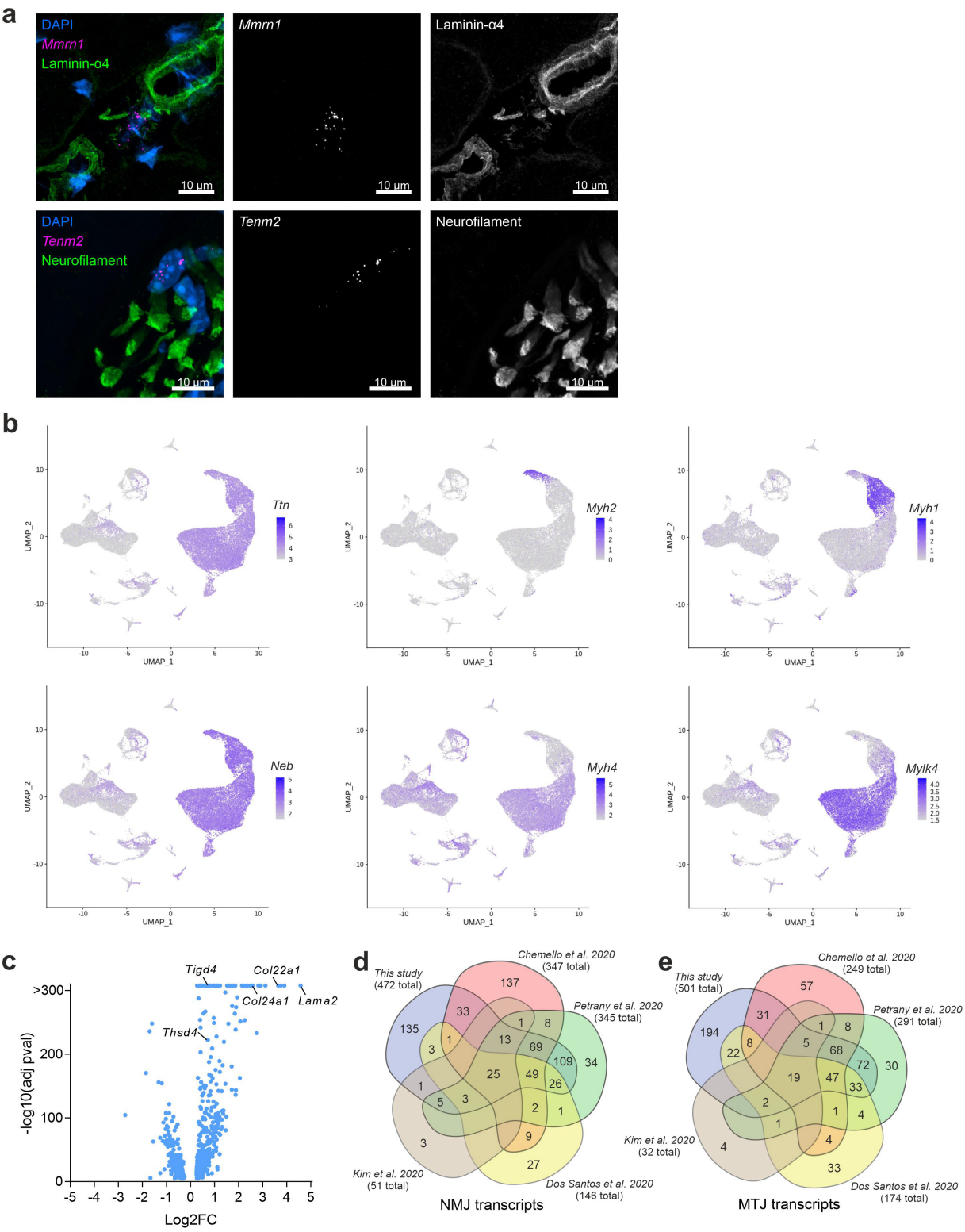

Figure S1: Additional data supporting the control skeletal muscle UMAP analysis.
a Single molecule fluorescent *in situ* hybridization (smFISH) of *Mmrn1* or *Tenm2* combined with an immunofluorescence staining of Laminin α4 or neurofilament on *gastrocnemius* (GAS) muscle. *Mmrn1*-expressing cells are located in close proximity to larger blood vessels and *Tenm2*-expressing cells are located near axons. **b** Feature plots visualizing muscle fiber-specific and muscle fiber type-specific markers. **c** Volcano plot of differentially expressed transcripts in MTJ myonuclei compared to body myonuclei (max. padj. cutoff at 2.23E-308). **d** NMJ- and **e** MTJ-enriched transcripts were compared to previously published NMJ and MTJ markers generated from snRNA-seq. Experiment in (**a**) was repeated once with similar results.

