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Expression of Distinct Classes of Titin Isoforms in Striated and Smooth Muscles by Alternative Splicing, and Their Conserved Interaction with Filamins

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While the role of titin as a sarcomeric protein is well established, its potential functional role(s) in smooth muscles and non-muscle tissues are controversial. We used a titin exon array to search for which part(s) of the human titin transcriptional unit encompassing 363 exons is(are) expressed in non-striated muscle tissues. Expression profiling of adult smooth muscle tissues (aorta, bladder, carotid, stomach) identified alternatively spliced titin isoforms, encompassing 80 to about 100 exons. These exons code for parts of the titin Z-disk, I-band and A-band regions, allowing the truncated smooth muscle titin isoform to link Z-disks/dense bodies together with thick filaments. Consistent with the array data, Western blot studies detected the expression of ~1 MDa smooth muscle titin in adult smooth muscles, reacting with selected Z-disc, I-band, and A-band titin antibodies. Immunofluorescence with these antibodies located smooth muscle titin in the cytoplasm of cultured human aortic smooth muscle cells and in the tunica media of intact adult bovine aorta. Real time PCR studies suggested that smooth muscle titins are expressed from a promoter located 35 kb or more upstream of the transcription initiation site used for striated muscle titin, driving expression of a bi-cistronic mRNA, coding 5′ for the anonymous gene FL39502, followed 3′ by titin, respectively. Our work showed that smooth muscle and striated muscle titins share in their conserved amino-terminal regions binding sites for α-actinin and filamins: Yeast two-hybrid screens using Z2-Zis1 titin baits identified prey clones coding for α-actinin-1 and filamin-A from smooth muscle, and α-actinin-2/3, filamin-C, and nebulin from skeletal muscle cDNA libraries, respectively. This suggests that the titin Z2-Zis1 domain can link filamins and α-actinin together in the periphery of the Z-line/dense bodies in a fashion that is conserved in smooth and striated muscles.

Keywords: smooth and striated muscle; titin; filamins; Z-disks and dense bodies; differential splicing

Introduction

Titin is a giant (up to ~3.7 MDa) and abundant intra-sarcomeric protein that has been well characterized in striated muscle, where it is critically important for sarcomere formation, maintenance of structural integrity of the myofibril and regulation of passive stiffness.[1–3] These unique properties of titin with regards to its size and elasticity have stimulated searches for titin or titin-like proteins in other tissue types where highly precise actomyosin complexes or supramolecular assemblies with elastic properties are assembled. This work identified a giant filamentous protein in the brush border duodenal epithelium.[4] Here, actomyosin filaments with precisely controlled lengths form contractile

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protrusions that organize the efficient transport and resorption of gastrointestinal fluids. This giant brush border protein cross-reacted with a subset of the tested titin antibodies,[4] and electron microscopy (EM) revealed filaments with a 4 nm beaded substructure and a globular end domain. These characteristics are intriguingly similar to titin isolated from skeletal muscle.[5] *In vitro*, the giant filamentous protein from the brush border regulated cellular myosin II assemblies.[6] Taken together, these studies suggest that cellular isoforms of titin exist and that they might regulate myosin II assemblies in non-muscle cells.[7]

A set of separate studies identified 700 kDa and 2000 kDa titin-like proteins from vascular and visceral smooth muscles in chicken, respectively. A 2000 kDa titin-like protein from smooth muscle referred to as smitin by Keller and colleagues (for smooth muscle titin-like protein) was shown to interact with myosin filaments and with smooth muscle α-actinin *in vitro*.[8,9] Smitin was therefore suggested to link thick filaments to dense bodies in avian vascular and visceral muscle. Similar to cellular titin, EM rotary shadowing visualized smitin as a 4 nm beaded polar filament, suggesting a relationship to titin.[8] However, in contrast to cellular titin, smitin failed to crossreact with titin antibodies, suggesting that smitin derives from a distinct gene. Maher and colleagues identified a ~700 kDa protein expressed in adult gizzard smooth muscles and in striated avian muscles, referred to as zeugmatin.[10] Cloning of partial zeugmatin sequences demonstrated that zeugmatin corresponds to an N-terminal fragment of titin.[11] Both the 700 kDa zeugmatin and 2000 kDa smitin proteins from avian smooth muscle were detected immunologically in dense bodies of smooth muscle and in stress fibers of non-muscle cells, implicating them in regulation of the actin cytoskeleton that is prominent in these structures.[8,10,12]

Finally, a titin-sized protein was identified in the rapidly dividing carcinoma HeLa cell line and becomes here an integral part of the chromosome during metaphase.[13] Titin could potentially regulate both the elastic properties of the metaphase chromosome as well as their structural assembly. So far, this chromosomal titin has been characterized more extensively in Drosophila, assuming that the *sls*/D-titin locus codes for a vertebrate titin-like protein: mutations in D-titin/sls result in aberrant metaphase chromosome assemblies and interfere with normal myoblast fusion during muscle development.[14] A more recent study identified a ~500 kDa titin-like protein in HeLa cell extracts and suggested that this protein corresponds to a nuclear isoform of titin involved in the regulation of nuclear envelope assembly: this 500 kDa protein included the M-line region of titin and interacted *in vitro* with lamin-A and lamin-B. Overexpression of the C-terminal region of titin disrupted normal nuclear envelope assembly in HeLa cells, presumably by dominant negative effects.[15]

In summary, numerous previous studies suggested that non-muscle cells also express giant titin-like proteins, potentially responsible for regulating the intracellular assembly of myosin II, metaphase chromosome condensation during anaphase and nuclear envelope assembly during interphase. In addition, smooth muscle tissues have been reported to express 700 kDa and 2,000 kDa titin-like proteins. However, insights into the molecular nature of cellular, chromosomal, and smooth muscle titin isoformss have been limited, because these proteins are considerably less abundant than conventional muscle titin, making it difficult to clarify their relationship with titin by Western blot studies. In particular, it is unclear whether these giant proteins correspond to titin-like homologous proteins, to specialized titin splice isoforms, or to proteolytic subfragments of titin. Here, we took advantage of the fact that titin is a single copy gene in the human genome and hybridized cDNAs from non-muscle and smooth muscle tissues to an array that displays the complete set of 363 titin exons.[16] Our experiments on a range of smooth muscle types demonstrate that mammalian smooth muscles express a specialized ~1 MDa titin splice isoform. Both smooth muscle and striated muscle titin isoforms include binding sites for filamins and α-actinin within their N-terminal region, suggesting conserved roles of titin for assembly of Z-disks and dense bodies in smooth and striated muscle tissues.

Results

Adult smooth muscle tissues express truncated titin isoforms by exon skipping

RNAs from a selected set of tissues were reverse-transcribed, labeled with biotin, and hybridized to the titin array, as described.[16] Thereby, we searched for novel titin splice isoforms in adult smooth muscles (aorta, bladder, carotids, stomach and uterus) as well as in mitotically active non-muscle cells: HeLa (from cervical carcinoma), HL (human leukemia), and HuH7 (liver carcinoma). As a control for sensitivity, we included adult skeletal muscle, and normalized signals to titin exon 4 (expressed both in smooth and striated muscles and not alternatively spliced); as a control for specificity, we used probes that included random five base pair mismatches in the 50-mer probe sequences (MM probes).[16] Under our experimental conditions, we detected 313 of the 363 exons in adult skeletal muscle (Figures 1(a) top and 2(a) top; for information about the probes see Tables 1 and 2). For HeLa and HL, none of the 363 probes detected a signal with one order of magnitude or more above MM background levels (data not shown). For each of the adult smooth muscle tissues analyzed, a subset of 70 to 100 exons was positive (Figure 1(a)). Comparison of the data sets from the different smooth muscle tissues established a consensus map of 34 exons that were positive in all smooth muscle tissues (Figure

