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Muscle type-specific expression of Zasp52 isoforms in *Drosophila*

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ABSTRACT

Zasp52 is a member of the PDZ-LIM domain protein family in *Drosophila*, which comprises Enigma, ENH, ZASP, Alp, CLP36, RIL, and Mystique in vertebrates. *Drosophila* Zasp52 colocalizes with integrins at myotendinous junctions and with α-actinin at Z-disks, and is required for muscle attachment as well as Z-disk assembly and maintenance. Here we document 13 *Zasp52* splice variants giving rise to six different LIM domains. We demonstrate stage- and tissue-specific expression in different muscle types for Zasp52 isoforms encoding different LIM domains. In particular, LIM1b is expressed only in heart muscle and certain somatic muscles, implying muscle-specific functions in Z-disk assembly or maintenance.

Vertebrate muscles are divided into three major types, skeletal, cardiac, and smooth muscle. Anatomically, they correspond in *Drosophila* to somatic (or body wall) muscle, heart, and visceral muscle. Vertebrate skeletal muscles are further subdivided into slow-twitch muscle and three classes of fast-twitch muscles, and *Drosophila* somatic muscles are similarly subdivided into asynchronous and synchronous muscles such as indirect flight muscle and thoracic tubular jump muscle, respectively (Sink, 2006). Common to all these muscle types is an actomyosin contractile system with thin filaments anchored at Z-disks. Crucial components of Z-disks are α-actinin, which anchors actin filaments at the Z-disk, and members of the Alp/Enigma family of PDZ-LIM domain proteins, which function in assembly and maintenance of Z-disks (Sheikh et al., 2007; Sparrow and Schöck, 2009). In vertebrates, the Alp/Enigma family comprises Clp36/Elfin/PDLIM1, Mystique/PDLIM2, ALP/PDLIM3, RIL/PDLIM4, ENH/PDLIM5, Zasp/Cypher/LDB3/PDLIM6, and Enigma/PDLIM7 (Zheng et al., 2010). All vertebrate family members have one N-terminal PDZ domain, a ZM motif and one or three C-terminal LIM domains. In *Drosophila*, Zasp52 is the major member of the Alp/Enigma family with a PDZ domain, ZM motif and four LIM domains; another member, Zasp66, lacks the LIM domains, but features a similar PDZ domain and a weakly similar Zasp-like (ZM) motif, and also localizes to Z-disks (Hudson et al., 2008; Jani and Schöck, 2007). Mutations in *Drosophila Zasp52* cause muscle detachment and defects in Z-disk assembly and maintenance (Benna et al., 2009; Jani and Schöck, 2007; Rui et al., 2010). Mutations of *Zasp52* orthologs in vertebrates cause similar defects, ranging from improper formation of somites and heart in zebrafish to fragmented Z-disks in skeletal and cardiac muscles in mice (van der Meer et al., 2006; Zhou et al., 2001). Mutations in the human ortholog ZASP result in variable phenotypes from congenital myopathies with fetal lethality to late-onset cardiomyopathy (Sheikh et al., 2007). All Zasp family members are alternatively spliced with four reported isoforms in *Caenorhabditis elegans* (McKeown et al., 2006), 13 in zebrafish (van der Meer et al., 2006), and six in mice and humans (Huang et al., 2003; Vatta et al., 2003). In *Drosophila*, we have previously reported two splice variants, called *Zasp52-RK* and *Zasp52-RE* in FlyBase (Jani and Schöck, 2007). Recently two additional splice variants, confirmed by size in Western blots, were reported in larvae (Benna et al., 2009).

Here we analyzed *Zasp52* splice variants by RT-PCR and EST sequencing, and verify their sizes with a newly raised antibody against the Zasp52 N-terminus. Altogether we identify 8 novel splice isoforms and three new exons or exon variants. Furthermore, we show larval and adult expression patterns of Zasp52 demonstrating its localization to Z-disks in all three major muscle types, somatic, heart, and visceral. Finally, we show muscle type-specific expression with antibodies against sequences encoded by exon 8, which encodes an alternative LIM domain, suggesting

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muscle-specific functions for Zasp52 isoforms in Z-disk assembly and maintenance.