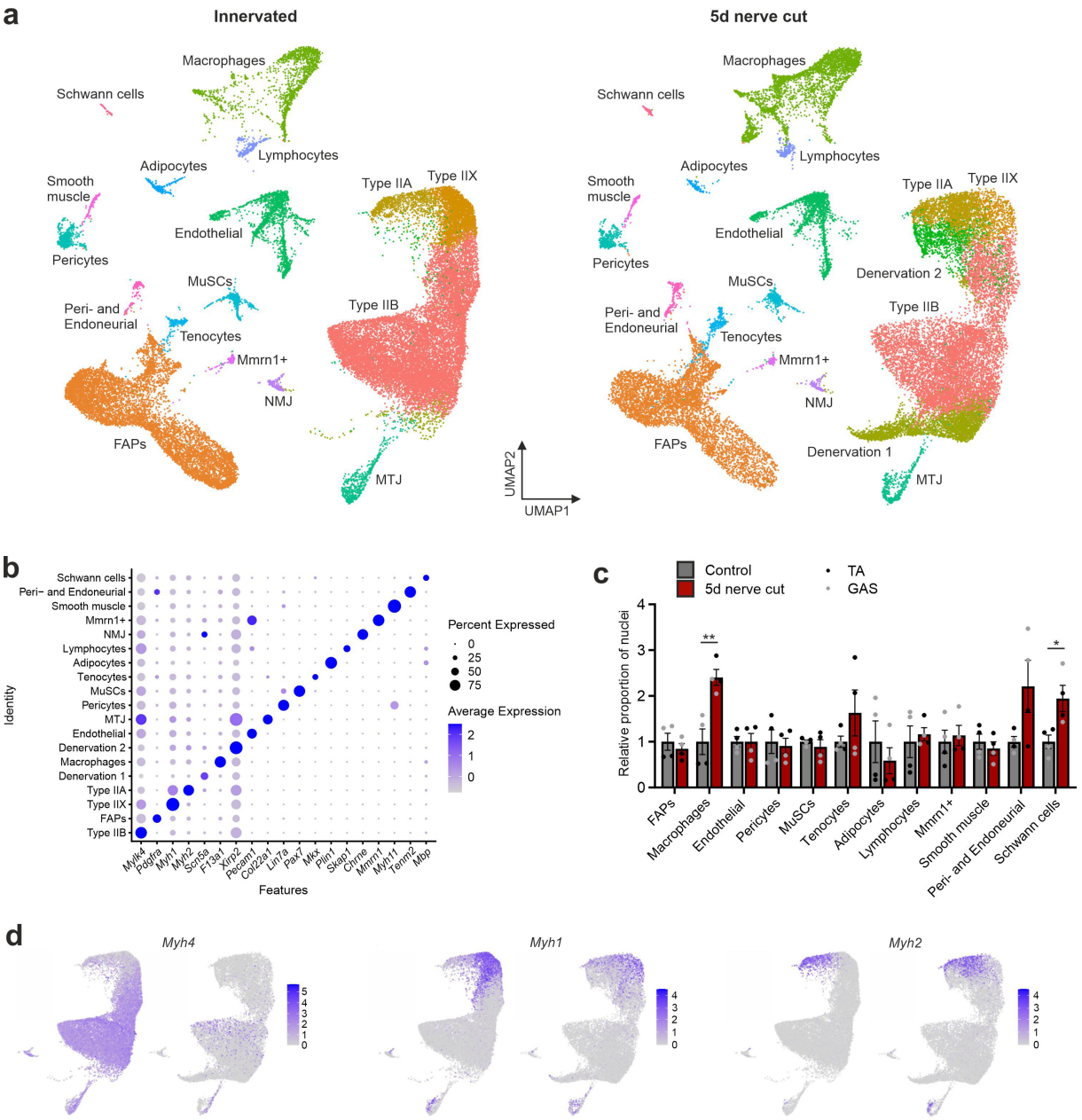

Figure S2: Sciatic nerve cut UMAP and supporting data

a Split UMAP of ~65,000 nuclei derived from five day denervated and control muscles. *n* = 2 for TA and GAS for both conditions. **b** Dot-plot visualizing the genes used to assign cell types to clusters in the UMAP in (**a**). **c** Relative proportion of nuclei of all mononucleated cell types in TA and GAS and the effect of denervation. **d** Feature plots of myonuclei visualizing the expression of different myosin heavy chain isoforms in control versus denervated muscle. In (**c**) the data is presented as mean ± SEM and a two-tailed t-test was performed ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***). Source data are provided as a Source Data file.

