Supporting Text

List of Primer Pairs for PEVK (Proline-Glutamate-Valine-Lysine) Constructs.
Titin PEVK and tandem Ig cDNA fragments were amplified from human soleus muscle cDNA by PCR (for detailed PCR amplication methods, see ref. 1). For amplification, synthetic 30- to 40mer synthetic oligonucleotides were designed to be complementary to domain junctions in X90568, X90569, and AJ277892. Small letters denote 5' mismatch sequences for the introduction of cloning sites and Cys-Cys tags. Capital letters (in groups of three) correspond to codons/reverse codons. S refers to sense primers, R for reverse primers. Exons numbers refer to AJ277892 (EMBL data library, annotated titin gene sequence).

Ex119-136 construct:
Ex119S: tttccatggcc-CCA GCT GTG CAT ACA AAG AAG ATG GTT ATTT;
Ex136R: tttggtacc-ta-aca-aca-TTT GAT GGG TTG AGG TTC TCT TTT TGG AGC;

Ex126-133 construct
Ex126S: tttccatg-GCA GTG CCA GAA ATA CCA AAG AAG AAA GTT;
Ex133R: tttggtacc-ta-GAG AGG TTC TTC CAT CTT AAT GAC TTT TGG;

Ex126-134
Ex126S: tttccatg-GCA GTG CCA GAA ATA CCA AAG AAG AAA GTT;
Ex134R: tttggtacc-ta-TTC CTC TGG CAC TCT TGC TTT TGG AGG CGC;

Ex119-128 construct:
Ex119S: tttccatggcc-CCA GCT GTG CAT ACA AAG AAG ATG GTT ATTT;
Ex128R: tttggtacc-ta-GGA TAG TTT TTC TTC AGC AAC AAA TCT CTT TTC;

Ex121-128 construct:
Ex121S (primer is in 3' end of Ex120): tttccatg- GTT GAA CCA CCA CCT AAA GTC CCT GAG CTA;
Ex128R: tttggtacc-ta-GGA TAG TTT TTC TTC AGC AAC AAA TCT CTT TTC;

When amplifying Ex119-136, we produced a construct that misses exon 135. Sequences verified that this construct is a splice variant containing exons 119 to 134, then spliced to 136 (exclusion of 135). We have studied this splice variant in our study.

Mutagenesis of Ex119-136.

Four glutamate residues in exon 129 were mutated to lysines by PCR assembly mutagenesis. Mutant codons are marked in red.
Ex129S-mutant primer:
GGT GTG TCC ATT TCA GTT TAT AGA AAA GAA GAA AGA GAG GAG GAG AAA GAA GCA
GAG GTT AC;
Ex129R mutant primer;
TCT ATA AAC TGA AAT GGA CAC ACC TTC CTT CTC TTC TGA GTA ACT CCA TTT CTC
CTC TGC AGA TAC TTC;

For assembly mutagenesis, mutant ex119-129 and ex129-136 subfragments were amplified separately, using ex119-S + 126R-mut and ex126S-mut + ex136-R primer combinations. Both fragments were then gel-purified, and 1 ng of each fragment mixed. Amplification with ex119-S+ex136-R for 25 cycles was used to assemble both subfragments in one piece. Cloning into pCR-4 and sequencing confirmed the mutation and the absence of other PCR mutation artifacts.

Addition of 3-Ig Domain Tags N- and C-Terminal to the PEVK Fragment.

The following 3-Ig domain fragments were amplified from skeletal human cDNA: Ig3/4/5, Ig8/9/10, Ig92/93/94, and Ig95/96/97. Fragments were restriction digested, and combinations of 3-Ig domain fragments and the PEVK Ex119-136 fragment were ligated together. After ligation, T4 DNA ligase was inactivated by a 5-min 70°C treatment. Ligation reactions were then digested for 2 h at 37°C with *Nco*I and *Kpn*I, and products were separated on 1% agarose gels. The desired size fraction was excised from the gel and subcloned into the *Nco*I-*Kpn*I-restricted pET9D His-tagged vector. Recombinant clones were verified by sequencing before being selected for expression studies.

Tandem Ig-tag primers

Ex-30S (Ig-3)
Tttccatg-GGT GCA GTT GTT GAG TTT GTG AAA GAA CTT CAG;
Ex-32R;
I-5 rev;
ttt-ggtacc-TTC TAC AGA GAG ATT ACA GTT GGT TTC AAC;

Ex119S; (*Kpn*I)
ttt-ggtacc- CCAGCTGTGCATACAAAGAAGATGGTTATTT;
Ex136R; (*Sac*I)
ttt-gagctc-TTT GAT GGG TTG AGG TTC TCT TTT TGG AGC;

Ex-35S (I8-S) (*Sac*I)

ttt-gagctc- GCT GTC AAA ATT AAG AAG ACT CTG AAG AAC CTC;
x392;
Ex37-R (I10-R);
tttggatcc- ta-aca-aca-GGT GAC TGT CAG GGT GGC ACT GAC TTG GTC;

Ex238-S; (Ig-95S);
tttccatg-GAT CGG GAA ATT AAA CTG GTG CGA CCC CTG;
X388a; Ig-97R;
tttggtacc-AGG CAC GAC ATT CAG GTT ACA GGA AGT CTT;

Ex235-S; (Ig92-S);
tttgagctc-GAA TTG CCT CTT ATC TTC ATC ACA CCT CTC AGT;
Ex237-R; (Ig-94R);
tttggatcc-ta-aca-aca- TCT AAT GTC AAG TTT TCC TGA GGT CTT ATC;

Protein Expression.