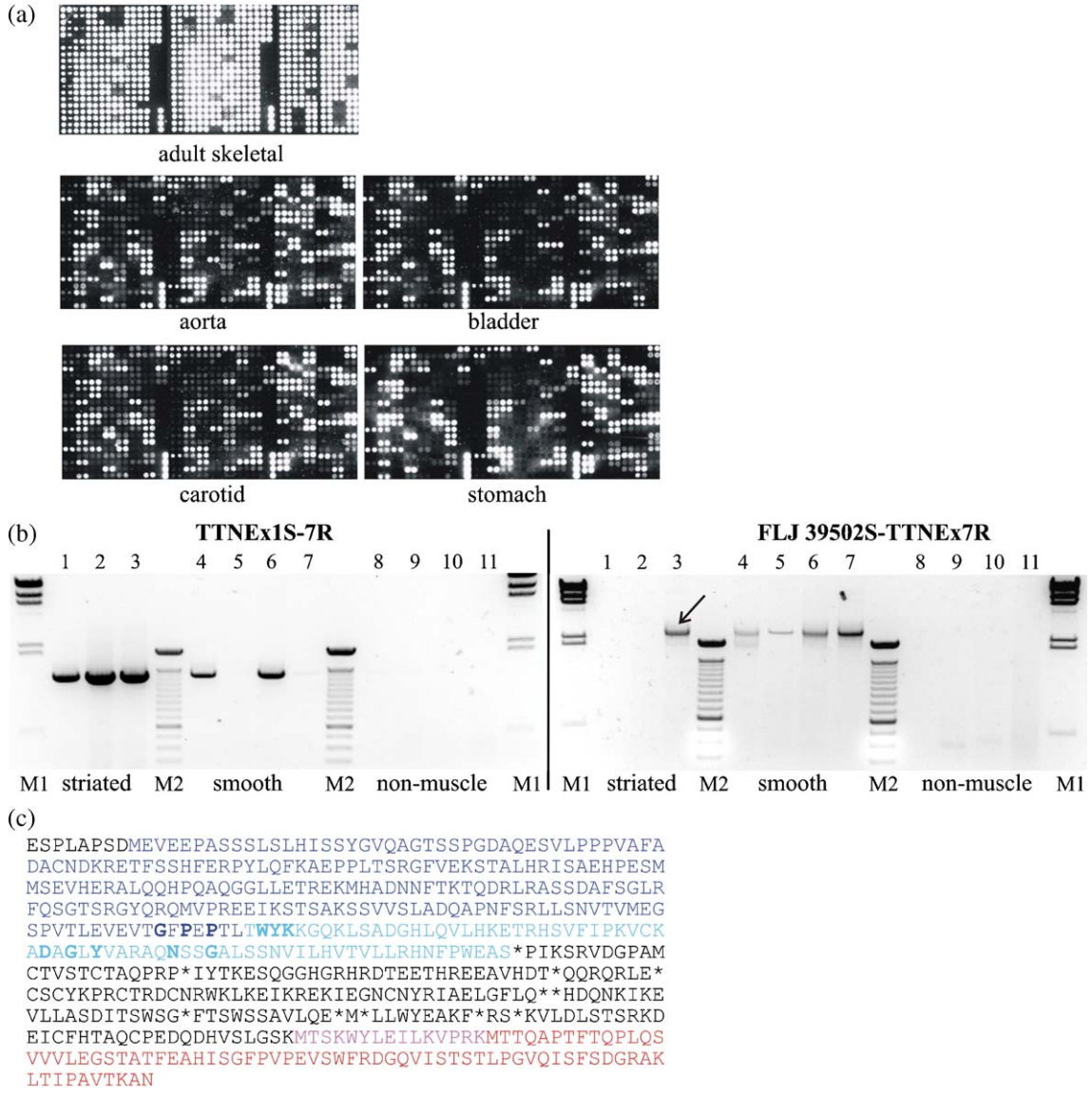


Figure 1. Titin expression profiling in adult smooth muscle and non-muscle tissues. (a) Biotinylated cDNA from four different human adult smooth muscle tissues (aorta, carotid artery, bladder, and stomach) were hybridized to the titin exon array. Smooth muscle tissues express about 80 of 363 titin exons (bottom), whereas skeletal muscle from adult (top) expresses 313 exons. (b) RT-PCR studies on striated, smooth and non-muscle tissues. Analyzed muscle tissues were from adult heart (1), adult skeletal muscle (2), fetal skeletal muscle (3), aorta (4), stomach (5), carotids (6), uterus (7), aorta endothelium (8), whole liver (9), spleen (10), and hepatocarcinomal cell line HuH7 (11; M1 and M2 are different DNA ladder size markers). Representative results for two different primer pairs are shown. Left: titin exons 1–7 are amplified from both smooth and striated muscle tissues. Non-muscle tissues and cell lines are negative. Anchored RT-PCR indicated that smooth muscle titins (compared to cardiac titin) include additional 5′ end sequences and start at an alternative 5′ ends. RT-PCR using an FLJ39502-sense primer located 35 kb upstream amplifies transcripts from smooth muscles and fetal skeletal muscle but not from adult striated muscle tissues. Aorta and carotids express both titin isoforms. (c) The 1.68 kb FLJ39502/titin linking cDNA fragment transcribed in aorta, carotids and fetal skeletal muscle is organized into eight exons on chromosome 2, spanning 35,338 kb. The amino-terminal 217 residues are identical with the peptide encoded by FLJ39502 (blue), followed by 71 residues from an alternatively spliced exon (sky blue; not included in FLJ39502; IG repeat consensus is highlighted). The carboxy-terminal 74 residues are identical with cardiac titin (X90568), and are encoded by titin exons 1 and 2 (see AJ277892). The 3′ UTR present in cardiac titin (exon 1) is skipped, and replaced by an alternative 5′ UTR exon, that also provides a potential upstream alternative start ATG. Both the FLJ39502 encoding frames and titin are separated by multiple stop codons in frames a, b, and c; (b and c translations not shown).

3(b)). To further analyze titin transcripts in smooth muscle and non-muscle tissues, we performed real time (RT)-PCR studies with a set of 36 primer pairs spanning the titin transcriptional unit (see Table 3). Titin could be amplified from tested smooth muscles (Figure 1(b), center). Sequencing of amplified fragments from aorta, carotids, and uterus (12 fragments each) indicated that they were spliced correctly (data not shown). In summary, our RT-PCR and array transcriptional profiling data consistently

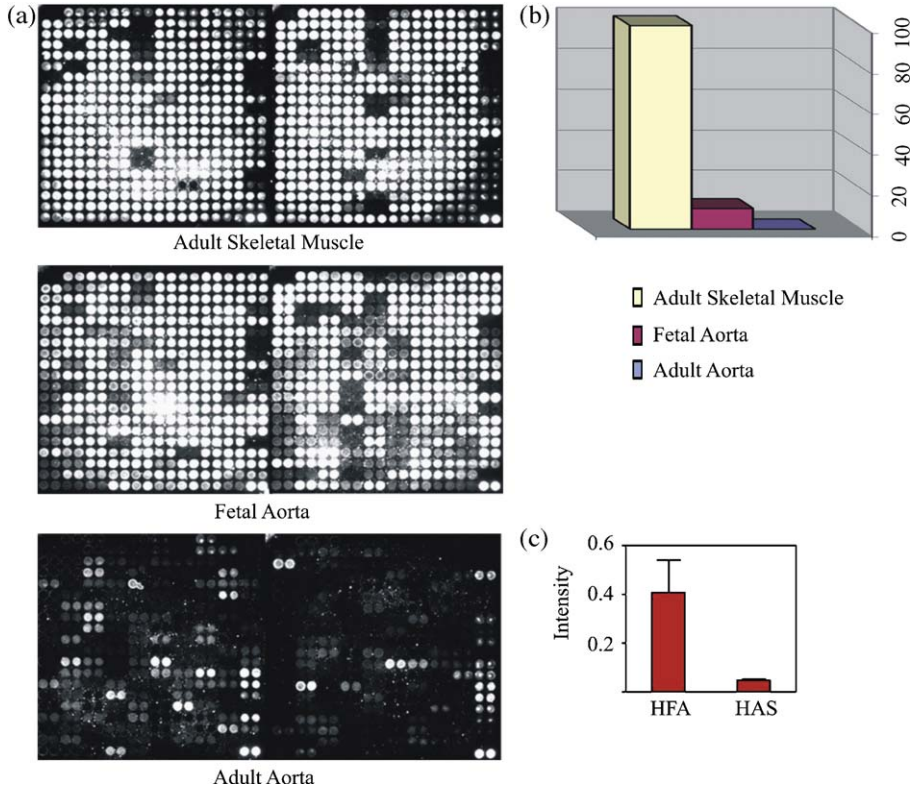


Figure 2. Developmental regulation of titin and MURF-2 transcription in aorta. (a) Titin exon expression patterns were compared in adult human skeletal muscle, human fetal and adult aorta. Skeletal muscle and fetal aorta express full-size titins, whereas adult aorta expresses the truncated smooth muscle titin species. (b) Real-time PCR experiments estimating the relative abundance of titin transcript in adult skeletal muscle, fetal aorta and adult aorta using exon 4 primers. The fetal aorta is tenfold less abundant than adult skeletal muscle transcript. Abundance of titin transcript was quantified by gene-specific standard curves and values were normalized to 18 S rRNA expression. (c) Differential expression of the titin-binding protein MURF-2 in human fetal aorta (HFA) and human adult skeletal muscle (HAS). MURF-2 is 8.1-fold up regulated in HFA ($p = 0.02$). This bar graph represents comparison of normalized intensity for the MURF-2 probe between HAS and HFA ($n = 3$). All MURF-2 intensities were normalized to β actin (normalization to GAPDH gave similar results).

identified a titin splice isoform in adult smooth muscle tissues that contains parts of the Z-disk and A-band regions of titin, and a shortened version of the I-band (Figure 3(c)).

Smooth and striated muscle titins have alternative 5′ ends

We performed anchored RT-PCR studies to amplify the 5′ end regions of titin expressed in skeletal and in smooth muscles, respectively. For this, we selected as anchored primer a reverse primer located in exon 4 (exon 4 is part of the consensus map of exons expressed in all tested smooth muscles; see also Table 1 and Figure 1(a)). This amplified from adult skeletal muscle a 132 bp 5′ untranslated region (UTR) fused to exons 1–4 that was identical with the previously determined 5′ end sequences of cardiac titin (EMBL data library accession X90568). From aorta, stomach, carotids, and uterus, anchored PCR amplified a 1.2 kb fragment further extending to the 5′ end. BLAST searches revealed that these additional 1.2 kb 5′ sequences overlap with the cDNA entry FLJ9502 (annotated as NM173648 as a separate gene). Further RT-PCR studies with sense primers from FLJ39502 and titin reverse primers amplified fragments from all tested smooth muscles, but not from adult striated muscles, or non-muscle tissues (Figure 1(b) right, see the arrow). Interestingly, FLJ39502/titin linking cDNAs were also amplified from fetal skeletal muscle (Figure 1(b), lane 3). Alignment of the FLJ39502/titin linking cDNA fragment with the human genome indicated that this linking fragment includes alternative splice events at the 3′ end of FLJ39502 and at the 5′ end of X90568, respectively, that provide additional coding information: FLJ39502 includes a complete Ig repeat at its C-terminal end, and in X90568/titin, an alternative exon 1a codes for an additional 15 amino-terminal residues (Figure 1(c)). The frames coding for FLJ39502 and titin are separated by multiple stop codons. These data are consistent with a model in which a separate promoter, located about 35 kb upstream from the initiation codon used in adult striated muscle tissues, drives a bi-cistronic fusion transcript coding for both FLJ39502 and titin in smooth muscle tissues.

Table 1. Human titin array

T1	T1	T41	T41	T81	T81	T121	T121	T161	T161	T201	T201	T241	T241	T281	T281
T3	T3	T43	T43	T83	T83	T123	T123	T163	T163	T203	T203	T243	T243	T283	T283
T5	T5	T45	T45	T85	T85	T125	T125	T165	T165	T205	T205	T245	T245	T285	T285
T7	T7	T47	T47	T87	T87	T127	T127	T167	T167	T207	T207	T247	T247	T287	T287
T9	T9	T49	T49	T89	T89	T129	T129	T169	T169	T209	T209	T249	T249	T289	T289
T11	T11	T51	T51	T91	T91	T131	T131	T171	T171	T211	T211	T251	T251	T291	T291
T13	T13	T53	T53	T93	T93	T133	T133	T173	T173	T213	T213	T253	T253		
T15	T15	T55	T55	T95	T95	T135	T135	T175	T175	T215	T215	T255	T255		
T17	T17	T57	T57	T97	T97	T137	T137	T177	T177	T217	T217	T257	T257		
T19	T19	T59	T59	T99	T99	T139	T139	T179	T179	T219	T219	T259	T259		
T21	T21	T61	T61	T101	T101	T141	T141	T181	T181	T221	T221	T261	T261		
T23	T23	T63	T63	T103	T103	T143	T143	T183	T183	T223	T223	T263	T263		
T25	T25	T65	T65	T105	T105	T145	T145	T185	T185	T225	T225	T265	T265		
T27	T27	T67	T67	T107	T107	T147	T147	T187	T187	T227	T227	T267	T267		
T29	T29	T69	T69	T109	T109	T149	T149	T189	T189	T229	T229	T269	T269		
T31	T31	T71	T71	T111	T111	T151	T151	T191	T191	T231	T231	T271	T271		
T33	T33	T73	T73	T113	T113	T153	T153	T193	T193	T233	T233	T273	T273		Biotin
T35	T35	T75	T75	T115	T115	T155	T155	T195	T195	T235	T235	T275	T275		Biotin
T37	T37	T77	T77	T117	T117	T157	T157	T197	T197	T237	T237	T277	T277		Biotin
T39	T39	T79	T79	T119	T119	T159	T159	T199	T199	T239	T239	T279	T279		Biotin
T2	T2	T42	T42	T82	T82	T122	T122	T162	T162	T202	T202	T242	T242	T282	T282
T4	T4	T44	T44	T84	T84	T124	T124	T164	T164	T204	T204	T244	T244	T284	T284
T6	T6	T46	T46	T86	T86	T126	T126	T166	T166	T206	T206	T246	T246	T286	T286
T8	T8	T48	T48	T88	T88	T128	T128	T168	T168	T208	T208	T248	T248	T288	T288
T10	T10	T50	T50	T90	T90	T130	T130	T170	T170	T210	T210	T250	T250	T290	T290
T12	T12	T52	T52	T92	T92	T132	T132	T172	T172	T212	T212	T252	T252	T292	T292
T14	T14	T54	T54	T94	T94	T134	T134	T174	T174	T214	T214	T254	T254		
T16	T16	T56	T56	T96	T96	T136	T136	T176	T176	T216	T216	T256	T256		
T18	T18	T58	T58	T98	T98	T138	T138	T178	T178	T218	T218	T258	T258		
T20	T20	T60	T60	T100	T100	T140	T140	T180	T180	T220	T220	T260	T260		
T22	T22	T62	T62	T102	T102	T142	T142	T182	T182	T222	T222	T262	T262		
T24	T24	T64	T64	T104	T104	T144	T144	T184	T184	T224	T224	T264	T264		
T26	T26	T66	T66	T106	T106	T146	T146	T186	T186	T226	T226	T266	T266		
T28	T28	T68	T68	T108	T108	T148	T148	T188	T188	T228	T228	T268	T268		
T30	T30	T70	T70	T110	T110	T150	T150	T190	T190	T230	T230	T270	T270		
T32	T32	T72	T72	T112	T112	T152	T152	T192	T192	T232	T232	T272	T272		
T34	T34	T74	T74	T114	T114	T154	T154	T194	T194	T234	T234	T274	T274		Biotin
T36	T36	T76	T76	T116	T116	T156	T156	T196	T196	T236	T236	T276	T276		Biotin
T38	T38	T78	T78	T118	T118	T158	T158	T198	T198	T238	T238	T278	T278		Biotin
T40	T40	T80	T80	T120	T120	T160	T160	T200	T200	T240	T240	T280	T280		Biotin