1. Results

1.1. Zasp52 locus expresses at least 13 splice variants

We performed RT-PCR with mRNA isolated from all stages of development and also sequenced all expressed sequence tags (ESTs) available from the Berkeley *Drosophila* Genome Project (Fig. 1 and Tables 1 and 2). Apart from *Zasp52-RE* and *Zasp52-RK*, which we reported previously (Jani and Schöck, 2007), we confirm FlyBase annotations for *Zasp52-RG* and *Zasp-52RI*, as well as one isoform recently identified in larvae (Benna et al., 2009) (isoform 4 in Fig. 1, available from GenBank under Accession No. JN034424), which is the most commonly detected variant by EST sequencing (Table 2). In addition, we identify eight novel splice variants not previously identified in other studies (available from GenBank under Accession Nos. JN034422, JN034423, JN034425, JN034426, JN034427, JN034428, JN034429 and JN034430). These splice variants contain one novel exon (6′), two novel exon variants (13b and 16b), and two novel transcription termination sites (3′ UTRs). There are likely additional splice variants involving exon 12 and 16, as suggested by additional FlyBase annotations and other reports (Benna et al., 2009; Machuca-Tzili et al., 2006).

1.2. Zasp52 splice variants encode six different LIM domains

In other species there is no evidence for alternative splicing of exons encoding LIM domains in Alp/Enigma family genes. In contrast, for *Zasp52*, alternative splicing affects two LIM domains, LIM1 and LIM2. The first nine amino acids of LIM domain 1 are encoded by exons 7 or 8, respectively, giving rise to two LIM1 domains (named LIM1 and LIM1b). Likewise, the first 9 or 10 amino acids of LIM domain 2 are encoded by exons 16 and 17, respectively (named LIM2 and LIM2b). Therefore, *Zasp52* encodes a total of six different LIM domains (Fig. 2).

1.3. Tissue- and stage-specific expression of Zasp52 isoforms

To determine if predicted Zasp52 isoforms of these splice variants correspond to observed bands on Western blots, we raised a novel antibody against 200 N-terminal amino acids of Zasp52 (Zasp-N, see Section 3), because our full-length antibody only works well in immunohistochemistry (Jani and Schöck, 2007). We first confirmed specificity by comparing immunoblots of late stage wild type embryos with *Zasp52* mutant embryos (Fig. 3A, see also Fig. 5A). We then assessed isoform composition at pupal and adult stages and in three different tissues, IFM, testes and ovaries (Fig. 3B). Many bands correspond in size to predicted isoforms; especially the two most frequent isoforms by EST sequencing (4 and 6) are present in all stages and tissues. In addition, several