The fragments were inserted into a modified pET9d vector to express a $(His)_6$ peptide. The constructs were transformed into *Escherichia coli* (BL21-codon + cells) (Stratagene, Heidelberg, Germany). Single transformed colonies were streaked out on kanamycin (Kan)/Chloramphenicol (CAM) plates (25 µg/10 µg per ml), and grown overnight. Next day, all cells from one plate were transferred into 1L LB, supplemented with Kan/CAM (25 µg of KAN/10 µg of CAM per ml). Typically 12-16 1-liter cultures were run simultaneously in 3L Erlenmeyer flasks with vigorous shaking (220 rpm).

Growth at 37°C was allowed for 4-5 h until O.D. was about A_{600} of 0.6─0.8. Then, expression was induced by addition of 200 µM isopropyl b-D-thiogalactoside (IPTG). Temperature was set to 37°C for expression. After 16 h, cells were harvested by centrifugation for 20 min at 4,000 rpm, and pelleted cells were resuspended in lysis buffer at 4°C, snap frozen in liquid nitrogen, and then stored at ─80°C. Cells were thawed on ice and then resuspended in 15 ml of lysis buffer (50 mM Tris•HCl, pH 8.0/200 mM NaCl/1mM 2-mercaptoethanol/10 mM imidazole/1 mM EDTA/1 mM DTT/1% (vol/vol) Triton X-100/1 mM PMSF/20 µg/ml leupeptin/40 µg/ml aprotinin/100 µl of lysozyme/DnaseI mix). The cells were then sonicated by sonifier 250 (Branson) (2 min on ice, duty cycle 40%, output control 4), and the lysate was centrifuged in an SS-34 rotor (Sorvall) at 20,000 rpm for 3 hours. This and all subsequent steps were performed at 4°C. The supernatant was separated from the pelleted cell debris, and the clarified lysate was loaded onto a chelating Sepharose column (Pharmacia) with 2 ml of Ni^{2+}. The column was equilibrated in lysis buffer and washed with buffer containing 20 mM imidazole, and the protein was eluted with 250 mM imidazole. The fractions containing the protein were pooled and further purified by loading 1 ml onto a Superdex 26/60 high load gel-filtration column (Amersham Pharmacia Biotech), equilibrated with MOPS buffer (25 mM MOPS/150 mM KCl/1mM EGTA/1 mM DTT, pH 7.4); 2-ml fractions

were collected. Proteins were eluted from the column, concentrated to 1 mg/ml, snap-frozen, and stored at −80°C. Standard SDS/PAGE analysis of protein samples was conducted using 8% (wt/vol) polyacrylamide gels. Broad-range protein marker was BenchMark Prestained Protein Ladder (Invitrogen). Protein samples were mixed with SDS loading buffer (100 mM Tris•HCl, pH 6.8/200 mM DTT/4% (wt/vol) SDS/0.2% (wt/vol) bromophenol blue/20% (wt/vol) glycerol) and boiled for 5 min before electrophoresis. Gels were stained with Coomassie brilliant blue.

Titin Isoform Studies.
For human titin isoform studies, a commercially available skeletal muscle cDNA library was amplified (prepared in pGAD10 prey vector, Clontech HL4010AB). Amplified cDNA aliquots (≈10 ng) were analyzed with exon 119- to exon 136-specific primers essentially as described (1). For rabbit titin isoform studies, tissues from psoas muscles were collected from adult and 24-h-old Myrtle's rabbits in compliance with IACUC guidelines (no. 3270). The psoas muscle was dissected and incubated in RNA later at 4°C for 24 h (Ambion, Austin, TX) and stored in RNA later at −20°C for less than 3 months until RNA was prepared. Total RNA was isolated from rabbit psoas muscle using Rneasy Fibrous Tissue Kit essentially as recommended by the manufacturer (Qiagen, Valencia, CA). For labeling, total RNA was converted to biotinylated cDNA using a random octamer, biotinylated d-CTP, and the Labelstar kit (Qiagen).

Oligonucleotide Array. An oligonucleotide, derived from titin's human exons 3 and 129, was printed using a MicroGrid arrayer (BioRobotics, Cambridge, U.K.). Synthetic oligonucleotides were obtained from Invitrogen. The exon-129 probe sequence was: TATCTGCAGAGGAGGAATGGAGTTACTCAGAAGAGGAGGAAGGTGTGTCC. The exon 3 probe sequence was: GTTTTCCAGTTCCTGAGGTGAGCTGGTTTAGGGATGGCCAGGTGATTTCC. Biotinylated target was hybridized to the oligonucleotide array in a humidified chamber at 55°C for 18 h. Detection chemistries and signal amplification were achieved using the TSA biotin System (Perkin−Elmer). The fluorescence intensity was measured using arrayWoRx^{e}TM Auto biochip reader (Applied Precision, Issaquah, WA). For data analysis and normalization, the softWoRx Tracker (Applied Precision) and GeneSifter (VizX Labs, Seattle, WA) was used. All probe intensities were normalized to human B actin (5'-GAGAAGATGACCCAGATCATGTTTGAGACCTTCAACACCCCAGCCATGTA).

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1. Centner, T., Fougerousse, F., Freiburg, A., Witt, C., Beckmann, J. S., Granzier, H., Trombitas, K., Gregorio, C. C. & Labeit, S. (2000) *Adv. Exp. Med. Biol.* **481,** 35–49.