Zasp is required for the assembly of functional integrin adhesion sites

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The integrin family of heterodimeric transmembrane receptors mediates cell–matrix adhesion. Integrins often localize in highly organized structures, such as focal adhesions in tissue culture and myotendinous junctions in muscles. Our RNA interference screen for genes that prevent integrin-dependent cell spreading identifies Z band alternatively spliced PDZ-motif protein (zasp), encoding the only known Drosophila melanogaster Alp/Enigma PDZ-LIM domain protein. Zasp localizes to integrin adhesion sites and its depletion disrupts integrin adhesion sites. In tissues, Zasp colocalizes with βPS integrin in myotendinous junctions and with α-actinin in muscle Z lines. Zasp also physically interacts with α-actinin. Fly larvae lacking Zasp do not form Z lines and fail to recruit α-actinin to the Z line. At the myotendinous junction, muscles detach in zasp mutants with the onset of contractility. Finally, Zasp interacts genetically with integrins, showing that it regulates integrin function. Our observations point to an important function for Zasp in the assembly of integrin adhesion sites both in cell culture and in tissues.

Introduction

Integrin-mediated adhesion between the ECM and the cytoskeleton is crucial for tissue interactions during development. Integrins are heterodimeric single-pass transmembrane receptors consisting of α and β subunits found in all animals from sponges to humans (Hughes, 2001). The globular extracellular domains of both subunits contribute to binding of ECM ligands. The short cytoplasmic carboxyl-terminal domains of integrins lack intrinsic catalytic activity. They organize the actin cytoskeleton through adaptor proteins and signal by associating with protein kinases and GTPases (Giancotti and Tarone, 2003). Disruption of the ECM, integrins, or their cytoskeletal adaptors affects integrin-mediated adhesion. Loss of integrin function leads to cell-spreadin defects, muscle detachment, and, in the human disease epidermolysis bullosa, the separation between epidermis and dermis (Bökel and Brown, 2002; Devenport et al., 2007).

Integrins typically localize in highly organized structures at sites of transmembrane linkage. The best characterized of these linkages is the focal adhesion found on mammalian fibroblasts in tissue culture (Burridge et al., 1988). In tissues, small adhesion sites mature during development into stable hemi-adherens junctions that connect epithelia to the basement membrane and into myotendinous junctions that connect the tips of striated muscles to the ECM. In striated muscles, actin filaments are anchored to myotendinous junctions and to Z lines, which border the smallest functional unit of muscles, the sarcomere (Clark et al., 2002). Z lines are laterally connected to the ECM by costameres (Garamvölgyi, 1965; Pardo et al., 1983; Ervasti, 2003). Connecting Z lines to other Z lines and to the surrounding connective tissue ensures synchronous, uniform muscle contraction. The Z line–costamere complex is morphologically similar to myotendinous junctions and contains many of the same proteins, among them integrins, which make the connection of the Z line to the ECM at the costamere (Pardo et al., 1983; Volk et al., 1990; Reedy and Beall, 1993; Ervasti, 2003). In mice, Drosophila melanogaster, and Caenorhabditis elegans, integrins are required for sarcomere assembly and Z line formation (Volk et al., 1990; Bloom and Brown, 1998; Schwander et al., 2003; Lecrois et al., 2007).

Tissue culture studies have revealed a large number of proteins implicated in intracellular signaling and adaptor functions at focal adhesions (Zaidel-Bar et al., 2007). How integrin adhesion sites form in vivo, however, is complex and the set of molecules required is not well defined. One class of proteins often found at focal adhesions and at related structures, such as myotendinous junctions, is the LIM domain family (Kadrmas and Beckerle, 2004). Most proteins containing LIM domains, notably the paxillin and zyxin families, have been implicated in cell adhesion and integrin signaling (Kadrmas and Beckerle, 2004). In contrast, the Alp/Enigma family, which is a group of proteins...
defined by an amino-terminal PDZ domain and one or three carboxyl-terminal LIM domains, maintains actin anchorage at the Z line of muscle cells together with α-actinin (Clark et al., 2002; te Velthuis et al., 2007). α-Actinin is a major component of Z lines. It cross-links antiparallel actin filaments from opposite sarcomeres. Flies lacking α-actinin die as first-instar larvae because of defects in Z line maintenance, yet they initially form normal striated muscles (Fyberg et al., 1998; Dubreuil and Wang, 2000).