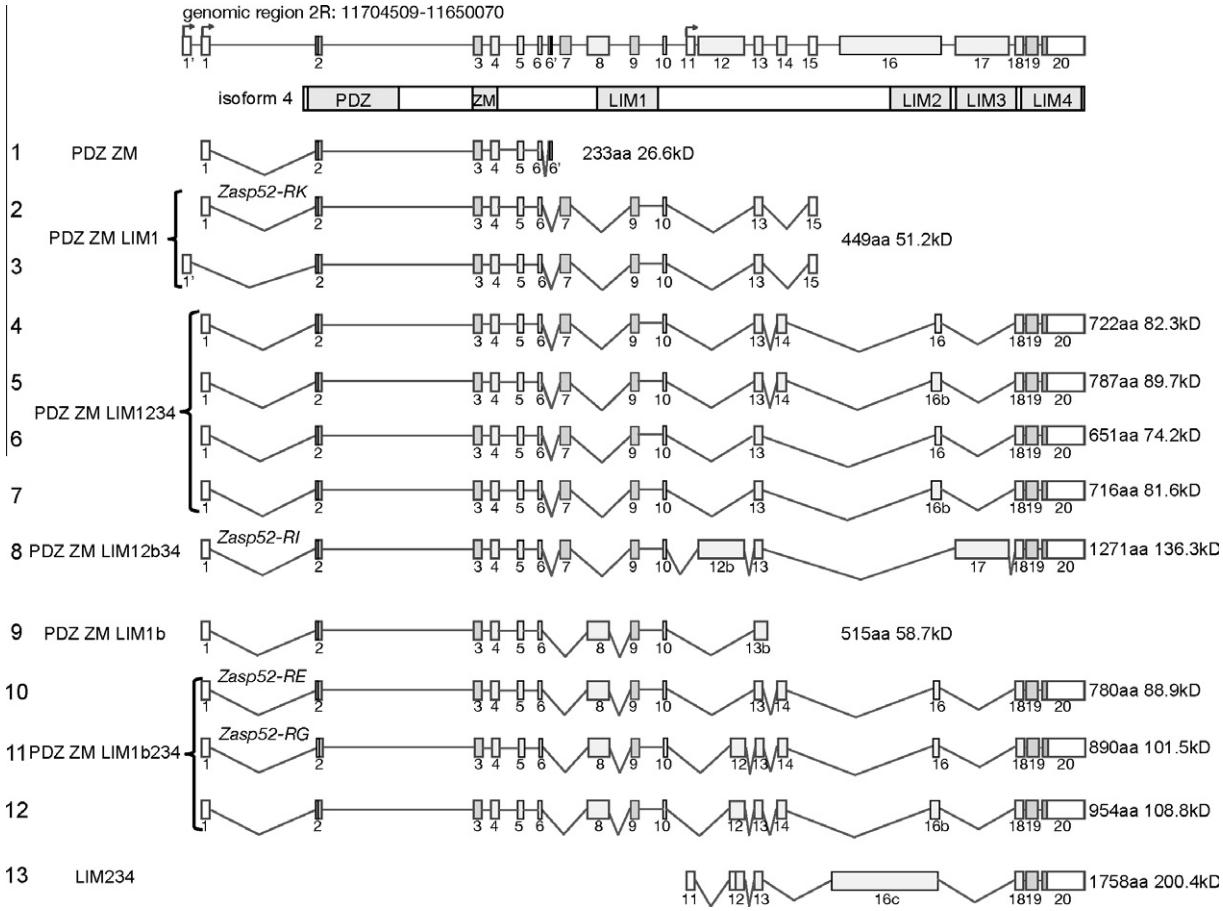

Fig. 1. *Zasp52* gene model. On top, genomic region of *Zasp52* with genomic coordinates. Arrows indicate alternative start sites. Middle, protein domains of the most common isoform, encoded by splice variant 4. Below, *Zasp52* splice variants 1–13. Straight lines indicate invariant splicing, diagonal lines indicate alternative splicing. White boxes, untranslated exons or part of exons; grey boxes, translated exons or part of exons. On the left, protein domains encoded by splice variants; on the right, amino acid length of splice isoforms and their predicted molecular weight in kiloDalton. Isoform 2 corresponds to Zasp^{Alp} (Jani and Schöck, 2007)/Zasp52-PK (FlyBase). Isoform 10 corresponds to Zasp^{Enigma} (Jani and Schöck, 2007)/Zasp52-PE (FlyBase). Isoforms 8 and 11 correspond to Zasp52-PI and Zasp52-PG in FlyBase. Splice variants 1, 3, 4, 5, 6, 7, 9, 12, and 13 are available from GenBank under Accession Nos. JN034422–JN034430.

Table 1
Zasp52 exons.

Genomic coordinates: *Drosophila melanogaster* chromosome 2R, NT_033778.3	Length in basepairs	Protein domains encoded
1′: 11704509/11704314–11704265	244/50	
1: 11702450/11702419/11702403–11702234	217/186/170	
2: 11690992–11690866	127	PDZ
3: 11670971–11670820	152	PDZ
4: 11670749–11670593	157	
5: 11669411–11669278	134	ZM
6: 11668584–11668490	95	
6′: 11668240–11668142	99	
7: 11667782–11667563	220	First 9 amino acids of LIM1
8: 11667163–11666770	394	First 9 amino acids of LIM1b
9: 11666051–11665857	195	LIM1/1b
10: 11664791–11664683	109	
11: 11663485–11663281	205	
12: 11662262–11661933	330	
12b: 11663012–11661933	1080	
13: 11661471–11661321	151	
13b: 11661471–11661060	412	
14: 11660758–11660546	213	
15: 11658652–11658456	197	
16: 11652757–11652628	130	First 9 amino acids of LIM2
16b: 11652952–11652628	325	First 9 amino acids of LIM2
16c: 11657056–11652628	4429	First 9 amino acids of LIM2
17: 11652340–11651431	910	First 10 amino acids of LIM2b
18: 11651334–11651151	184	LIM2/2b and LIM3
19: 11651094–11650855	240	LIM3 and LIM4
20: 11650775–11650070	706	LIM4

Table 2
ESTs in support of predicted *Zasp52* splice variants.

Splice variant	Sequenced ESTs
1	GH05237
2 (*Zasp52-RK*)	LP01361, LP01550, MIP09364, RE06836
3	LP12966
4	GH09105, GH16307, GH25611, GH28449, LP11454, LP15026, LP20934, RE08540, RE32053, RE58207, RE73562
5	GH18981
6	LP18979, RE19447, RE38156, RE49166
7	RE53516, RE55390
8 (*Zasp52-RI*)	GH26874
9	GH12986, GH19004
10 (*Zasp52-RE*)	RH02578, RH03424, RH03452
11 (*Zasp52-RG*)	IP01285
12	GH22268
13	GH04526

unaccounted bands suggest a few additional isoforms. Spatial and temporal changes in isoform expression are evident. Noteworthy are the differences between IFM, a somatic muscle, and ovaries and testes, which are solely surrounded by visceral muscles. The four major bands in ovaries and testes appear to correspond by size to exon 7-containing isoforms, suggesting that exon 8 is not expressed in visceral muscles (Fig. 3B).