T293	T293	T333	T333	I27I84	27I84	T294	T294	T334	T334	POLYA	POLYA
T295	T295	T335	T335	T4	T4	T296	T296	T336	T336	T4MM4	T4MM4
T297	T297	T337	T337	T4MM5	T4MM5	T298	T298	T338	T338	T5	T5
T299	T299	T339	T339	T5MM	T5MM	T300	T300	T340	T340	T5MMC	T5MMC
T301	T301	T341	T341	T7	T7	T302	T302	T342	T342	T7MM2	T7MM2
T303	T303	T343	T343	T7MM3	T7MM3	T304	T304	T344	T344	T7MM4	T7MM4
T305	T305	T345	T345	T7MM5	T7MM5	T306	T306	T346	T346	T49	T49
T307	T307	T347	T347	T49MM	T49MM	T308	T308	T348	T348	T50	T50
T309	T309	T349	T349	T50MM5	T50MM5	T310	T310	T350	T350	T224	T224
T311	T311	T351	T351	T224MM	T224MM	T312	T312	T352	T352	T358	T358
T313	T313	T353	T353	T358MM	T358MM	T314	T314	T354	T354	Hum B actin	Hum B actin
T315	T315	T355	T355	H.GAPD	H.GAPD	T316	T316	T356	T356	Hum.Su.M. Mysoin	Hum.Su.M. Myosin
T317	T317	T357	T357	T41202	T41202	T318	T318	T358	T358	T110603	T110603
T319	T319	T359	T359	T490202	T490202	T320	T320	T360	T360		
T321	T321	T361	T361			T322	T322	T362	T362		
T323	T323	T363	T363			T324	T324	SUMO1	SUMO1		
T325	T325	SUMO2	SUMO2			T326	T326	SUMO3	SUMO3		

Design of the array for exon-specific expression profiling of titin. The human titin gene encompasses 363 exons. From each exon, a 50-mer sequence was selected as probe and printed onto a glass slide in duplicate (T1 to T363, respectively). Biotin is included as a positive control for the labeling chemistry, GAPDH and β-actin as a positive control for the RNA preparation, reverse transcription, hybridization, and labeling chemistry (probes highlighted in yellow; see also Lahmers *et al.*[16]). Exon 4 and exon 7 probes (detected in both striated and smooth muscle tissues) were included as MM5 mismatch probes as controls for specificity of hybridizations. (For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

Developmental regulation of titin transcription in aorta

Our RT-PCR studies suggest that FLJ39502/titin fusion transcript expression is regulated developmentally in skeletal muscle: fusion transcripts could be amplified from fetal skeletal muscle, but not from adult skeletal muscle (see Figure 1(b)). Therefore, we compared titin expression in fetal and adult human aorta by quantitative RT-PCR.

Table 2. Human titin array

		39	39	79	79	119	119	159	159	199	199	239	239	279	279	319	319	359	359
1	1	41	41	81	81	121	121	161	161	201	201	241	241	281	281	321	321	361	361
3	3	43	43	83	83	123	123	163	163	203	203	243	243	283	283	323	323	363	363
5	5	45	45	85	85	125	125	165	165	205	205	245	245	285	285	325	325	5MM	5MM
7	7	47	47	87	87	127	127	167	167	207	207	247	247	287	287	327	327	4MM4	4MM4
9	9	49	49	89	89	129	129	169	169	209	209	249	249	289	289	329	329	SUMO2	SUMO2
11	11	51	51	91	91	131	131	171	171	211	211	251	251	291	291	331	331	MURF1	MURF1
13	13	53	53	93	93	133	133	173	173	213	213	253	253	293	293	333	333	MURF3	MURF3
15	15	55	55	95	95	135	135	175	175	215	215	255	255	295	295	335	335	N2BA	N2BA
17	17	57	57	97	97	137	137	177	177	217	217	257	257	297	297	337	337	p94	p94
19	19	59	59	99	99	139	139	179	179	219	219	259	259	299	299	339	339	Nebulin 1	Nebulin 1
21	21	61	61	101	101	141	141	181	181	221	221	261	261	301	301	341	341	Nebulin 149	Nebulin 149
23	23	63	63	103	103	143	143	183	183	223	223	263	263	303	303	343	343	Bactin	Bactin
25	25	65	65	105	105	145	145	185	185	225	225	265	265	305	305	345	345	mouse B actin	mouse B actin
27	27	67	67	107	107	147	147	187	187	227	227	267	267	307	307	347	347	Hum GAPDH	Hum GAPDH
29	29	69	69	109	109	149	149	189	189	229	229	269	269	309	309	349	349	cat Bactin	cat Bactin
31	31	71	71	111	111	151	151	191	191	231	231	271	271	311	311	351	351	k9 B actin	k9 B actin
33	33	73	73	113	113	153	153	193	193	233	233	273	273	313	313	353	353	326	326
35	35	75	75	115	115	155	155	195	195	235	235	275	275	315	315	355	355	328	328
37	37	77	77	117	117	157	157	197	197	237	237	277	277	317	317	357	357	biotin	biotin
		40	40	80	80	120	120	160	160	200	200	240	240	280	280	320	320	360	360
2	2	42	42	82	82	122	122	162	162	202	202	242	242	282	282	322	322	362	362
4	4	44	44	84	84	124	124	164	164	204	204	244	244	284	284	324	324	5MMC	5MMC
6	6	46	46	86	86	126	126	166	166	206	206	246	246	286	286	326	326	4MM5	4MM5
8	8	48	48	88	88	128	128	168	168	208	208	248	248	288	288	328	328	SUMO1	SUMO1
10	10	50	50	90	90	130	130	170	170	210	210	250	250	290	290	330	330	SUMO3	SUMO3
12	12	52	52	92	92	132	132	172	172	212	212	252	252	292	292	332	332	MURF 2	MURF 2
14	14	54	54	94	94	134	134	174	174	214	214	254	254	294	294	334	334	PolyA	PolyA
16	16	56	56	96	96	136	136	176	176	216	216	256	256	296	296	336	336	N2B	N2B
18	18	58	58	98	98	138	138	178	178	218	218	258	258	298	298	338	338	PIA	PIA
20	20	60	60	100	100	140	140	180	180	220	220	260	260	300	300	340	340	Nebulin 10	Nebulin 11
22	22	62	62	102	102	142	142	182	182	222	222	262	262	302	302	342	342	GAPDH	GAPDH
24	24	64	64	104	104	144	144	184	184	224	224	264	264	304	304	344	344	human sm. M. myosin	human sm. M. myosin
26	26	66	66	106	106	146	146	186	186	226	226	266	266	306	306	346	346	rat B actin	rat B actin
28	28	68	68	108	108	148	148	188	188	228	228	268	268	308	308	348	348	rabbit B actin	rabbit B actin
30	30	70	70	110	110	150	150	190	190	230	230	270	270	310	310	350	350	bovine B actin	bovine B actin
32	32	72	72	112	112	152	152	192	192	232	232	272	272	312	312	352	352	325	325
34	34	74	74	114	114	154	154	194	194	234	234	274	274	314	314	354	354	327	327
36	36	76	76	116	116	156	156	196	196	236	236	276	276	316	316	356	356		
38	38	78	78	118	118	158	158	198	198	238	238	278	278	318	318	358	358	biotin	biotin

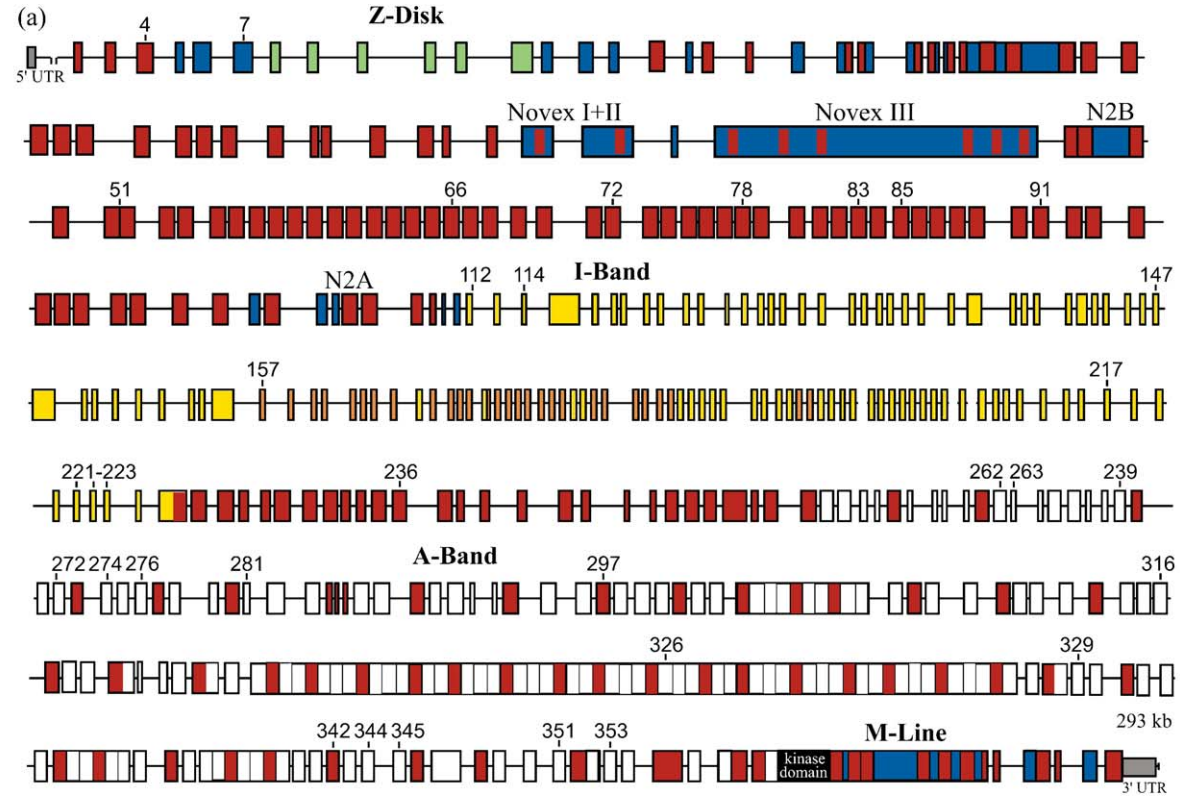
To compare titin and titin ligand expression side-by-side, a number of probes specific for titin-binding proteins such as MURF-1,2,3 were included on the same glass slide. The results (see Figure 2) show that the expression of MURF-2 is regulated during myogenesis.