Alp/Enigma family proteins cooperate with α-actinin in Z line maintenance. Alp and Cypher/Z band alternatively spliced PDZ-motif protein (ZASP), the best characterized members of the family, colocalize with α-actinin at Z lines and their PDZ domain directly interacts with the carboxyl terminus of α-actinin (Xia et al., 1997; Faulkner et al., 1999; Pomies et al., 1999; Zhou et al., 1999; Klaavuniemi et al., 2004). Mutations in Alp and Cypher/ZASP demonstrate their function in Z line maintenance. Mice that lack Alp or Cypher function develop fragmented Z lines and cardiomyopathy or congenital myopathy, respectively (Pashmforoush et al., 2001; Zhou et al., 2001). Likewise, mutations in ZASP, the human Cypher orthologue, result in dilated cardiomyopathy (Vatta et al., 2003; Arimura et al., 2004).

In this study, we analyze Zasp, the only member of the Alp/Enigma family in D. melanogaster, and identify novel roles for Alp/Enigma family proteins, both in tissue culture cells and flies. We show that Zasp is required for the formation of three different integrin adhesion sites: integrin adhesion sites in tissue culture and Z lines and functional myotendinous junctions in muscles.

Results

S2R+ cells are a model system to study integrin-mediated adhesion

To identify novel genes involved in integrin-mediated cell adhesion and spreading, we investigated two D. melanogaster cell lines, S2 and S2R+ cells, for their ability to uncover such genes using RNAi. Both cell lines are believed to be derived from embryonic hemocytes but exhibit differences in their ability to spread on substrates (Schneider, 1972; Yanagawa et al., 1998). In routine culture conditions, S2 cells are small and spherical, which is typical of unspread cells. However, they can spread when plated on the lectin concanavalin A. Concanavalin A–induced spreading is controlled by remodeling of the actin cytoskeleton upon binding of lectins to the polysaccharide side chains of plasma membrane proteins and lipids (Rogers et al., 2003). In contrast, S2R+ cells are large, flat, and strongly adherent even in the absence of concanavalin A or any other externally supplied ECM substrate. Incubation of S2R+ cells with mxy (encoding βPS integrin) double-stranded RNA (dsRNA) disrupts cell spreading and causes rounding up, indicating that this ability to spread is integrin dependent (Kiger et al., 2003).

We therefore sought to test whether S2 and S2R+ cells can be used to differentiate lectin-mediated cell spreading from integrin-mediated cell spreading.

As both cell lines express βPS integrin, we first assessed its subcellular localization in spreading S2R+ and S2 cells. In S2R+ cells stained with anti–βPS integrin antibody, we observed integrin staining typical of integrin adhesion sites, with bright foci along the cell edge and streaks in areas of potentially increased local forces (Fig. 1 A). In contrast, S2 cells spread on concanavalin A do not exhibit these integrin adhesion sites (Fig. 1 B). Instead of distinct foci and streaks, βPS integrin is exclusively localized intracellularly, most likely because S2 cells do not express the αPS1 and αPS2 integrin subunits (Gotwals et al., 1994), and without a heterodimerization partner, βPS integrin does not translocate to the plasma membrane. To further support the notion that we observed functional adhesion sites in S2R+ cells, we stained them with antibodies against other markers commonly found in focal adhesions, such as talin, vinculin, paxillin, and phosphotyrosine. In each case, we observed similar clusters at the cell edge in S2R+ cells but not in S2 cells (Fig. 1, C–F; and not depicted).

We then determined whether talin, vinculin, and integrin localize to the same clusters. We observed colocalization of vinculin and αPS2 integrin as well as that of talin and βPS integrin (Fig. 1, G–L). These observations point to a major role for integrins in S2R+ cell spreading and indicate that S2 cells spread on concanavalin A by an integrin-independent mechanism.

