'MMP'.
Q18:	Please confirm that edits to the sentence beginning 'Consistent with perturbed titin-based...' have retained the intended meaning.
Q19:	In the sentence beginning 'Our work demonstrates the use...', please check whether 'TEVpe' should be changed to 'TEVp'.
Q20:	In the sentence beginning 'Viral particles were purified by...', please write 'MWCO' in full.

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Q25:	In the sentence beginning 'For the co-localization test of...', please provide the version number used for Huygens deconvolution software.
Q26:	In the sentence beginning 'For the analysis of myonuclei...', please write 'ROI' in full.
Q27:	Please confirm that edits to the sentence beginning 'For electron microscopy, EDL muscle...' have retained the intended meaning.
Q28:	In the Acknowledgements, please check whether 'Welcome Trust' should be changed to 'Wellcome Trust'.
Q29:	Figure Panel 'd' is present in the figure legend , but it is not mentioned in the artwork of Extended Data Fig. 7. Please provide a corresponding text of the panel.
Q30:	For ref. 90, please add the title, the year and the editor(s), if any.
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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☐ ☒ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection

ZEN 2.6 \ Zeiss \ <https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html#zenversions>

JPK Data Analysis software v.8.0.159 \ Bruker \ <https://www.bruker.com/es/products-and-solutions/microscopes/bioafm/jpk-nanowizard-4-xp-bioscience.html>

610A Dynamic Muscle Control LabBook v.17.0 \ Aurora Scientific \ <https://aurorascientific.com/products/muscle-physiology/muscle-daq-software/615a-dynamic-muscle-control/>

Data analysis

Scaffold software v.5 \ Proteome Software \ <https://www.proteomesoftware.com/products/scaffold-5>

Proteome Discoverer Software v.1.4 \ Thermo Scientific \ <https://www.thermofisher.com/order/catalog/product/B51001479>

Fiji (v.1.54p) \ ImageJ \ <https://fiji.sc/>

JPK Data Analysis software v.8.0.159 \ Bruker \ <https://www.bruker.com/es/products-and-solutions/microscopes/bioafm/jpk-nanowizard-4-xp-bioscience.html>

611A Dynamic Muscle Analysis v5.501 \ Aurora Scientific \ <https://aurorascientific.com/products/muscle-physiology/muscle-daq-software/615a-dynamic-muscle-control/>

Cellpose 2.0 \ GitHub \ <https://www.cellpose.org/>

StarDistv.3.0 \ GitHub \ <https://github.com/stardist/stardist>

CellCounter v.3.0 \ ImageJ \ <https://imagej.net/ij/plugins/cell-counter.html>

Huygens Deconvolution Software \ Science Volume Imaging \ <https://svi.nl/Huygens-Software>

FastQC v.0.74 \ Babraham Bioinformatics \ <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

CCutadapt v.5.0 \ <https://cutadapt.readthedocs.io/en/stable/>

RSEM v.1.3.1 \ <https://github.com/deweylab/RSEM>

Limma v.3.46.0 \ <https://bioconductor.org/packages/release/bioc/html/limma.html>

Gene set enrichment analysis v.4.4.0 \ <https://www.gsea-msigdb.org/gsea/index.jsp>

Galaxy Web Platform v.0 \ <https://usegalaxy.org/>

STAR v.2.7.11a \ <https://usegalaxy.org/>

FeatureCounts v.2.0.8 \ <https://usegalaxy.org/>

DESeq2 v.2.11.40.8 \ <https://usegalaxy.org/>

PANTHER v.19.0 \ <https://pantherdb.org/>

Graphpad PRISM 10 \ GraphPad \ <https://www.graphpad.com/>

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Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The main data supporting the results in this study are available within the paper and its Supplementary Information. Non-commercial materials are available from the corresponding authors. Transcriptomic data from bulk RNA-seq are available from the Gene Expression Omnibus (GEO) with GSE271916 accession code and transcriptomic data of EDL and psoas muscles was obtained from GSE158283 and GSE210263.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