1.4. Zasp52 localization in larval and adult tissues

We previously analyzed Zasp52 expression and localization in the embryo with a rabbit Zasp-FL antibody (Jani and Schöck, 2007). This polyclonal antibody likely detects all isoforms, because the antigen used to generate the antibody significantly overlaps all predicted isoforms. We stained third instar larval body wall muscles and midgut, as well as male adult tissues comprising indirect flight muscles (IFM), thoracic tubular jump muscles (TDT), abdominal muscles, heart, and midgut with Zasp-FL and Zasp-N antibodies (Fig. 4, and data not shown). Zasp52 localizes to Z-disks in all muscle tissues, heart, visceral and somatic. The complete overlap of expression detected with both Zasp-FL and Zasp-N antibody also suggests that isoform 13, which is not detected by Zasp-N antibody, is co-expressed with other isoforms in the tissues analyzed.

1.5. Exon 8 encoding LIM1b is not expressed in visceral muscles

We were particularly interested in the expression of exons encoding alternatively spliced LIM domains, because variable LIM domains suggest functional differences. We therefore raised an antibody against exon 8-coding sequence (see Section 3), and obtained an antibody against exon 16-coding sequence (Benna et al., 2009).

We confirmed specificity of the Zasp-E8 antibody with an RNAi transgene (JF01133) expressed in muscles and targeting *Zasp52* exons three to six. All isoforms detectable with Zasp-N antibody by Western blotting are depleted, demonstrating that exon 8-containing isoforms are indeed depleted in this RNAi line, and also confirming the specificity of the Zasp-N antibody in adults (Fig. 5A). As expected, Zasp-E8 antibody staining is strongly reduced in exon 8-depleted IFM compared to wild type (Fig. 5B).

We then stained the same set of tissues with anti-Zasp-E16 and anti-Zasp-E8 antibody. Zasp-E16 antibody stainings are identical to Zasp-N antibody stainings in all tissues analyzed (larval body wall and larval midgut, adult midgut, IFM, TDT, adult heart and abdominal muscles, data not shown), indicating that long and short isoforms of Zasp52 are co-expressed in the same muscle types. In contrast, with Zasp-E8 antibody stainings, we observe expression in adult heart and certain somatic muscles (IFM and TDT), but not in larval and adult midgut, larval body wall muscles and abdominal muscles, indicating muscle type-specific functions of the LIM1b domain (Fig. 6). We also performed a Zasp-E8 antibody staining with stage 17 embryos, but observed no staining (data not shown), consistent with the absence of exon 8 mRNA expression during embryonic stages (Graveley et al., 2011).

2. Discussion

In this study we report 13 *Zasp52* splice variants, which encode a PDZ domain, a ZM motif and six LIM domains. We show that Zasp52 localizes to Z-disks in all muscle types, somatic, heart, and visceral muscles, which corresponds to skeletal, heart, and smooth muscles in vertebrates. We demonstrate changes in isoform expression in development and in different tissues, in particular, we demonstrate the specificity of exon 8-encoding isoforms for somatic and heart muscles.

Three additional isoforms are annotated in FlyBase, and 10 additional larval isoforms were recently proposed (Benna et al., 2009), although splice variants from our study are the only ones supported by full-length cDNA inserts and immunoblotting data. Still, additional isoforms likely exist, given that some bands in Zasp immunoblots could not be assigned to any of the 13 isoforms. Recently the modENCODE project has made a huge contribution to identifying additional transcribed regions (exons) in *Drosophila* (Graveley et al., 2011). Although currently not annotated, exon 6′, 13b and 16b are present in RNA-seq data of modENCODE, further validating our isoforms.

The localization of Zasp52 to Z-disks in heart muscles suggests that it also plays a role in heart function and maintenance in *Drosophila*, a role that has been abundantly documented for vertebrate

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LIM1   CTECERLIT-G-VFVRIKDKNLHVECFKCAT--CGTSLKNQGYYNFNNKLYCDIHAKQAA 56
LIM1b  CQLCGVGIV-G-VFVRIKDKNLHVECFKCAT--CGTSLKNQGYYNFNNKLYCDIHAKQAA 56
LIM2   CNSCNVQIR-G-PFITALGRIWCPDHFICVNGNCRRPLQDIGFVEEKGDLYCEYCFEKYL 58
LIM2b  CCQCNKEITSG-PFITALGRIWCPDHFICVNGNCRRPLQDIGFVEEKGDLYCEYCFEKYL 59
LIM3   CSKCAGKIK-G-DCLNAIGKHFHPECFTCGQ--CGKIFGNRPFFLEDGNAYCEADWNELF 56
LIM4   CFACGFPVEAGDRWVEALNHNYHSQCFNCTF--CKQNLEGQSFYNKGGRPFCKNHAR--- 55
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Fig. 2. Alignment of LIM domains encoded by Zasp52 isoforms. Alternative splicing results in six different LIM domains, which were aligned with CLUSTAL 2.1. Black background indicates identical amino acids, grey background indicates similar amino acids. Amino acid length of each domain is indicated on the right.