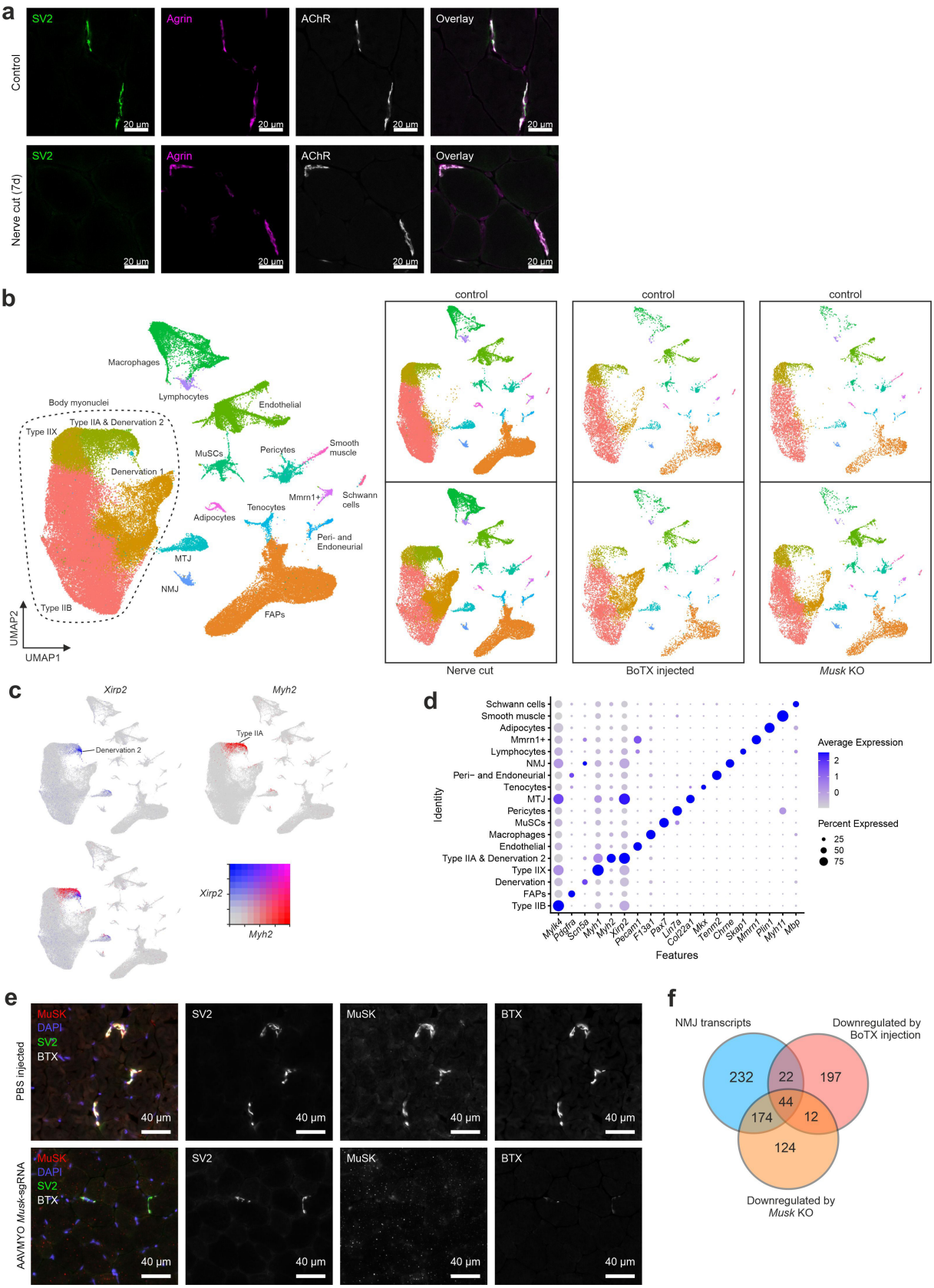

Figure S3: Nerve cut, BoTX injection and *Musk* knockout supporting data

a IF staining of NMJs on TA cross-sections in control mice and seven days after sciatic nerve cut. Nerve terminals were visualized with antibodies to synaptic vesicle protein 2 (SV2), synaptic basal lamina with antibodies to agrin and postsynaptic AChR clusters with α-bungarotoxin. Note that agrin remains bound at the synaptic basal lamina and that AChRs remain clustered at the NMJ despite loss of motor neuron axons (lack of SV2). **b** UMAP depicting ~100,000 nuclei isolated from TA and GAS muscles after nerve cut, BoTX injection and MuSK depletion (*Musk*

KO). The different conditions and respective controls are additionally visualized as split UMAPs to the right. **c** Dual gene feature plot depicting a *Myh2* devoid region within the type IIA cluster expressing the denervation 2 marker *Xirp2* (see Fig. 2). **d** Dot-plot visualizing the genes used to assign cell types to clusters in the UMAP in (**b**). **e** Cross-sections of EDL muscles injected with either PBS or AAVMYO encoding *Musk*-sgRNAs. Lack of MuSK and AChR clusters, stained with α-bungarotoxin (BTX), confirms successful KO of *Musk*. **f** Venn diagram visualizing how many NMJ transcripts are downregulated in NMJ myonuclei after BoTX injection or *Musk* KO. In (**f**) transcripts were categorized as significantly changed when p-val. < 0.01 and log2FC ±0.25.