This suggested that titin transcription in fetal aorta weeks 16–20 corresponds to about 10% and in adult aorta to about 1% of skeletal titin (when normalizing input cDNA to 18 S rRNA, see Figure 2(b)). In addition, array exon profile comparison of fetal and adult aorta titin indicated a developmental isoform transition: In fetal aorta, a full-size titin transcript was detected, whereas in adult aorta, a titin isoform including ~80 exons was detected (Figure 2(a) middle and bottom; array exon grid in Table 2). This developmental down-regulation of titin transcription in aorta was accompanied by a transition from a ~3.7 MDa titin (similar in size to skeletal muscle titin, and including the kinase and the M-line region of titin) to ~1.0 MDa truncated titin isoforms (missing the titin kinase domain).

To determine if this developmental regulation of titin primary structure in aorta correlates with developmental regulation of its ligands, we quantified the relative transcription levels of MURF-1, MURF-2, and MURF-3 in fetal and adult aorta, since these ubiquitin ligases are associated with the titin filament,[17,18] and might be involved in the control of muscle turnover (relevant array sequences are given in Table 4).[19] In fetal aorta, MURF-2 was expressed at about eightfold higher levels (relative to adult skeletal muscle; Figure 2(c)) whereas MURF-1 and MURF 3 were not expressed differently (data not shown).

Western blot studies on titin isoforms expressed in smooth muscles

The array data suggest the expression of a titin splice isoform with an estimated mass of ~1.0 MDa in adult smooth-muscles. Consistent with this prediction (see Figure 3(b)), a ~1.0 MDa protein was identified in adult porcine aorta and stomach (Figure 4(a)). The consensus array map predicts the inclusion of tandem-Ig domains and of PEVK repeats in smooth muscle titin (see Figure 3(b) and (c)). Antibodies directed to the tandem Ig domains I40/41 and to PEVK repeats (9D10)[20] indeed



(b)

Exon	Size	Domains
4	288	Z2
7	331	Zis-1
51	564	Ig-I28/29
66	279	Ig-I44
72	282	Ig-I50
78	288	Ig-I56
83	279	Ig-I61
85	279	Ig-I63
91	279	Ig-I69
112	84	PEVK r.
114	48	PEVK
147	84	PEVK r.
157	81	PEVK r.
217	111	PEVK r.
221	90	PEVK r.
222	72	PEVK r.
223	90	PEVK r.
236	267	Ig-I93
262	297	FN3-I113

Exon	Size	Domains
263	187	Fn3-I114 (5')
269	306	Fn-3 I118
272	303	A3 – FN3
274	303	A5- Fn3
276	297	A7-Fn3
281	191	A12-Fn3 (5')
297	282	A25-Ig
316	306	A53-Fn3
326	17106	A64 (Fn3)-A121 (Fn3)
329	303	A125 (Fn3)
342	291	Ig-A149
344	306	A151 (Fn3)
345	306	A152 (Fn3)
351	303	A159 (Fn3)
353	306	A162 (Fn3)
TOTAL:	**25180**	**bp**
	1,072	**kDa**

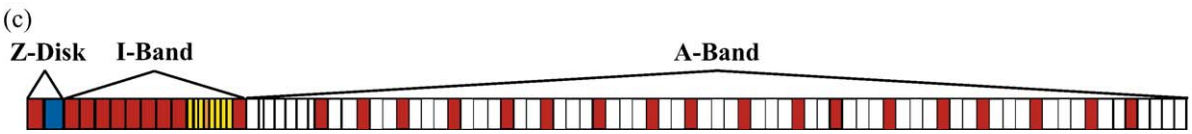


Figure 3. Structure of the smooth muscle titin isoform. (a) Previous sequencing studies indicated that the ~300 kb human titin gene contains 363 exons (rectangles), coding for a 4200 kDa polypeptide.[18,22] Color indicates the protein motifs encoded by the respective exons (red, IgG domains; white, FN3 domains; yellow, PEVK repeats; green, titin Z-repeats, blue, unique domains, black, titin kinase). (b) Array analysis (see Figures 1(a) and 2(a)) indicated that 34 exons are expressed consistently in adult smooth muscle tissues. Exons included in the consensus transcript are from multiple experiments ($n = 3$ for each tissue) and include only the exons that have an intensity greater than the mismatch probes in all tissues evaluated. (c) A consensus transcript of titin domains included in all adult smooth muscle tissues. Together, these 34 exons code for a ~1 MDa polypeptide. The consensus transcript includes Z-disk, I-band and A-band exons.

Table 3. Panel of primer pairs used to amplify titin exons and titin ligands

#	Name	Sequence	Gene/Exon	Length
1a	X112	ttt ccatg gCA ACT CAA GCA CCG ACG TTT		
	X113	ttt ggtacc tca ACC TTG AAC CAG TAA TTC AGC	Z1+Z2	500 bp
1	X146	tttccatg GCG GGG AAT GAC CGT GTG CAG ACC ATC AT		
	X149	tttggtacc TCC AGC TCC TTA CTG GTG TCC TTC TTC CTT	MURF-1	530 bp
2	X156	ttctcgagc GCA TCA GGG GGC CGA TTC CGC TGC CCA		
	X157	tttacgcgt CTC AGC CAG GCA GAC AGA GGG GCA GCA	MURF-2	1.1 kb
3	X226	ttt ctcgagc CCA CTG CCC ACC ATT TAC AAA CGC CAG AAG		
	X227	ttt acgcgt ta TCG AGC AGG GTC GGG TCG GCG CAG GC	MURF-3	800 bp
4	X235	ttt-agatct-GAC AAC CTG GAG AAG CAG CTC ATC TGC CCC		
	X236	ttt-gtcgac- TCG AGC AGG GTC GGG TCG GCG CAG GC	MURF-3 full length	1.1 kb
5	x247	tttctcgagct- GAA CCA ATT TCC TCA AAA CCA GTA ATT GTT AC		
	x248	tttggatCC CTC TTA AAT GGA TCG AAT ATG TAT ATT CAC	M8-M10	1.6 kb
6	X253	ttt tctaga-GAA AAA CTC AGG ATC GTA GTT CCT CTT AAG GAC		
	X254	ttt ggtaccta-AGG GCC TGG AAC ATC ATA TTT ACT CCT TGC	I93-98	1.8 kb
7	X255	ttt tctaga- GTA CCT GAC CCA CCA GAG AAT GTT AAA TGG AGA		
	X256	tttggtaccta-TGG TCC AGG AGT GGT AAA GGT GTC TTT GGC	I103-108	1.8 kb
8	X267	tttccATG GCT ACC TCA GAG CTG AGC TGC GAG GTG TCG GAG GAG AAC TGT GAGC		
	X268a	tttgaattc-TCA GCC TCT CTG TGC TTC CTG GGA CAT GGA GC	T-cap	500 bp
9	X271	ttt ctcgagct CCA CCA TCA TTT TCT CGA CAA TTG AGA GAT GTTC		
	X272	ttt ggatcc ta GGT TGC TGG GGC CAC AGC TGG TTT GTC	N2A	1.2 kb
10	X277	ttt ctcgagct CCA CCA GAA GAA ATC CCT CCT GTG GTT GCT		
	X278	ttt ggatcc ta AGC TCG GGG AGG AGG AGC TTT CTT AGC GAC	PEVK region	1.5 kb
11	X295	tttccatggct-CCT CTG TCA AGC CTC AGG GTG CAC AAC GGG GA		
	X298	tttggtacc-ta-GTT AGC ATT TGC TCT CCA TCC ACT GTA TGC	novex1+2	600 bp
12	X297	tttccatggct-AAG TAC AGT ATT ACT GCA CTT AAA GTA AGT		
	X300	tttggtacc-ta-TGC TGA CGT TGT CCT CTC TCC ACA GTC ATT GTG		600 bp
13	X321	tttg-aat-tct-CCA ACA ATT GAC CTG GAG ACT CAT GAC ATT		
	X322a	ttt-ctcgag-cta-CAC AGC TGG AGG CTC TTG TGG TTC AGC CAC	A1-A7	1.8 kb
14	X325	tttg-aat-tct-AAG CCA CGG TCC ATG ACC GTC TAC GAG GGC		
	X326	ttt-ctcgag-cta-GGA AGC TGT AGC TGA ACA CTG GCC ACG GAA	M5-M9	2 kb
15	X347	tttccatg- GTT GAA CCA CCA CCT AAA GTC CCT GAG CTA		
	X348	tttggtacc-ta-GGA TAG TTT TTC TTC AGC AAC AAA TCT CTT TTC	PEVK exons 120-135	600 bp
16	X349	tttccatg gcc- CAA AGA GTG GAA GTC ACG CGG C		
	X350	tttggtacc-ta-CTC CTC TAT TTT AGC AGG AAT TTT TGG	PEVK exons 120-135	300 bp
17	X351	tttccatg-GCA GTG CCA GAA ATA CCA AAG AAG AAA GTT		
	X352	tttggtacc-ta-GAG AGG TTC TTC CAT CTT AAT GAC TTT TGG	PEVK region exons 120-135	700 bp
18	X353	tttccatg-GGG- AGA ACT GTC CTT GAA GAA AAA GTA TCA		
	X354	tttggtacc-ta- ACC TTT AGG TGG AGC TTT TGG TTT TTC AAA TAC	PEVK region exon 154	
19	X369	tttccatg-gaa att aag aag aaa gtg aca gag aag aaa gtg gtc		
	X370	tttggtacc-ta-CAC TTC AGG GGG AGG ACT TTC CGG TTT GGG	PEVK exons 149-174	600 bp
20	X379	tttccatg-GAT CGA GAC ATT GCT CCA TTT TTT ACC AAA CCC		
	X380	tttacgcgt-ta-CCT CTT AAA GTC ACC CTG GCA CTG CAG A	exons 56-59	
21	X385	tttccatg-GAA GCT GTC TTT ACC AAA AAT CTT GCC AAC		
	X386	tttggtacc-ta-CAA TAA CTT TTA AAT TGA TGA ATC CTT CTG CTT	I99-I105	1.8 kb
22	X387	tttccatg-GGT gca gtt gtt gag ttt gtg aaa gaa ctt cag		
	X388	ttt-ggtacc-TTC TAC AGA GAG ATT ACA GTT GGT TTC AAC	I3-I5	900 bp
23	X389a	ttt-ggtacc- CCAGCTGTGCATACAAAGAAGATGGTTATTT		
	X390a	ttt-gagctc-TTT GAT GGG TTG AGG TTC TCT TTT TGG AGC	PEVK exons 119-136	1.8 kb
24	X391	ttt-gagctc- gct gtc aaa att aag aag act ctg aag aac ctc		
	X392	tttggatcc- ta-aca-aca-GGT GAC TGT CAG GGT GGC ACT GAC TTG GTC	I8-I10	900 bp
25	X387a	tttccatg-gat cgg gaa att aaa ctg gtg cga ccc ctg		
	X388a	tttggtacc-AGG CAC GAC ATT CAG GTT ACA GGA AGT CTT	I95-97	900 bp
26	X391a	tttgagctc-Gaa ttg cct ctt atc ttc atc aca cct ctc agt		
	X392a	tttggatcc-ta-aca-aca- TCT AAT GTC AAG TTT TCC TGA GGT CTT ATC	I92-I94	900 bp
27	X393	tttgaattc-GCA CCA CAC TTT AAA GAG GAA CTG AGA AATC		
	X397	tttggatccta-AAC CGG GTC AAA GAC CCG TCT ATT ACG TAC	A168-kinase	2 kb
28	X405	tttcc-ATG gCA AAC TGG GGC GGA GGC GCA AAA TGT		
	X406	tttggat-CCTCT TCA TTC TTT CTT TTC CAC TTG TTG TGT	MLP	600 bp
29	X409a	tttcca-atg gta ctg aaa gta gag gaa ctg gtc act		
	X410a	tttggtacc-TCA GAA TGT AGC TAT GCG AGA GGT CTT GTA	CARP	1 kb
30	X415	tttccatg-GGG CCA CCT ACT GGA CCA ATC AAA TTT G		
	X416	tttagatct-cta-TCT GTC AAG AAC TTT GAC ATT GAA AAT GTG	A69-A72	1.2 kb
31	X425	tttccatg-GAT CGA GAC ATT GCT CCA TTT TTT ACC AAA CCC		
	X426	ttggatcc-ta-TTT GAC TAT TAC AAT GCT ACT GCA GTG GTC ACT GCC	I34-36	900 bp
32	X427	tttccatg-GAT CTT ATC ATA CCT CCT TCA TTC ACC AAA		
	X428	tttggatcc-ta-TTT CAC TTT GAG TTC AAT GCT GCA GCT CGC	I40-42	900 bp