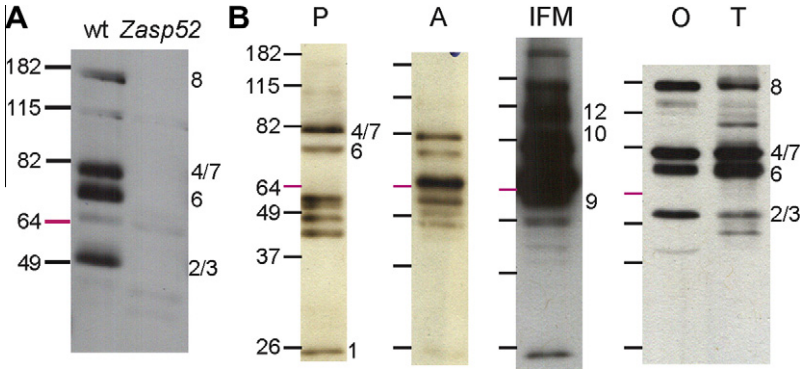

Fig. 3. Zasp52 isoforms at different developmental stages and in different tissues. (A) Western blot of wild type and *Zasp52*$^{\Delta}$ stage 17 embryonic extracts probed with anti-Zasp-N antibody. (B) Western blot of wild type extracts from pupae (P), adults (A), indirect flight muscle (IFM), ovaries (O), and testes (T) probed with anti-Zasp-N antibody. Some of the bands closely corresponding to predicted isoforms are labeled on the right. Molecular weight marker is indicated on the left in kiloDalton.

Enigma and Alp family proteins (Pashmforoush et al., 2001; Sheikh et al., 2007; Vatta et al., 2003).

Our EST sequencing, Western blotting and immunostaining data indicate that LIM1 and LIM2 domains encoded by exons 7 and 16, respectively, are expressed in all muscles, whereas LIM1b and LIM2b encoded by exons 8 and 17, are restricted to specific muscles. This in turn suggests differences in Z-disk structure, maintenance or signaling mediated by these LIM domains. There is accumulating evidence that individual splice isoforms play crucial roles in muscle function and maintenance: first, mutations in splicing factors that affect Zasp52 or human Alp splicing cause severe muscle disorders (Artero et al., 1998; Machuca-Tzili et al., 2006; Ohsawa et al., 2011). More importantly, splice isoform-specific mutations in human ZASP or mouse Cypher also result in myopathies (Arimura et al., 2004; Cheng et al., 2011; Vatta et al., 2003). We propose that some of the variety that is encoded by different genes in mammals is encoded by the large number of isoforms of *Zasp52* in *Drosophila*. This is also consistent with the large number of LIM domains, six in total, encoded by different isoforms in *Drosophila*. Vertebrate PDZ-LIM domain proteins only encode one LIM domain in the Alp subfamily or three LIM domains in the Enigma subfamily, and alternative splicing does not affect the number or sequence of LIM domains. The multitude of Zasp52 splice isoforms may be sufficient to ensure proper assembly and maintenance of the many different muscles in *Drosophila*. Our study is an important first step to identify the binding partners of different splice isoforms of Zasp52 and their function, which in turn will provide insights into the function of PDZ-LIM domain proteins in development, muscle maintenance and disease in vertebrates.

3. Experimental procedures

3.1. Fly stocks and molecular biology

We used the following stocks: OreR, Dmef2-Gal4 and P{TRiP.JF01133}attP2 obtained from the Bloomington *Drosophila* stock center and *Zasp52*$^{\Delta}$ (Jani and Schöck, 2007).

For RT-PCR, mRNA was isolated from a mix of embryos, larvae, pupae, and adults using Trizol (Invitrogen) according to the manufacturer's instructions. We reverse transcribed mRNA with oligo(dT)12–18 (Invitrogen), and confirmed alternative splicing by PCR reactions with exon-specific primers using Taq polymerase (New England Biolabs).