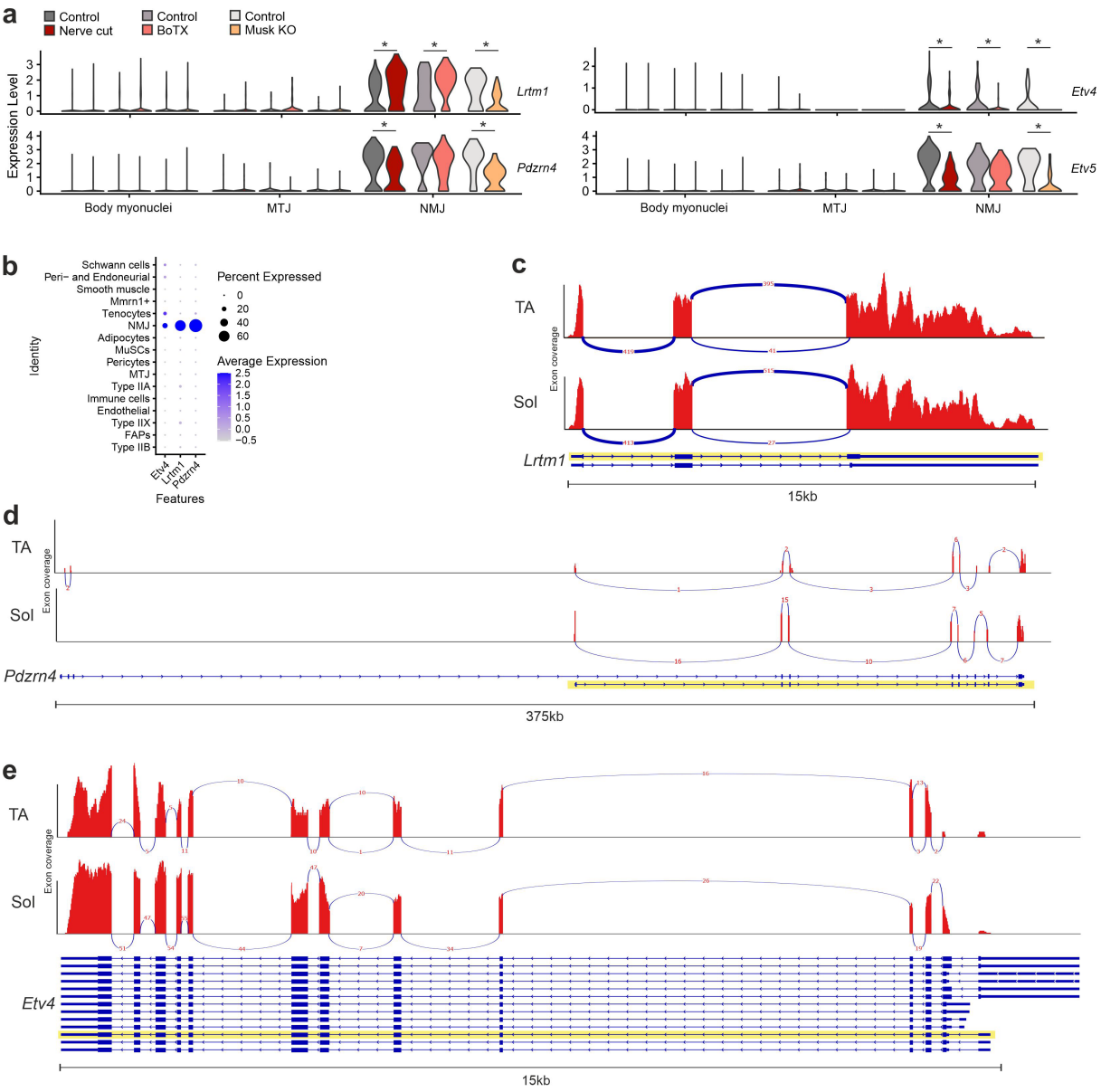

Figure S4: Data supporting NMJ gene target selection

a Violin plots generated from the snRNA-seq data shown in Fig. 3a. **b** Dot-plot generated from the snRNA-seq data depicted in Fig. 1a showing expression of *Etv4*, *Lrtm1* and *Pdzrn4* in the different nuclei populations in innervated muscle. **c**, **d** and **e** bulk RNA-seq reads from TA and *soleus* (Sol) for *Lrtm1* (**c**), *Pdzrn4* (**d**) and *Etv4* (**e**) were extracted and visualized as Sashimi plots using integrative genomics viewer. The numbers between splice junctions show the number of reads mapped between splice-sites. The dominant splice isoform is highlighted in yellow. The NCBI nucleotide ID is NM_176920 for *Lrtm1*, NM_001164594 for *Pdzrn4* and NM_001316365 for *Etv4*.