(continued on next page)

Table 3 (*continued*)

#	Name	Sequence	Gene/Exon	Length
33	X154	gttcctgttcagaaaaaaga ggcaccccca		
	X354	tttggtacc-ta- ACC TTT AGG TGG AGC TTT TGG TTT TTC AAA TAC	Ex154S + X354 (exons 154-156)	
34		tggccctggagaacctgcatatgttgatga		
		CAGGATCCAGTCTTTCAACCCAGTATCCTG	Ex258S + EX-263R	
35		tattagtgaaccttctcgggctactcctcc		
		GAAGGTGGACTGAACTTTCCAGGTCCAGCT	ex272S + ex-275R	
36		cacttgactggaaagagccccgcagtaatg		
		CCAGCTCACTAGCATTGATCCTTTGGTGCG	ex279S + EX-282R	
37		gtcctagaaagtcggctcattgggactgag		
		AGGGGTGTGATTGGCTCTTCTCCACTCTTC	ex296S + EX-300R	
38		actggatatatagtggagaagcgagatctacc		
		GGAACAACGAACTGAGTGATTCTGAGGGCG	ex323S + EX-326R	
39	X433	tttctcgagc-gtg aaa caa gat gct gac aaa agt gca gct		
	X434	tttacgcgt-cta-TGA GGC TGG ACG TTG GGG AGG CTC AGC TAC	EXONS 8-14	
40	X435	ttt cca tg GTG CCA CTG AAG TTT GTG AAA GAA ATC AAA GAC		
	X436	tttggtacc tta ACG CTC TCC AAG TGT CAG CTT TGC AGG GTA	Ig84-86; exons 225S + 227R	

All primer pairs are from human. For those primers 30-mers or longer, the recommended annealing temperature is 68 °C. Lower case letters are 5′ mismatch tags for cloning. Upper case letters are matching titin or titin ligands. For primer pairs 9, 15, 16, 20, 22-24, 31, 35, and 40, efficient amplification was obtained from both skeletal and carotid cDNAs. DNA sequence analysis confirmed their correct splicing.

recognized the 1 MDa species in Western blots; in addition, anti-MIR (directed to the A/I junction)[18] recognized the 1 MDa protein present in aorta and stomach (Figure 4(a)). Since the titin 9D10 antibody is known to cross-react with another giant protein, AHNAK, we performed additional Western blots with antibodies to AHNAK.[21] Finally, we included antibodies to Novex-3 titin, a ~650 kDa splice isoform of titin co-expressed in striated muscles with conventional half-sarcomere spanning titins.[22] The antibodies to AHNAK and anti-Novex 3 antibodies did not recognize the 1 MDa species present in smooth muscles (Figure 4(a), right).

We performed Western blot studies on cultured adult human aortic smooth muscle cells to determine if the titin protein is indeed present in smooth

Table 4. List of probe sequences to monitor the expression of titin ligands

Grid	#	Gene/Exon	Sequence
Box IV - E8	348	titin exon 16	ctgactctgtttccatgtaggcatcaatagccggtagtgctattgccaca
Box IV - E9	349	titin exon 15	atcaagaaaactacagatctaacaacggaaagattagtccatgtggataa
Box IV - E10	350	titin exon 14	gtggacgttggaaaaaaggctgaagctgtagcaacagttgtt
Box IV - F1	351	titin exon 13	atgagaaaggaagccgagaaaactgccttgtctacaatagcagttgctac
Box IV - F2	352	titin exon 12	ataatgaaggaaactaggaaaacagttgtacctaaagtcatagttgccac
Box IV - F3	353	titin exon 11	ataagacaggaaactgagataactgctgcatccatggtggtagttgccac
Box IV - F4	354	titin exon 10	taagaaaagaaactgaaaaaacatttgtaccaaaggtagtaatttccgca
Box IV - F5	355	titin exon 9	gtaagaaaggaagcggagaagactgctgtaactaaggtagtagtggccgc
Box IV - F6	356	titin exon 8	gtgaaacaagatgctgacaaaagtgcagctgttgcgactgttgttgctgc
Box IV - F7	357	titin exon 7	gtccgtgtctccagcagcaagaatctccacatcccccatcaggtctgtta
Box IV - F8	358	titin exon 6	aagattgaagcccactttgatgccagatcaattgcaacagttgagatggt
Box IV - F9	359	titin exon 5	gtgaagaagaagtacctgctaaaaagacaaagacaattgtttcgactgct
Box IV - F10	360	titin exon 5MMc	gtgaagaagaagtacctgctaaaatttttaagacaattgtttcgactgct
Box IV - G1	361	titin exon 5 MM	gtgaagccgaagtacctgctaaaattacaaagacaattccttcgactgct
Box IV - G2	362	titin exon 5 MM6	gtgaagaagaaccccctgctaaaaagacaaagacaattccctcgactgct
Box IV - G3	363	titin exon 4	ctgagacagcaccacccaacttcgttcaacgactgcagagcatgaccgtg
Box IV - G4	364	titin exon 3	gttttccagttcctgaggtgagctggtttagggatggccaggtgatttcc
Box IV - G5	365	titin exon 2	agtgcctagaaagatgacaactcaagcaccgacgtttacgcagccgttac
Box IV - G6	366	titin exon 1	ggattagaggctcaccgattcatgtcggagatggtcagaaaaaccaactc
Box IV - G7	367	titin exon 1MMc	ggattagaggctcaccgattcaaaaaagagatggtcagaaaaaccaactc
Box IV - G8	368	titin exon 1MM	ggattagagaatcaccgattcaaatcggagatggtcccaaaaaccaactc
Box IV - G9	369	titin exon 1MM6	ggattagaggctcggggattcatgtcggagatgggggaaaaaccaactc
Box IV - G10	370	Sumo 1	aaccttcaactgaggacttgggggataagaaggaaggtgaatatattaaa
Box IV - H1	371	Sumo 2	tgtgaagacagagaatgaccacatcaacctgaaggtggccgggcaggacg
Box IV - H2	372	Sumo 3	aagactgagaacaacgatcatattaatttgaaggtggcggggcaggatgg
Box IV - H3	373	MURF-1	ttctcaggtactttatcggacctctcacatggctgcatgcccagaaatgt
Box IV - H4	374	MURF-2	aaacaatgtcctccaccgagagaaacgtaaaggacacttgatcacacaat
Box IV - H5	375	MURF-3	atgaacttcacagtgggtttcaagccgctgctaggggatgcacacagcat
Box IV - H6	376	PIAS	agaactctttgcagtcaaacctacccaacccatccaggagctgctacaca
Box IV - H7	377	p94/calpain-3	atatcagttagcctggtttcactatacagtacatcattttgcttaaagtc

The MURF-2 probe revealed the developmentally regulated expression in skeletal muscle from fetus and adult (see Figure 2).

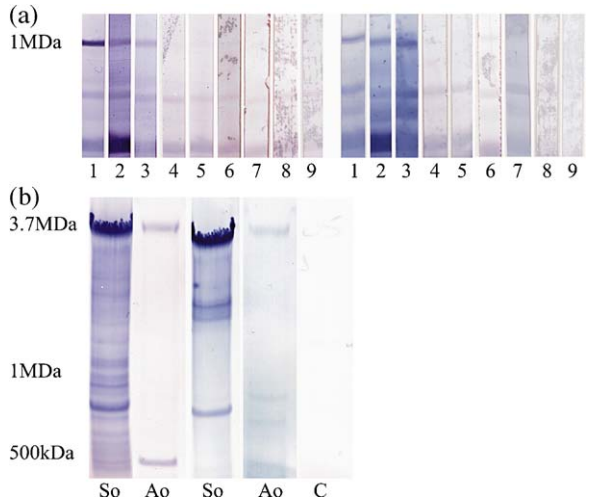


Figure 4. Western blot studies on smooth muscle tissues with titin antibodies. (a) Western blot of protein isolated from pig aorta (left) and pig stomach (right). The blot set (lanes 1–3) represents immunoreactivity of a ~1 MDa protein with titin antibodies to multiple titin domains. Controls in lanes 6–9 include antibodies to other giant proteins as a negative control. Titin antibodies: lane 1, 9D10-anti PEVK anti-titin antibody; lane 2, anti-Ig40-42 anti-titin antibody; lane 3, MIR-anti A/I junction anti-titin antibody; lane 4, no primary control rabbit; lane 5, no primary control mouse. Controls: lane 6, anti-AHNAK antibody; lane 7, anti-Novex-3 antibody; lane 8, no primary control rabbit; lane 9, no primary control mouse. (b) Western blot detecting a ~3.7 MDa protein isolated from human soleus (So) and human aortic smooth muscle cells (Ao). Human soleus blots are included for size estimates and positive controls. Z1/Z2 antibody raised to titin N terminal domains (left two lanes) and MIR (right two lanes) are immunoreactive with a similarly sized protein (~3.7 MDa) in soleus and human aortic smooth muscle cells. A ~500 kDa protein is identified in human aortic smooth muscle cells with the Z1/Z2 antibody but not with MIR, suggesting a truncated/differently spliced protein. A no primary antibody (rabbit) control (C) is included for human aortic smooth muscle cells.

muscle cells rather than in perivascular connective tissue (Figure 4(b)). Human soleus was included in this study as a reference for protein size estimates and relative protein abundance. Expression of a 3.7 MDa protein (similar in size to human soleus titin) was detected in cultured human aortic smooth muscle cells. This protein was immunoreactive with titin anti-Z1/Z2 and MIR antibodies (Figure 4(b)). In addition, a ~500 kDa species was detected in cultured aortic cells that reacted with Z1-Z2 antibodies. When comparing with soleus and using myosin as the reference for loading, the titin protein was about 10–20-fold less abundant in aorta (data not shown). In summary, our Western blot studies suggest the expression of a truncated titin-related species ~1 MDa in size in all smooth muscle tissues tested. In the case of cultured aorta smooth muscle cells, co-expression of a ~400 kDa titin isoform and a full-size titin species was detected, suggesting that cultured smooth muscle cells, similar to fetal aorta tissues, can express a truncated species and a full-size titin.