3.2. Antibody generation and Western blotting

We prepared a rat polyclonal antibody against full-length Zasp52-PE called rat anti-Zasp-FL antibody as described previously (Jani and Schöck, 2007). We also prepared rat and rabbit polyclonal antibodies against the first 200 amino acids of Zasp52-PE (Zasp-N antibody). For Zasp-N, cDNA was amplified from EST RH03424 as a template with CACCATGGCCCAACCACAGCTGCTG and CTCGGAGCGATCGCCCTGGTA as primers and cloned into the Gateway pENTR/D-TOPO vector (Invitrogen). Recombination between the entry clone and the Gateway pDEST17 destination vector generated expression clones. Expression was induced with 0.2% L-arabinose. We purified the recombinant protein under denaturing conditions on Ni^{2+}-affinity columns (Qiagen) according to the manufacturer's instructions. An antibody against 129 amino acids encoded by exon 8 (Zasp-E8 antibody) was commercially generated using a proprietary mix of peptides coupled to keyhole limpet hemocyanin (GenScript). We tested antibody specificity by Western blotting or immunofluorescence detection comparing wild type and *Zasp52*$^{\Delta}$ first instar larvae or wild type and Dmef2-Gal4 JF01133 adults.

We resolved proteins by 8% SDS–PAGE and transferred them onto Hybond-C extra nitrocellulose membrane (GE Healthcare) for detection with rabbit anti-Zasp-N antibody (1:5000) and rat anti-actin MAC237 antibody (1:5000; Babraham Institute). Anti-rabbit and anti-rat IgG horseradish peroxidase-linked secondary antibody (1:5000) was used together with the ECL detection kit for visualization (GE Healthcare).