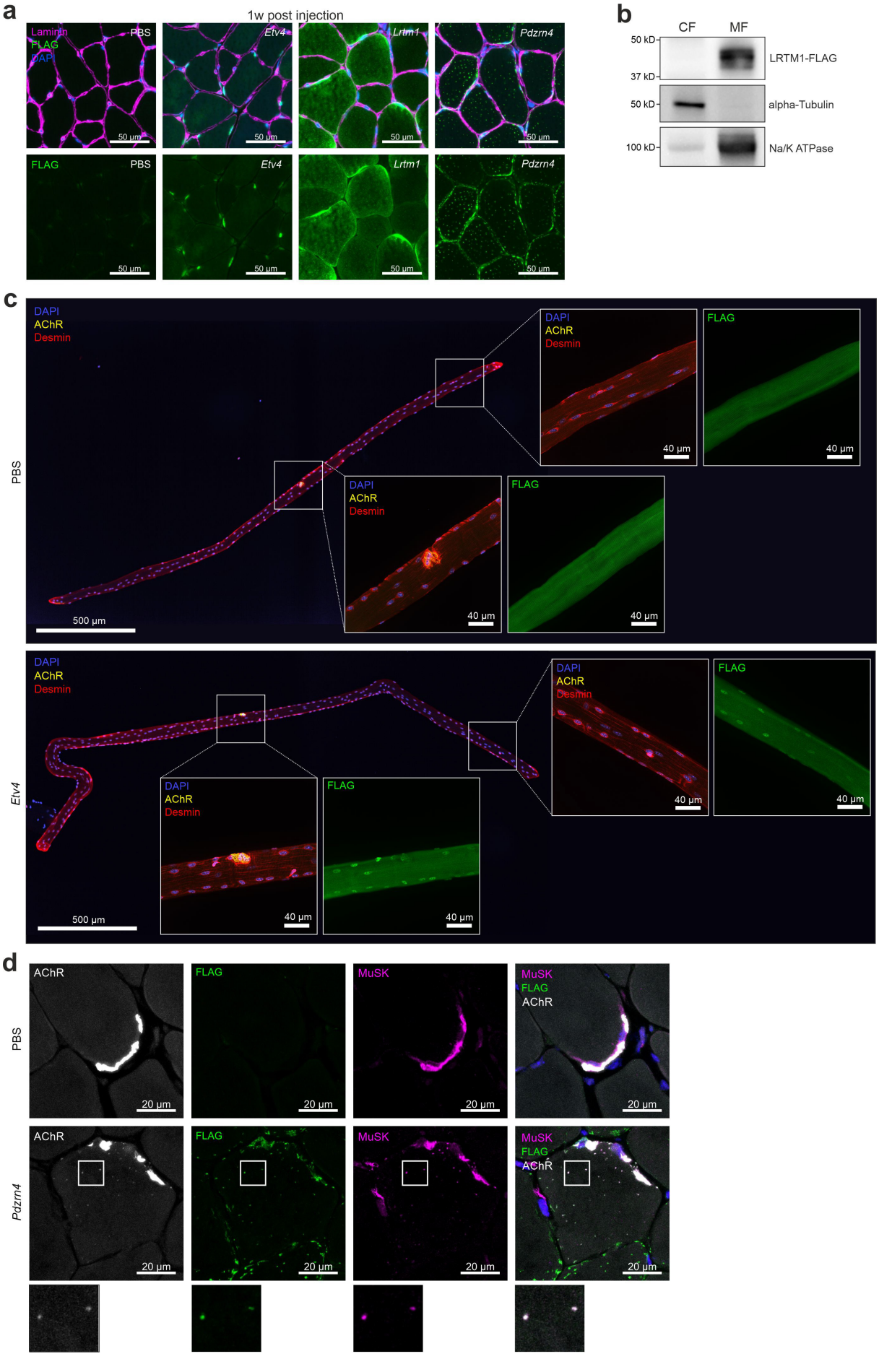
a
1w post injection
Laminin
FLAG
DAPI
PBS
Etv4
Lrtm1
Pdzrn4
50 μm
b
CF
MF
50 kD
37 kD
50 kD
100 kD
LRTM1-FLAG
alpha-Tubulin
Na/K ATPase
c
PBS
Etv4
DAPI
AChR
Desmin
FLAG
500 μm
40 μm
d
PBS
Pdzrn4
AChR
FLAG
MuSK
20 μm

Figure S5: Overexpression of *Etv4*, *Lrtm1* and *Pdzrn4* in mouse muscle

a Immunofluorescence staining against the FLAG-tag on TA cross-sections one-week post AAVMYO or PBS injection. ETV4 localizes to myonuclei, LRTM1 is mainly found at the muscle fiber periphery and PDZRN4 is detected near the sarcolemma and in vesicle-like structures within muscle fibers. **b** Western blot analysis of a TA muscle overexpressing LRTM1 that was separated into a cytosolic fraction (CF) and a membrane fraction (MF). Alpha tubulin and Na/K ATPase are positive controls. **c** Immunofluorescence staining on single muscle fibers isolated from EDL muscle one week post intramuscular injection of either PBS or AAVMYO encoding *Etv4-Flag*. ETV4 was detected in most nuclei across the entire muscle fiber. **d** Immunofluorescence staining on TA cross-sections showing co-localization of AChRs and MuSK within the vesicle-like structures positive for PDZRN4. Source data are provided as a Source Data file.