Z2-Zis-1 shared by smooth and striated muscle titins interacts with filamin and α-actinin

At their N-terminal ends, smooth muscle and skeletal muscle titins share the Z2-Zis1 domains (encoded by exons 4–7, see annotations to AJ277892). To gain insights into the functions of Z2-Zis1, we inserted this fragment into a yeast two-hybrid (YTH) bait vector and screened both smooth muscle from aorta and skeletal muscle cDNA libraries. In skeletal muscle, this identified the C-terminal region of nebulin as an interaction partner of titin Zis1 (described elsewhere in detail)[23] In both the smooth muscle aorta and in the skeletal muscle cDNA library screens, Z2-Zis1 identified prey clones coding for filamin and for α-actinin: the aorta screen pulled out filamin-A and α-actinin-1 clones, whereas the skeletal cDNA library screen isolated α-actinin-2, α-actinin-3 and filamin-C prey clones, respectively (see Figure 5(a) and (b)). The region of overlap in the multiple prey clones suggests that the C-terminal two Ig repeats of filamin-A or filamin-C are required for interaction with titin Z2-Zis1, whereas the C-terminal 70 kDa (spectrin repeats 2, 3 ,and 4, and C-terminal domain) of α-actinin-1, α-actinin-2 or α-actinin-3 are required for interaction with titin (Figure 5(a) and (b)). The interaction of titin with filamin-A and with α-actinin-1 was tested further by a pulldown assay. For this, titin Z2-Zis1 was fused to maltose-binding protein (MBP), and co-expressed with filamin-A (see Materials and Methods for further details). Co-purification of titin-Z2-is1 MBP fusion proteins and filamin-A on amylose agarose confirmed the interaction of titin with filamin-A (Figure 5(c)).

Localization of titin in intact smooth muscle tissues and cultured aortic smooth muscle cells

To further localize the smooth muscle titin isoform, confocal microscopy was performed on intact bovine aorta, human uterus, and cultured human aortic smooth muscle cells. For this, we used titin antibodies positive in our Western blot studies and directed to epitopes included in smooth muscle titin according to our array data. In intact bovine aorta, antibody labeling was present diffusely in the tunica media layer at low magnification (4×) (Figure 6(a) left), whereas higher magnification (60×) indicated labeling within the cytosol of the smooth muscle cells (Figure 6(a) right). There was no labeling in the endothelial or tunica adventitia layers of the aorta (Figure 6(a)).

We used a uterus sample from late-stage pregnancy obtained in a Cesarean section to analyze an active and well-vascularized smooth muscle tissue type. Here, titin antibodies did not label the

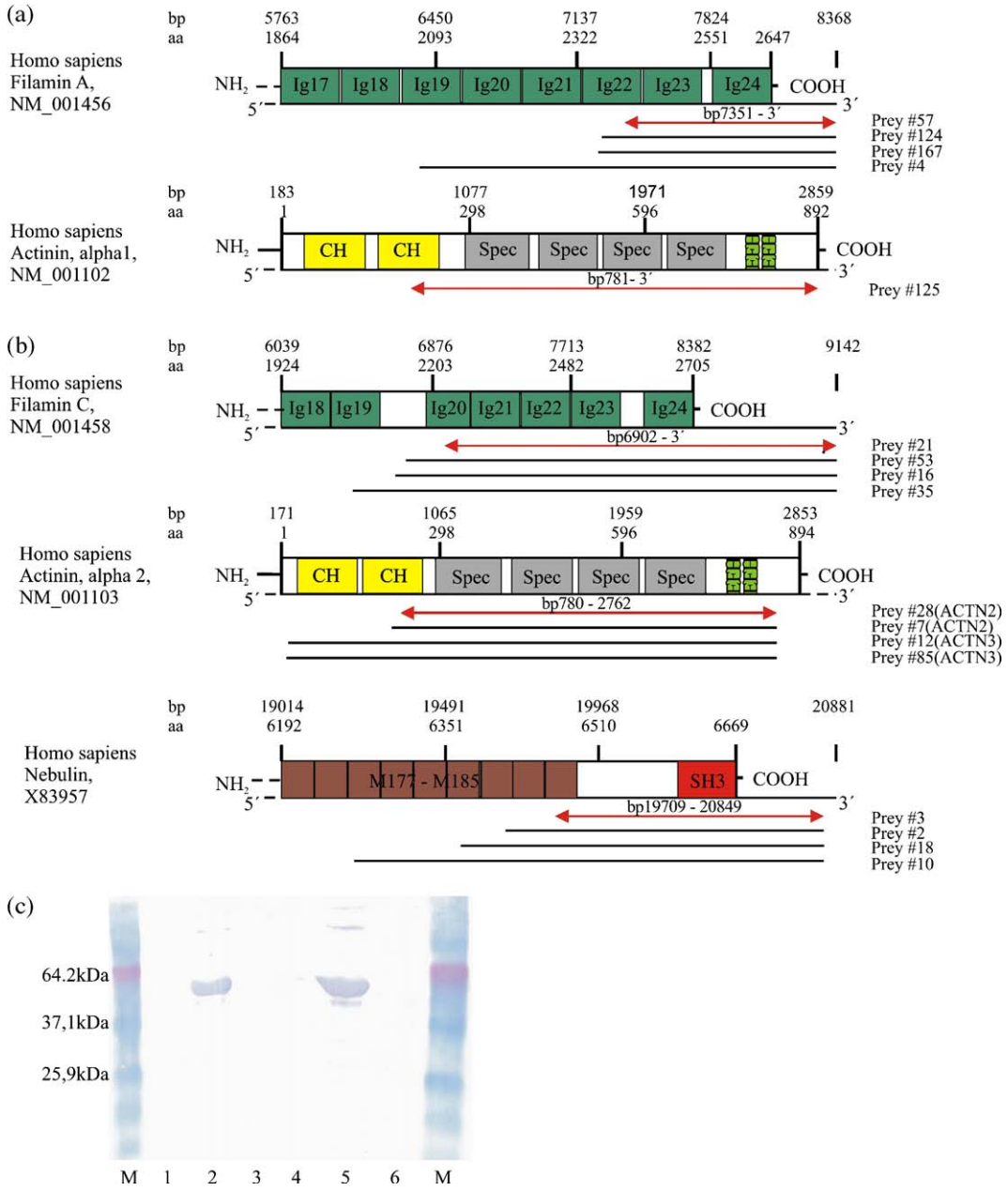


Figure 5. Identification of filamin and α-actinin binding sites shared between smooth and striated muscle titins. (a) and (b) A fragment coding for titin segment Z2-Zis1 (exons 4–7) was used as a bait to screen aorta smooth muscle and skeletal muscle cDNA libraries. Identified prey clones indicate the interaction with filamin-A and α-actinin-1 ((a) from smooth muscle), and filamin-C, α-actinin-2, 3 and nebulin ((b) from skeletal muscle). A second binding site for filamin (here filamin-C) was detected by a YTH screen using the titin kinase M-line region in a distinct screen. (c) Co-expression of titin Z2-Zis1–MBP (maltose-binding protein) fusion proteins with filamin-A and co-purification on amylose resins confirmed the interaction of the filamin-A C terminus with titin-Z2-Zis1 (lane 2) as detected by Western blots of eluted fractions (antibody directed against filamin). Controls: lane 1, Z2-Zis1-MPB alone; lane 3, filamin-C terminus alone; lane 4, co-expression of filamin-A and MPB; lane 5, filamin-A fragment input before column; and lane 6, BSA. M, marker.

muscularis of small arteries included in the uterus tissues (Figure 6(b)). Therefore, while our array, Western blot and histology studies could detect a vascular titin in the muscularis of large arteries, we could not detect titin in small peripheral blood vessels. In contrast, titin antibodies stained the myometrium (Figure 6(b)). Double-labeling studies performed with anti-titin Ig34-36 and anti-filamin suggested that titin and filamin co-localize in the myometrium (Figure 6(c)).

We performed double-labeling studies with the same set of antibodies in skeletal muscle. This verified the co-localization of titin I34-36 and filamin (Figure 6(d)). In addition, filamin labeled the membrane intensely, consistent with its potential function as a linker of the myofibril and the sarcolemnal membrane (Figure 6(d) middle).[24,25] Finally, we localized the smooth muscle titin protein to the cytosol in cultured aortic cells (Figure 6(d)).