We next tested if this difference in spreading can be exploited to screen for novel regulators of integrin-mediated cell spreading. We therefore compared cell spreading of S2 cells on concanavalin A and S2R+ cells in the absence of Abi, a known regulator of cytoskeletal remodeling that acts through SCAR, and rhea (encoding talin), the major linker of integrins to the actin cytoskeleton (Brown et al., 2002; Kunda et al., 2003; Rogers et al., 2003; Ginsberg et al., 2005). Although RNAi with Abi causes spreading defects, which are characterized by a star-shaped morphology, in both S2 and S2R+ cells, RNAi with rhea results in cell spreading defects exclusively in S2R+ cells (Fig. 2, A–F). The exclusive spreading defects of S2R+ cells in the absence of talin indicate that we can screen for novel genes involved in integrin regulation by comparing S2R+ and S2 cell spreading and that S2+ cells are a suitable model system to study integrin-mediated adhesion.

We then conducted a pilot screen for novel genes required for cell spreading with 72 candidate genes selected from a genome-wide screen for cell shape changes in S2R+ cells (unpublished data). We selected candidate genes with phenotypes potentially related to cell spreading defects such as a round, star-shaped, or rough-edged cell shape. Parallel RNAi treatment of S2 and S2R+ cells uncovered 12 genes that show an exclusive phenotype in S2R+ cells, suggesting they are specifically involved in integrin-mediated processes and not in general cytoskeletal remodeling or cell viability (Tables S1 and S2 and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb .200707045/DC1). As expected, this group contains the genes encoding βPS integrin and αPS2 integrin and genes with a well-characterized role in regulating integrin adhesion like talin and Rap1 GTPase. Among the novel genes we identified, we chose to focus on CG30084.

The Alp/Enigma family has a single member in D. melanogaster

CG30084 represents the single member of the D. melanogaster Alp/Enigma family of PDZ-LIM domain proteins. We named
because the major predicted splice variant encodes a protein highly similar to human ZASP (Fig. 2 H; Faulkner et al., 1999). In C. elegans, the Alp and Enigma subfamilies are encoded as splice variants of a single gene (McKeown et al., 2006). To test if zasp encodes both Alp and Enigma splice variants, we sequenced 21 ESTs (Table S3, available at http://www.jcb.org/cgi/content/full/jcb.200707045/DC1). Together with data from the Berkeley D. melanogaster Genome Project, we predict that zasp contains 20 exons and is transcribed into two major transcripts (Fig. 2, G and H). The majority of ESTs (20/29) encode Enigma-like proteins (zasp-RA). We also found three ESTs corresponding to the Alp subfamily (zasp-RB, available from GenBank/EMBL/DDBJ under accession no. EF221635). LIM1 is truncated in the Enigma-like protein ZaspEnigma, most likely resulting in only three functional LIM domains as in vertebrate Enigma proteins. Our sequence data confirm the predictions of a recent bioinformatics analysis (te Velthuis et al., 2007). LIM1 is most closely related to the LIM domain of the Alp subfamily, whereas LIM2–4 are most closely related to the LIM domains of the Enigma subfamily (te Velthuis et al., 2007).
Figure 2. Zasp is required for integrin-dependent spreading of S2R+ cells. (A–F) S2R+ cells spreading without addition of external ligand (A–C) and S2 cells spreading on concanavalin A (D–F). A and D, no RNAi treatment; B and E, treatment with Abi RNAi; C and F, treatment with rhea (talin) RNAi. Cells were stained with Alexa 594–phalloidin for filamentous actin. Abi-depleted cells show star-shaped phenotypes in both cell lines (B and E), whereas talin depletion results in rounding up only in S2R+ cells (C). (G) Schematic presentation of the zasp gene. Translated exons are shown in gray and untranslated exons in white. piggyBac insertions used to generate the zasp− deletion and dsRNAs used to target the zasp gene are indicated. Only the two major splice variants are shown. [H] The D. melanogaster zasp gene encodes two major proteins. ZaspEnigma is the Enigma-like protein and ZaspAlp is the Alp-like protein. Numbers represent the amino acid length of each protein. Below three conserved domains, we show the percent identity between Zasp and its human orthologue. [I and J] zasp exon 5 RNAi targeting zasp-RA and zasp-RB. Cells are stained with Alexa 594–phalloidin to visualize the actin cytoskeleton. (I) S2R+ cells round up and exhibit many filopodia-like processes. (J) S2 cells spread on concanavalin A show no phenotype. (K) RT-PCR analysis of zasp dsRNA-treated cells compared with untreated ones. zasp mRNA (153 bp exon 5 amplicon) is depleted in both S2R+ and S2 cells. Control PCR was done with primers against an untargeted gene. Bars, 15 μm.
Loss of Zasp disrupts integrin adhesion sites in S2R+ cells