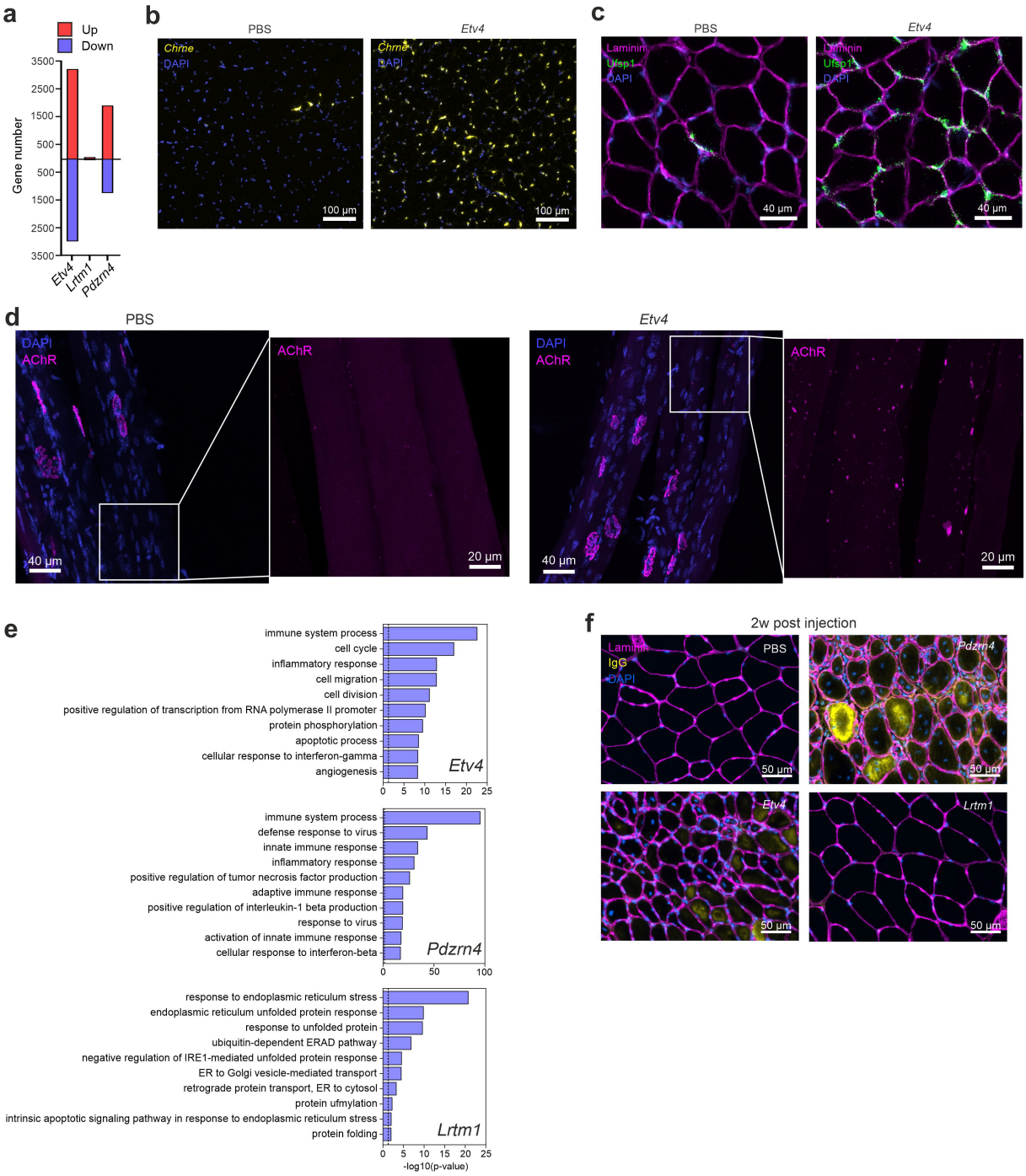

Figure S6: Overexpression of *Etv4*, *Lrtm1* and *Pdzrn4* in mouse muscle

a Number of differentially expressed genes in bulk RNA-seq one-week post AAVMYO injection (padj < 0.05, Log2FC ± 0.25). **b** SmFISH of *Chrne* transcripts at low magnification that depict its widespread expression across the entire EDL muscle upon overexpression of *Etv4*. **c** SmFISH of the NMJ transcript *Ufsp1* combined with an IF staining for laminin on TA cross-sections. Similar to *Chrne*, overexpression of *Etv4* causes widespread *Ufsp1* expression in many myonuclei. **d** Whole-mount preparations of two-week denervated EDL muscles that had been injected with either PBS or AAVMYO one week before tissue collection. Note the presence of many, extrasynaptic AChR clusters in *Etv4*-overexpressing (*Etv4*) but not in control muscle (PBS). **e** Top 10 DAVID GO-terms (biological processes) associated with upregulated genes in the indicated samples. **f** Immunofluorescence staining against mouse IgG on TA cross-sections two weeks after AAVMYO or PBS injection. *Etv4* and *Pdzrn4* overexpression causes de- and regeneration of muscle fibers, indicated by the presence of centralized nuclei and of IgG-positivity of muscle fibers.