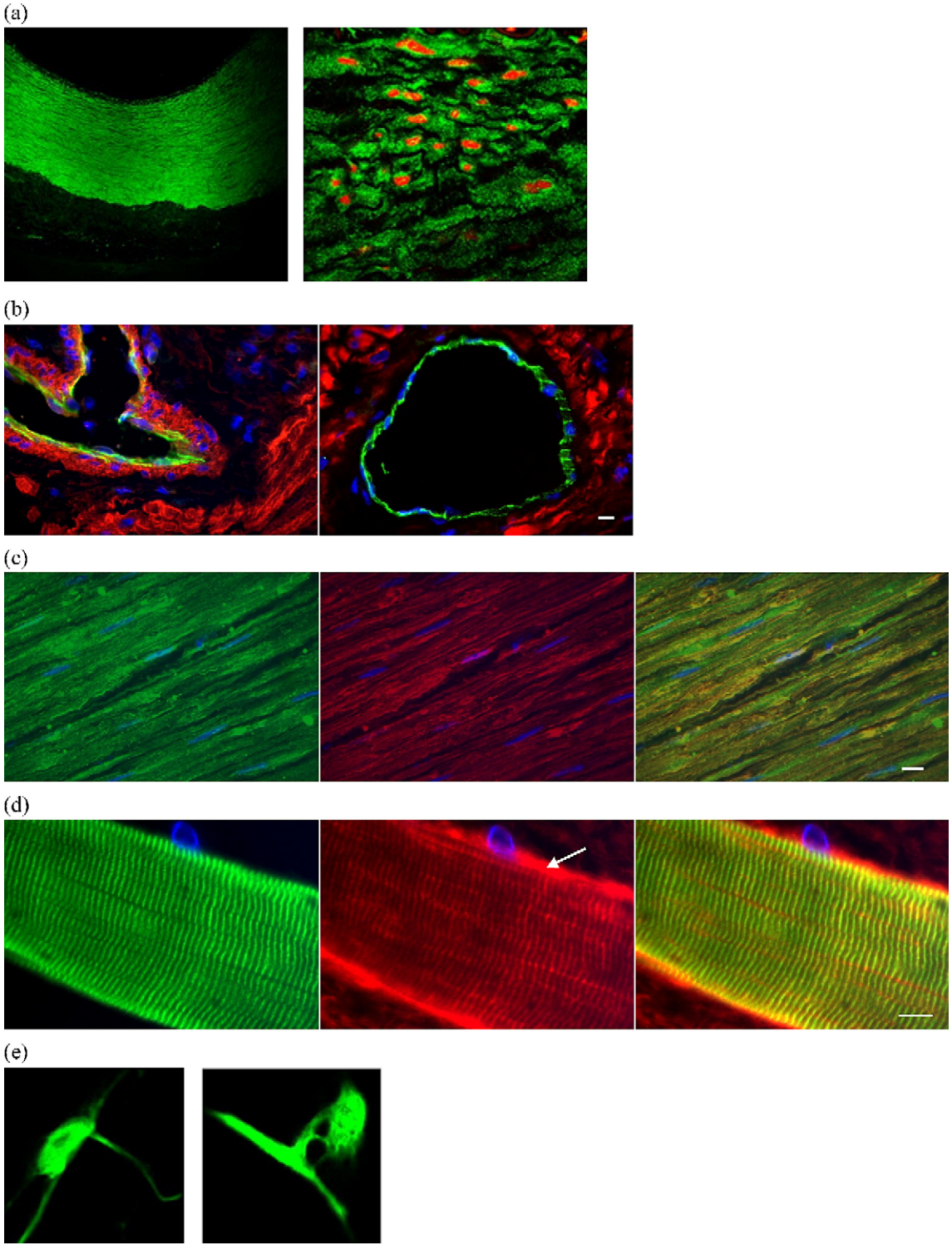


Figure 6. Immunofluorescence-based localization of titins and filamins in smooth and striated muscle. Antibodies directed to different titin epitopes and antibodies directed to the C-terminal region of filamin were used to stain striated and smooth muscle tissues. (a) In intact bovine aorta, titin anti-MIR labels the smooth muscle cell layer (tunica media, (left 4×) and their cytosol (right, 60×). Nuclear labeling with propidium iodide (red) is noted (right). (b) Left: In intact human uterus, filamin (red) is detected in the muscularis of small arteries, and in the myometrium. Right: Antibodies directed to titin Z-repeats 4–6 (red) also recognize the myometrium but not the arterial muscularis (green, PECAM-1 as enthothelial marker; blue, DAPI nuclear labeling). The scale bar represents 10 μm. (c) In the myometrium, titin Ig34-36 (green) and filamin (red) locate in striations and co-localize here. The scale bar represents 10 μm. (d) In human skeletal muscle anti-titin Ig34-36 (green) and anti-filamin (red) label myofibrils in a striated pattern. Anti-filamin also labels the sarcolemnal plasma membrane (see arrow). Right: Double labeling of filamin and titin Ig34-36 demonstrates co-localization of I-band titin epitopes and filamin. The scale bar represents 10 μm. (e) Confocal microscopy of cultured human aortic smooth muscle cells indicates labeling of the cytosol with the MIR (left) and 9D10 (right) titin antibodies.

Discussion

Putative non-muscle titin has been identified in platelets,[26] in fibroblasts, in HeLa cells,[13,15], in the intestinal epithelial brush border,[4,6] and in avian smooth muscle.[8,10,12] In contrast to striated muscle titin, the giant protein species from smooth muscle and non-muscle tissues have been difficult to characterize due to an overall low level of their expression. Moreover, Western blot studies observing failure to react with titin antibodies may be explained by a splice isoform lacking the respective titin epitope, whereas a positive reaction might be caused by a cross-reactivity of titin to different proteins such as AHNAK.[21] Here, we screened human tissues with an exon array specific for the single-copy titin gene[16,22] to determine which tissues in addition to cardiac and skeletal muscle express titin isoforms at significant levels.

For smooth muscle tissues, the array data provide a framework to interpret previous data on 700-2000 kDa titin-like proteins identified in smooth muscle tissues.[8,10,12] In human adult smooth muscle tissues a specialized titin splice isoform of about 1 MDa is expressed in which ~280 exons of the titin complete 363 exon set are excluded. For example, the seven titin Z-repeats (corresponding to exons 8–14 each coding for a 45-residue Z-repeat) are excluded from smooth muscle titin according to our array typing (Figure 1(a)) and immuno-histological characterization of smooth muscles tissues (Figure 6(b)). Z-repeats provide attachment sites for α-actinin.[27–29] Since, near the N terminus, smooth muscle titin isoforms include within exons 4–7/Z2-Zis-1 an additional binding site for α-actinin (Figure 7), our data are consistent with the α-actinin-binding properties of smooth muscle titin.[9] In their C-terminal regions, smooth muscle titin isoforms include Fn-3 repeats (Figure 3(c)) that can provide binding sites for myosin.[30] Therefore, smooth muscle titins may link together in an elastic fashion α-actinin from dense bodies and stress fibers with myosin filaments, involving Zis1 and Fn-3 repeats at their respective end regions (because of the inclusion of spring elements; see Figure 3(c)). Smooth muscle titin may therefore contribute to smooth muscle tissue elasticity, such as in the developing aorta where they are expressed at higher levels (Figure 2). Interestingly, these compliant titin isoforms from the developing aorta are predicted to include the titin kinase domain, whereas adult-type smooth muscle titin is predicted to be less compliant and to omit the titin kinase domain.

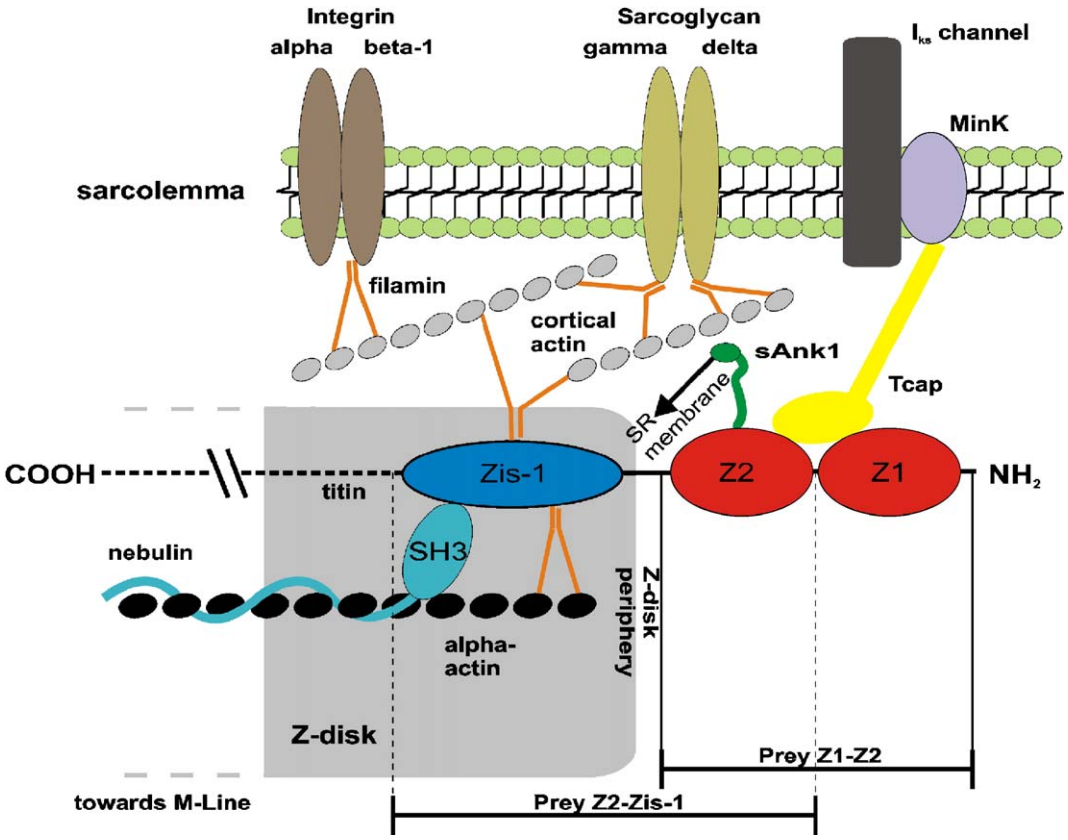


Figure 7. Model for the connections made by Z1-Z2-Zis1 in the myofibrillar titin filament as identified by previous YTH screens with titin-Z1-Z2 and here with Z2-is1. Z-is1 interacts with nebulin, filamin and α-actinin in the periphery of the Z-line region and has the potential to coordinate their assembly. Z1-Z2 attaches the myofibril to the sarcolemnal membrane by interacting with the adaptor proteins sAnk1 and T-cap;[32,39] Z2-Zis1 can link to the cortical actin filament and to integrin-β-1 *via* interaction with filamin. Interaction of Zis1 with α-actinin and nebulin links the titin filament to the thin filaments inserting into the Z-disks (α-actinin is omitted from the cartoon for simplicity, see Discussion for details).

For skeletal muscle, it remains to be studied how the unique α-actinin-binding site (exons 4–7/Z-is1) and the repetitive α-actinin-binding sites (Z-repeats, exons 8–14) functionally cooperate. The unique α-actinin-binding site in Z-is1 is located close to the binding sites for filamin, nebulin and potentially developmentally regulated phosphorylation sites (Figure 7), whereas the central Z-repeats are dispensable, as indicated by splicing out in fast fiber-type skeletal muscle tissues.[29] Therefore, we speculate that the unique α-actinin-binding site in Z-is1 may have an important coordinating role during Z-disk assembly, while Z-repeats may serve a more structural role to provide mechanical stability within the Z-disc lattice.

The N-terminal domains Z2- Zis1 shared by striated and smooth muscle titin isoforms also provide binding sites for filamins: in striated muscles, our YTH screens with a Z2-Zis1 bait identified filamin-C in skeletal muscle cDNA library screens, and filamin-A from aorta cDNA library screens (Figure 5(a) and (b)). Deletion mapping experiments indicated that the C-terminal ~30 kDa of both filamin-C and filamin-A are sufficient for the interaction with titin (Figure 5(a) and (b)). This C-terminal segment shares >80 % sequence identity between the seven distinct members of the human filamin gene family (as identified by BLAST searches‡). Therefore, our deletion mapping experiments, together with the high C-terminal conservation of members of the filamin gene family, favor a model in which members of the filamin family such as filamin-A and filamin-C interact with the titin Zis1 domain in a conserved fashion.

For investigating the association between titin N-terminal and filamin C-terminal epitopes, we studied skeletal muscle myofibrils that allow more informative double-labeling studies than in smooth muscles: anti-filamin labeled the myofibrillar Z-disk and I-band portions (Figure 6(d), consistent with the *in vitro* binding studies using purified filamins and isolated myofibrils.[31] In addition, our anti-filamin antibody labeled the perimyofibrillar plasma membrane (consistent with the interaction of filamin with integrin-β).[24,25] Taken together, these staining and previous *in vitro* binding data are consistent with a model in which titin Z-is1 specifies attachment of the C-terminal region of filamin to the myofibrillar Z-line (or dense bodies in smooth muscles), that in turn can make connections to the sarcolemnal membrane *via* their interaction with integrins (Figure 7).[25] Myozenin (calsarcin/FATZ) and myotilin are likely to be additional regulatory components of this myofibril–membrane linking complex.[24]

In addition to the plasma membrane, striated muscle contains two specialized internal membrane systems, the sarcoplasmic reticulum (SR) and the T-tubular systems. The SR has been proposed to connect to the myofibrils at the level of both the Z-disk and the M-line involving titin and obscurin in docking these specialized membrane to myofibril connections.[32,33] Our YTH screens of skeletal muscle cDNA libraries using baits covering the titin M-line region identified the interaction of filamin-C with titin exon 358 (data not shown). Exon 358 codes for A169-A170, the titin kinase, and M1–M3.[22] *In situ*, these sequences locate in the periphery of the M-line region.[34] Interestingly, both Zis1 located in the periphery of the Z-disk region, and the titin kinase region located in the periphery of the M-line region are phosphorylated early during myogenesis: a PIRMSP motif in Zis1 and KSP repeats in exon 361, respectively, are both substrates for serine-threonine kinases.[35,36] Together with the early expression of both titin and filamin (both are detected at day 8 *post coitum* in the emerging heart in mice embryos,[37,38]) it is an attractive hypothesis that regulated co-assembly of titins and filamins during early myogenesis specifies the positions where membrane-myofibrillar connections are made, subject to potential further differentiation to specialized SR/T-tubular internal membrane systems during differentiation. The different membrane–myofibril connections identified so far might provide functionally redundant information, or perhaps more likely, may transmit distinct signals across the sarcolemnal membrane: in the Z-disk region, the complex of sAnk1, T-cap, and filamin with titin Z1-Z2-Zis1 is likely to transmit stretch-dependent regulation of the ion channel subunit minK (T-cap)[39] and of gene expression *via* MLP,[40] and myozenin-1/calcarcin-2/FATZ-1.[24] Anchoring obscurin/sAnk1 to the M-line region might connect Rho/GEF signaling pathways to the myofibril.[33,41]

Functionally, the smooth muscle titin described here, novex-III titin (another splice variant of titin),[22] and obscurin[41] share a number of similarities. All three filamentous giant proteins are associated with the myofibril and with membrane components, are expressed at sub-stoichiometric levels, and their expression levels are highly regulated (for down-regulation of smooth muscle titin during development, see Figure 2(a); for up-regulation of novex-III titin and obscurin in chronic cardiac disease, see Wu *et al.*[42] and Borisov *et al.*[43] Future studies are warranted to determine whether obscurin, novex-III titin, and smooth muscle titin can cooperate in the co-assembly of the myofibrillar to membrane connectivity system. Future studies will be mandatory to further clarify the potential role of titin in the nucleus. Studies using the array profiling approach described here are in progress to determine if the 3700 kDa[14] and 500 kDa[15] titin-like proteins from HeLa cell lines correspond indeed to specialized titin splice isoforms.