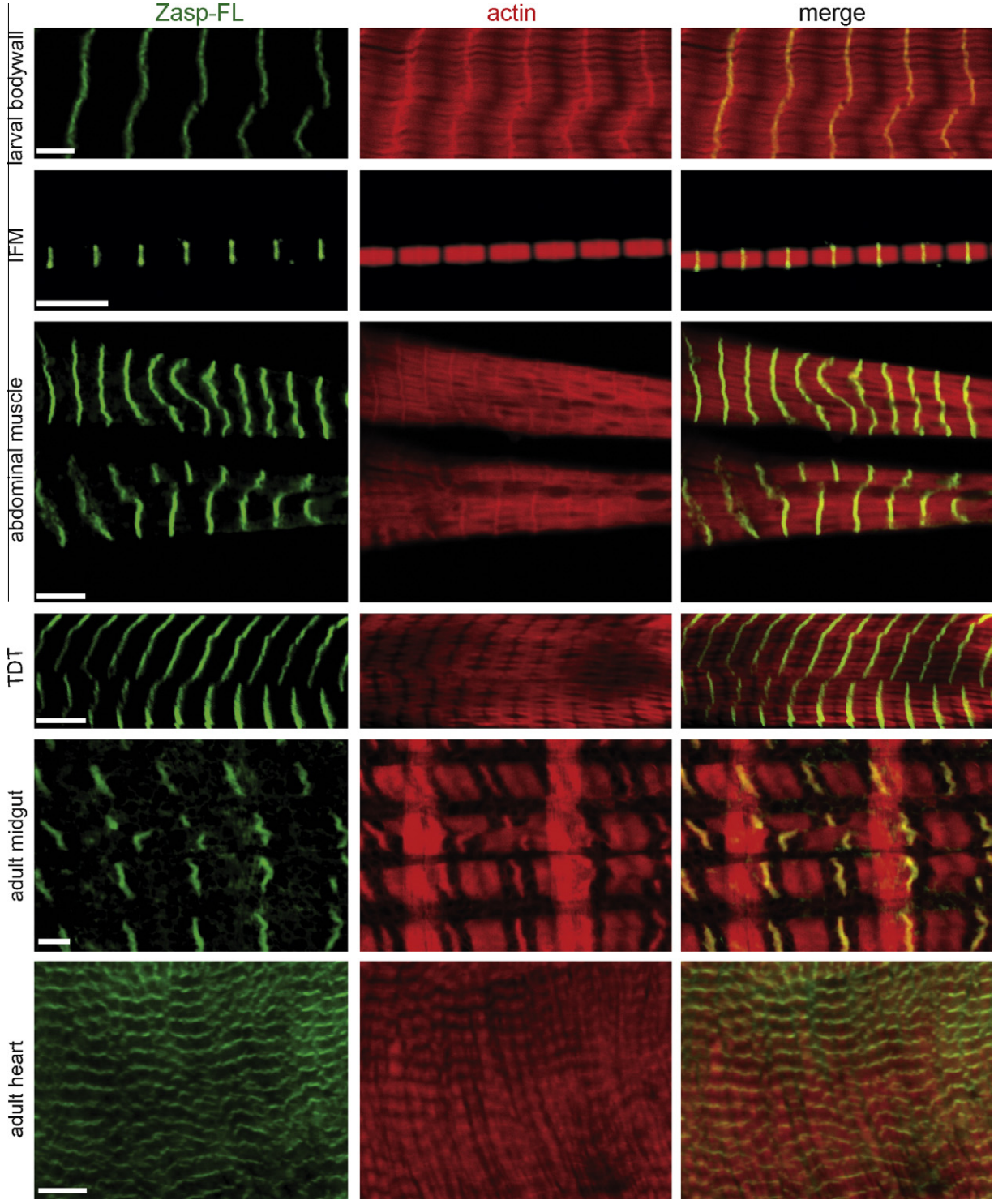

Fig. 4. Zasp52 localizes to Z-disks in all muscle types. Wild type tissues double-stained with anti-Zasp-FL antibody (green, left panels) and phalloidin-Alexa 594 (red, middle panels). Merge is shown on the right. IFM, indirect flight muscle; TDT, tubular jump muscle, also called tergal depressor of the trochanter. Bar, 5 μm.

3.3. Immunohistochemistry

We used the following primary antibodies for immunofluorescent stainings of muscle tissues: rabbit anti-Zasp-FL (1:200) (Jani and Schöck, 2007), rabbit anti-Zasp-N (1:200), rabbit anti-Zasp-E8 (1:50), and mouse anti-Zasp-E16 (1:50) (Benna et al., 2009). Alexa Fluor 488 goat anti-rabbit and goat anti-mouse (1:500, Invitrogen) were used as secondary antibodies. Actin was labeled with Alexa Fluor 594-conjugated phalloidin (1:1000, Invitrogen).

For staining of IFM and TDT, fly thoraces were cut in half along the longitudinal axis and incubated in relaxing solution (20 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM ATP, 5 mM DTT) with protease inhibitors, 50% glycerol and 0.5% Triton X-100 for 2 h on ice. IFMs and TDTs were then dissected from the thorax and washed in relaxing solution without glycerol and without Triton. IFMs were separated into single myofibrils by gently homogenizing them. IFMs and TDTs were incubated in primary antibodies diluted in relaxing solution overnight at 4 °C.

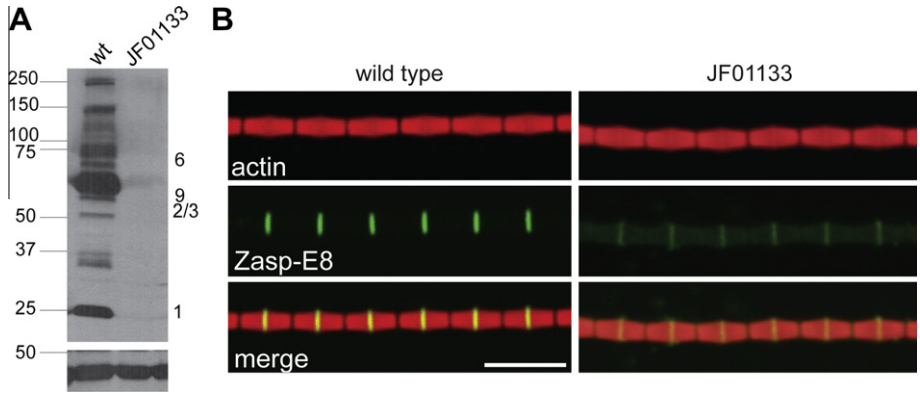

Fig. 5. Zasp-E8 antibody is specific. (A) Western blot of IFM extracts of wild type (wt) and Dmef2-Gal4 JF01133 targeting exons 3–6 probed with anti-Zasp-N antibody. Isoforms detected in wild type extracts are depleted in mutant IFM. Molecular weight marker is indicated on the left in kiloDalton. Loading control is shown at the bottom (anti-actin antibody). (B) Mutant IFM (JF01133, right panels) show a strong reduction in Zasp-E8 staining. IFM are double-stained with phalloidin-Alexa 594 (red, top) and anti-Zasp-E8 antibody (green, middle). Merge is shown at the bottom. IFM, indirect flight muscle. Bar, 5 μm.