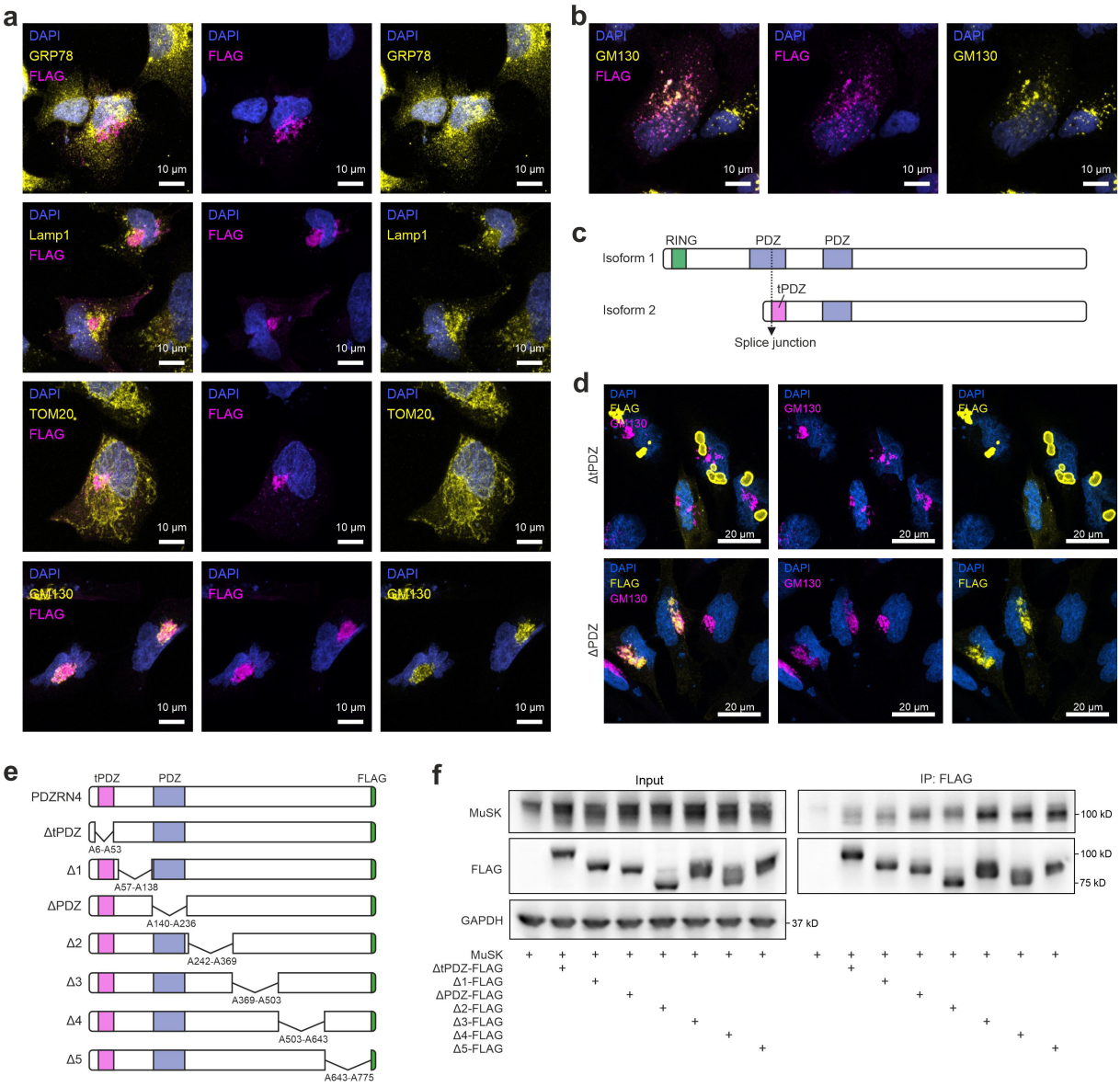

Figure S7: PDZRN4 localizes to the Golgi apparatus in HeLa cells and binds to MuSK

a HeLa cells were transfected with constructs coding for PDZRN4-FLAG, fixed 24 h later and stained with antibodies for the endoplasmic reticulum (GRP78), lysosomes (Lamp1), mitochondria (TOM20) and the Golgi apparatus (GM130). Note the precise overlap between anti-FLAG and anti-GM130 staining. **b** Even during mitosis, when the Golgi is disassembled, PDZRN4 co-localized with GM130. **c** Schematic of the two PDZRN4 isoforms. Skeletal muscle only expresses isoform 2 that lacks the RING domain and contains a truncated PDZ domain (termed tPDZ). **d** IF staining on HeLa cells overexpressing different deletion mutants of PDZRN4. The mutant lacking tPDZ does not localize to the Golgi while the ΔPDZ mutant show a Golgi localization like full-length PDZRN4. **e** Schematic of deletion mutants of PDZRN4. **f** Co-immunoprecipitation experiments using deletion mutants expressed in HEK 293T cells together with MuSK using antibodies against the FLAG-tag. MuSK is present in all precipitates to a different degree. Source data are provided as a Source Data file.