In summary, our titin array-based profiling studies have identified a specialized ~1 MDa splice isoform expressed in smooth muscle tissues that potentially can contribute to smooth muscle tissue elasticity. Its interaction with filamins is conserved with striated muscle titin, and provides a novel link between early myofibril assembly and early myogenesis: Previous studies in *Caenorhabditis*

‡ www.ensembl.org

elegans have identified an essential role of α and β-integrins for induction of early myogenesis in response to signals from the extracellular matrix§, whereas titin has been suggested to be essential for myofibrillogenesis.[1,45] Since Z2-Zis1 also interacts with α-actinin and with nebulin,[23] it will be highly interesting to study how Z2-Zis1 regulates myofibrillogenesis during development.

Materials and Methods

Muscle specimen

Bovine (Angus cross) and porcine (Yorkshire) aortas were collected from adult (ten months to 1.5 years old) animals. Animals were killed humanely by captive bolt and exsanguination in accordance with NIH and Washington State University IACUC guidelines. Human uterine tissues were collected during Cesarean section and quick-frozen in liquid nitrogen. Tissue samples were stored at –80 °C (according to the ethics proposal "Titin in smooth muscle" #13/05 of the University of Heidelberg, Faculty of Clinical Medicine, Mannheim).

Titin expression profiling

Microarray experiments were performed essentially as described but with minor modifications.[16] Commercially available total RNA was utilized for all microarray experiments (Stratagene, La Jolla, CA) (Ambion, Austin, TX) (Biochain, Hayward, CA). Multiple sources were used to obtain total RNA samples from multiple individuals. As target RNAs, 40 μg from smooth or non-muscle tissues, or 5 μg from skeletal tissues were biotinylated and reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA) using random octamers as primers (Operon Biotechnologies, Cologne, Germany) and biotin dUTP (Roche, Penzberg, Germany). Biotinylated cDNAs were mixed with 2× hybridization buffer, and heat-denatured and hybridized to titin exon arrays at 55 °C overnight. The arrays displayed 50-mer synthetic oligonucleotide probes representing each titin exon. In addition, MURF probes and selected housekeeping genes were included as positive control probes for detection chemistry and reverse transcriptase reactions (biotin, β-actin, and GAPDH, respectively). For the control of hybridization specificity, we used 50-mers including 3 to 5 bp mismatches to selected titin exons. Hybridization signals were amplified with a TSA™ system (PerkinElmer, Boston, MA) by including Alexa Fluor 555 (Invitrogen, Carlsbad, CA) in the fluorescent amplification step. Titin exon profiling was performed at least three times for each tissue type. Median hybridization intensity and SEM of the median intensity were measured and calculated for all probes with array WoRx™ auto biochip reader and SoftWoRx™ Tracker software (Applied Precision, LLC, Issaquah, WA). Exons with hybridization intensity greater than the mismatch probe (corrected for background) were considered positive. For comparisons between tissues, all titin exons were normalized to a constitutively expressed titin exon 5. All non-titin gene probes were normalized to β-actin and GAPDH. Comparisons between tissue types were made using Genesifter custom analysis package (VizX Labs LLC, Seattle, WA). Results were compared with a two-tailed Student's t test for samples of unequal variance and $P<0.05$ was used as the criterion for statistical significance.

For RT-PCR studies, commercially available total RNAs (Stratagene, La Jolla, CA) or total RNAs isolated from human aortic smooth muscle cell cultures (Cell Applications, San Diego, CA) using the Rneasy Kit (Qiagen, Valencia, CA) were reverse-transcribed essentially as described.[38] For qualitative RT-PCRs, input cDNA was normalized by a GAPDH control primer pair to obtain ~50 ng of amplified GAPDH fragment after 22 cycles. For PCR amplification of striated muscle cDNAs, 30 cycles were used, for smooth muscle and non-muscle cDNAs, 35 cycles were used. The 40 titin primer pairs used are given in Table 3.

For real time PCR, primers were designed using the IDT software (IDT, Coralville, IA). Commercially available total RNA (Stratagene, La Jolla, CA) or total RNAs isolated from human aortic smooth muscle cell cultures were used as the template for the reverse transcriptase reaction. iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was used for amplification of cDNA with a total reaction volume of 40 μl. Melt curves were performed for all runs to assess product purity. No amplification was observed after 45 cycles of PCR in control reactions. For each tissue, results were compared to a gene-specific standard curve and normalized to expression of 18 S rRNA in the same sample.

For anchored PCR extensions in search of 5′ titin ends, the Marathon extension kit was used (BD Biosciences, #639325). Smooth muscle-specific 5′ sequences were further analyzed using an FLJ39502-sense primer in combination with titin exon 2–7 reverse primer‖. For amplification of FLJ39502/titin fusions transcripts, the following sense primer from FLJ39502 was used: CTGAGTCCCCCCTTGCACCATCTGACATG.

Yeast two-hybrid studies and confirmation of interaction using expressed materials

A titin exon 4–7 spanning fragment was amplified with the following primer pair from total human skeletal muscle cDNA:

TTN-4S: tttccatg-GCACCACCCAACTTCGTTCAACGA
TTN-7R: tttggatcc-ta-CACCTCTTTAGCACCAGTGGC

The 1.1 kb cDNA fragment obtained was inserted into pGBKT7, and YTH screens of cDNA libraries inserted into pACT2 (from total human skeletal muscle, BD Biosciences #638818; from human aorta, BD Biosciences #638813) were performed essentially as described.[17]

For confirmation of the interaction of filamin and titin, a cDNA fragment coding for the filamin A carboxy-terminal region was amplified with the following primer pair (derived from BC014654):

2070S: tttctcgagc-CCTCGGGAGAATGGCGTTTA-CCTGATTGAC
2840R: tttacgcgt-CAGACTCAGGGCACCACAAC-GCGGTAGG

§ www.wormbook.org

‖ www.titin.org

The 770 bp fragment obtained was inserted into pET8c (ampicillin resistance), whereas the 1.1 kb titin exon 4–7 fragment was inserted into pETM-44 (kanamycin resistance; expresses an MBP fusion). Both constructs were transformed into BL21 cells, and double-resistant cell lines selected on Kan+Amp LB plates. Co-expression of filamin-A and titin was induced by addition of IPTG and protein expression was performed essentially as described.[23] Co-purification of the titin/filamin complex over amylose resin was performed essentially as recommended by the manufacturer (New England Biolabs).

Cell culture

Adult human aortic smooth muscle cells at second passage were obtained commercially (Cell Applications, San Diego, CA). For RNA studies, cells were plated at a density of 10,000cells/cm^2 in T75 flasks with 15 ml of Smooth Muscle Cell Growth Medium (Cell Applications, San Diego, CA). The medium was changed every other day and the volume was increased to 30 ml when >60% confluent. Cells were examined daily for normal cellular appearance. Once confluence was reached at >80%, cells were washed with Hank's buffered salt solution (HBSS) and detached by trypsinization (trypsin/EDTA). Trypsin activity was stopped with trypsin-neutralizing solution. Free cells were centrifuged at 220*g*. The cell pellet was resuspended in 2 ml of smooth muscle cell growth medium for cell counting. Cells were centrifuged again at 220*g* and the cell pellet was quick-frozen in liquid nitrogen. Cells at passage 3 to 5 were used for all experiments.

Immunofluorescence microscopy

For confocal studies, adult human aortic smooth muscle cells were plated in 8-well Lab-tek™ chambered coverglass (Fisher Scientific International, Pittsburgh, PA) at a density of $\sim 2 \times 10^4$ cells/well in 400 μl of Smooth Muscle Cell Growth Medium (Cell Applications, San Diego, CA). At 24–48 h after plating, human aortic smooth muscle cells were washed with phosphate-buffered saline (PBS) (pre and post-formaldehyde), fixed with 3.7 % (v/v) formaldehyde in PBS (pH 7), and blocked with PBS containing 1% (w/v) bovine serum albumin (BSA) for 30 min. Cells were incubated overnight at 4 °C with the primary antibody diluted in PBS, 1% BSA to 1:100 (v/v). A no primary antibody control was used for each experiment. Cells were incubated at 4 °C with 1:50 (v/v) dilution of anti-rabbit fluorescence secondary (Molecular Probes Inc., Eugene, OR) for 2 h. The cells were stored with Vectashield® mounting medium (Vector Laboratories, Burlingame, CA). Intact bovine aorta samples were cut using a Leica microtome (Leica Microsystems Inc, Bannockburn, IL) and processed for fixation and labeling in a manner similar to that used for the cultured cells. Microscopy was done on a Nikon Eclipse TE 300 microscope and images were produced using a Bio-Rad MRC 1024 confocal laser scanning microscope using LaserSHARP 2000 software package (Hercules, CA). Immunofluorescence microscopy of human skeletal muscle and uterus probes were performed essentially as described.[16,23]

Titin antibodies and Western blot studies

Western blots were probed with the following rabbit affinity-purified polyclonal antibodies to unique titin regions: Z1/Z2 raised to Z1/Z2 region of titin, and MIR raised to the A/I junction of titin;[44] 9D10 obtained from DSH Iowa is directed to the PEVK domain.[20] A rabbit polyclonal antibody directed to titin Ig repeats I40/41 was raised using these expressed titin domains from *E. coli*.

Samples were solubilized, electrophoresed and transferred to nitrocellulose in a manner similar to that described.[45] Briefly, tissue were ground rapidly to a fine powder in a Dounce homogenizer while cooled in liquid nitrogen, and homogenized in buffer containing 8 M urea at a 40:1 (v/v) and 50 % (v/v) glycerol with protease inhibitors. Samples were subjected to electrophoresis on a vertical agarose gel electrophoresis system.[46] Blots were first blocked with 0.5 % (w/v)casein blocking solution and then incubated overnight at 4 °C in 1:500 (v/v) dilution with the primary antibody in PBS with 0.1% (v/v) Tween®20. Secondary anti-rabbit antibody was used at a 1:10,000 (v/v) dilution followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG from Vectastain®ABC kit (Vector Laboratories, Burlingame, CA). Immunoreactive bands were visualized using detection buffer containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate according to the manufacturer's instructions (MP Biomedicals, LLC, Aurora, OH).

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