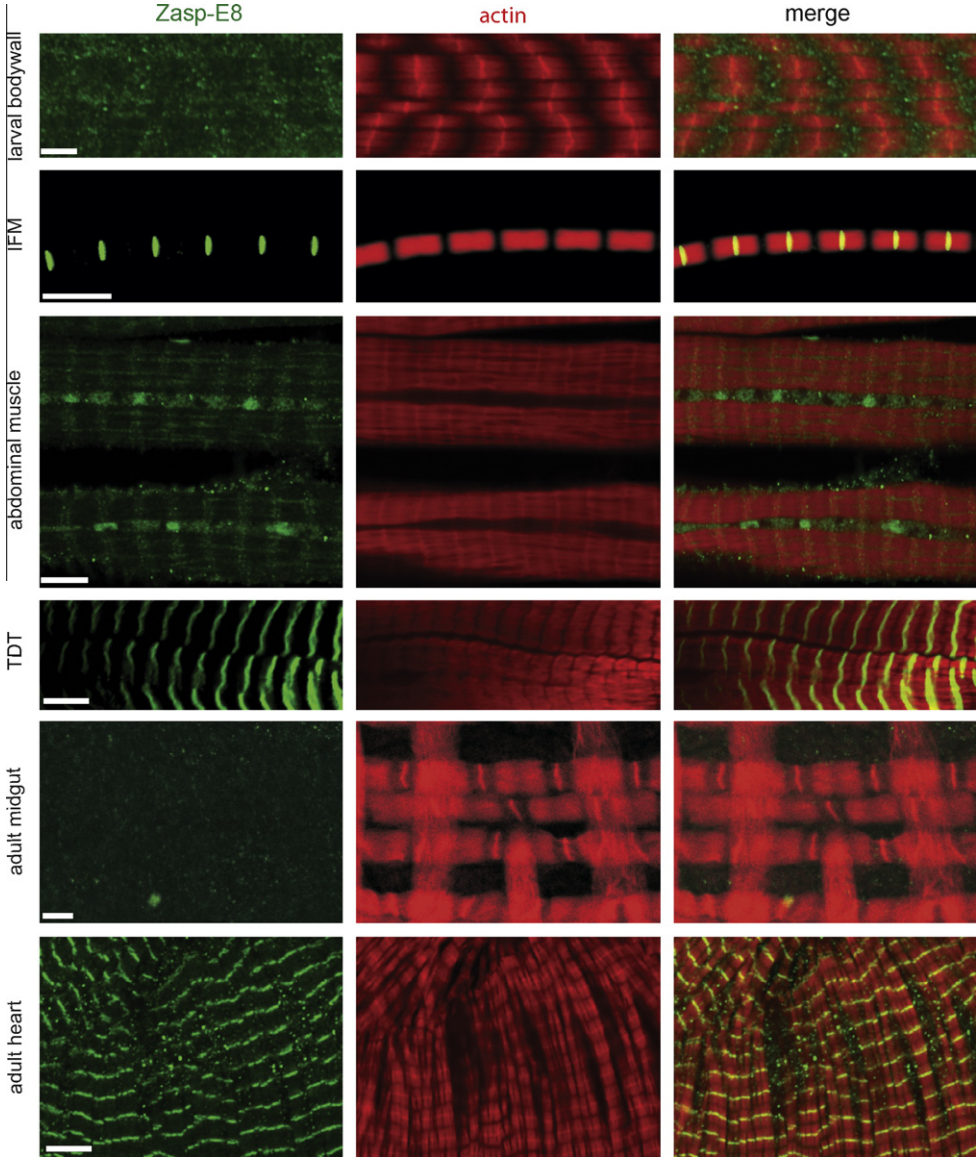

Fig. 6. Zasp52 LIM1b expression is restricted to heart and somatic muscles. Wild type tissues double-stained with anti-Zasp-E8 antibody (green, left panels) and phalloidin-Alexa 594 (red, middle panels). Merge is shown on the right. IFM, indirect flight muscle; TDT, tubular jump muscle, also called tergal depressor of the trochanter. Bar, 5 μm.

Samples were then washed with relaxing solution and incubated with secondary antibodies in relaxing solution for 1 h at room temperature. Samples were washed, fixed in 4% paraformaldehyde in relaxing solution, and finally mounted using ProLong Gold antifade (Invitrogen). Adult midguts and third instar larvae were dissected and fixed in 4% paraformaldehyde in PBS for 7 min at room temperature. Samples were then washed three times for 10 min with PBS/0.1% Triton X-100 (PBT). Primary antibody incubation in PBT was carried out overnight at 4 °C, followed by secondary antibody incubation for 1 h at room temperature. Adult hearts and abdominal muscles were dissected and processed as described previously (Alayari et al., 2009). Images were obtained on a Zeiss LSM 510 Meta inverted confocal microscope using a 63× 1.4 NA Plan Apo oil immersion objective and LSM imaging software.

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