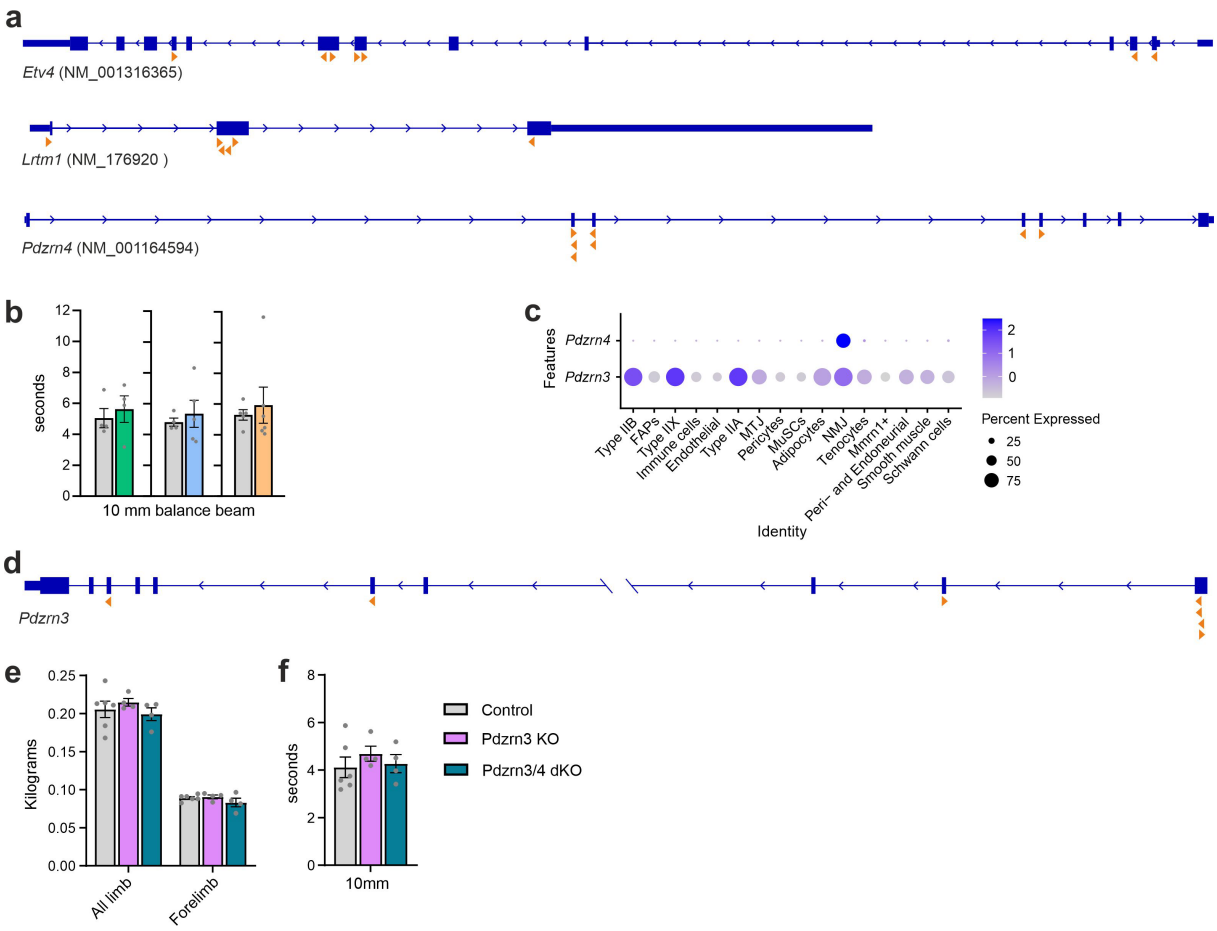

Figure S8: Single guide RNA localization and balance beam experiments
a Exon map with orange arrow heads indicating the location and direction of each single guide RNA (sgRNA). **b** Balance beam performance of knockout mice. After an initial training to walk across a 12 mm narrow metal bar, mice were timed to traverse a 10 mm balance beam, covering a distance of 80 cm. **c** Dot-plot generated from the snRNA-seq data depicted in Fig. 1a showing expression of *Pdzrn4* and *Pdzrn3* in the different nuclei populations in innervated muscle. **d** Exon map of *Pdzrn3* with orange arrowheads indicating the location and direction of each sgRNA. **e** Grip strength and **f** balance beam performance of *Pdzrn3* (*Pdzrn3* KO) and *Pdzrn3/4* double knockout (*Pdzrn3/4* dKO) mice. The data in (**b**, **e**, **f**) is presented as mean ± SEM. Source data are provided as a Source Data file.