n/a

Reporting on race, ethnicity, or other socially relevant groupings

n/a

Population characteristics

n/a

Recruitment

n/a

Ethics oversight

n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences

☐ Behavioural & social sciences

☐ Ecological, evolutionary & environmental sciences

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes were estimated according to previous experience from the investigators considering availability of animals and using the least number of animals possible
Data exclusions	One WT and one TEVs-TTN legs at 21 dpi were discarded due to abnormally excessive GFP-TEV overexpression leading to massive myofiber turnover
Replication	For animal studies, mice used came from different litters and different batches of viruses have been used (unless for MMP2 and GFP studies where only one batch has been used). All replicates in the manuscript are biological (independent).
Randomization	The legs from the mice were randomly injected in order to avoid differences arising from the skill of the researcher to inject leg or right legs. For every injection day, the injection leg was alternated from the left to the right starting from the mouse with the lower identification code to the higher one thus removing any bias on genotype/preferred leg.
Blinding	All experiments were performed and analyzed in a blinded manner, and the investigators were unaware of the genotype of the mice nor the condition of the leg studied.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	GFP antibody Abcam Cat#: ab290; RRID: AB_303395 cTEVsite antibody Novus Biologicals Cat#: NBP2-37831 ANKRD1 antibody MYOMEDIX Cat#: Ankrd1-1 Calpain-3 antibody MYOMEDIX Cat#: CAPN3 FHL1 antibody Aviva Cat#: ARP34378_T100; RRID: AB_842144 HSP27 antibody DSHB Cat#: CPTC-HSPB1; RRID: AB_2617268 αβ-crystallin DSHB Cat#: CPTC-CRYAB-2; RRID: AB_1553792 MuRF1 antibody MYOMEDIX Cat#: MuRF1-3 Ubiquitin antibody Santa Cruz Cat# sc-8017; RRID: AV_628423 GFP antibody Thermo Cat#: A10262; RRID: AB_2534023 Desmin antibody Abcam Cat#: ab15200; RRID: AB_301744 PEVK-Titin antibody DSHB Cat#: D910 α-actinin Sigma Cat#: A7732; RRID: AB_2221571 HA tag antibody Cell signaling Cat#37245; AB_1549585 Halo-TMR ligand Promega Cat#: G8251 Wheat germ agglutinin, Alexa Fluor 488 Thermo Cat#: W11261 Wheat germ agglutinin, Alexa Fluor 647 Thermo Cat#: W32466 Goat anti-Rabbit IgG (H+L) Secondary Antibody, Horseradish peroxidase Sigma Cat#: A0545; RRID: AB_257896 Goat anti-Mouse IgG (H+L) Secondary Antibody, Horseradish peroxidase Thermo Cat#: 31430; RRID: AB_228307 Goat anti-Chicken IgY (H+L) Secondary Antibody, Horseradish peroxidase Thermo Cat#: A16054; RRID: AB_2534727 Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 488 Thermo Cat#: A-11039; RRID: AB_2534096 Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 647 Thermo Cat#: A-21245; RRID: AB_2535813 Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 Thermo Cat#: A-11034; RRID: AB_2576217 Goat anti-Mouse IgM (H) Secondary Antibody, Alexa Fluor 546 Thermo Cat#: A-21045; RRID: AB_2535714 Chicken anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 647 Thermo Cat#: A-21463; RRID: AB_2535869 Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 568 Thermo Cat#: A-11031; RRID: AB_144696 Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 Thermo Cat#: A-32732; RRID: AB_2633281
Validation	Immunofluorescences were always run in parallel with negative controls lacking incubation with primary antibodies.

Validation

The validation of the commercial antibodies is available on manufacturer's websites. MYOMEDIX antibodies are validated using KO samples.

All antibodies were validated in control (wild-type) murine tibialis anterior tissue, as well as by western blot or by Immunofluorescence, according to manufacturers' instructions and recommendations that can be found on the manufacturers' website as well as in previous publications. We used www.benchsci.com to select antibodies that have shown to work in conditions using the same species, application and tissue.

Additionally, cTEVsite antibody was used in this manuscript for a different application than previously reported. We show in the extended data figures that it recognizes the cleavage of TEV site.

For GFP antibody, we validated the antibody using recombinant GFP protein for western blot or AAV-driven overexpression of GFP for immunofluorescence. For the remaining antibodies, we relied on validation by the providers (more info in RRID website).

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	HEK293T cells (ATCC, CRL-3216)
Authentication	All cell lines came with authentication documentation. Each cell line was also identified and verified by its characteristic morphology or way of growth.
Mycoplasma contamination	Cell lines are weekly tested for myoplasm contamination and they were always negative.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used in this study.

Animals and other research organisms

Policy information about [studies involving animals; ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	C57BL/6J, female mice were used for phenotyping. Strain used were TEVs-TTN (doi.org/10.1038/s41467-020-15465-9), Pax7CreERT (JAX line #017763) and Rosa26tdTomato (JAX line #007914). Animals were housed under specific pathogen-free conditions at CNIC. Mice were maintained in racks with individual ventilation cages according to current Spanish and European legislation. All animals had food and water "ab libitum" in conditions of 45-65% of relative humidity, temperature 21-24 C and a light/dark cycle of 12:12 hours. Mice used in this study were injected with AAV at 10-12 weeks of age.
Wild animals	No wild animals were used in this study.
Reporting on sex	Only female mice were used in this study. The effect of titin cleavage is not expected to differentially affect male mice and the results obtained in this study would be equivalent in males. Indeed, systemic injection of AAV encoding TEV protease has been performed in a parallel study in the host lab and the results are equivalent in male mice.
Field-collected samples	No field collected samples were used in this study.
Ethics oversight	All experimental procedures were done in accordance with Spanish and European legislation and approved by Comunidad de Madrid committee (protocols PROEX 042/18 and PROEX 107.8/23).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	n/a
Novel plant genotypes	n/a
Authentication	n/a