Treating S2R+ cells with zasp dsRNA targeting exon 5, which depletes ZaspAb and ZaspEnigma, results in severe spreading defects often associated with the formation of filopodia-like processes (Fig. 2 I). S2R+ cells round up similar to mys- or rhea-depleted cells. In contrast, we observed no phenotype in S2 cells (Fig. 2 J). The absence of zasp mRNA after dsRNA treatment in both cell types was verified by RT-PCR (Fig. 2 K). We observed an identical phenotype with dsRNA targeting exon 3, confirming the specificity of the spreading defect (unpublished data).

We next determined if integrin adhesion sites are affected in Zasp-depleted S2R+ cells. zasp exon 3 and exon 5 dsRNA-treated S2R+ cells show no integrin adhesion sites (unpublished data). Treating S2R+ cells with zasp exon 7 dsRNA, which only depletes ZaspAb, strongly impairs integrin adhesion sites. In severe cases, integrin adhesion sites are either completely absent or hardly visible (Fig. 3 B). Loss of integrin adhesion sites is also observed in Zasp-depleted cells that are partially spread, indicating that this defect is not secondary to changes in cell shape (Fig. 3 C).

Finally, as loss of Zasp disrupts integrin adhesion sites, we wanted to know if Zasp colocalizes with βPS integrin in integrin adhesion sites. For this purpose, we raised an antibody against ZaspEnigma, which recognizes both ZaspAb and ZaspEnigma. Like βPS integrin, Zasp localizes to the same bright foci and streaks at the cell edge (Fig. 3 C). Furthermore, the absence of any localized Zasp staining in S2 cells spread on concanavalin A (Fig. 3, G–I) argues that functional integrin heterodimers are required to localize Zasp in S2R+ cells. The distinct spreading defects in the absence of Zasp and its localization in integrin adhesion sites indicate that Zasp is a novel regulator of integrin-mediated cell spreading.

Figure 3. Zasp localizes to integrin adhesion sites and Zasp depletion disrupts integrin adhesion sites. (A–C) Anti–βPS integrin antibody (green) and Alexa 594–phalloidin (red) co-staining of wild-type S2R+ cells or cells treated with zasp exon 7 dsRNA (B and C). Typically, integrin adhesion sites are very small or absent (B). In milder cases, integrin adhesion sites are reduced in number and the cell retracts its edge between two integrin adhesion sites (C). (D–I) Anti–βPS integrin antibody (green) and anti-Zasp antibody (red) co-immunostaining of S2R+ (D–F) and S2 (G–I) cells spread on concanavalin A. Zasp colocalizes with βPS integrin in foci and streaks in S2R+ cells (D–F). Bar, 15 μm.
Zasp mRNA and protein expression is similar to that of βPS integrin

In mammals, most Alp/Enigma family members function in Z line maintenance (Xia et al., 1997; Faulkner et al., 1999; Pomies et al., 1999; Zhou et al., 1999, 2001; Pashmforoush et al., 2001). To learn more about Zasp function and its potential involvement in the formation of integrin adhesion sites, we investigated its expression profile during D. melanogaster embryogenesis. mRNA and protein expression largely overlap (Fig. 4). Preblastoderm-stage embryos show weak staining, indicating a maternal contribution (Fig. 4, A and B). Zygotically, Zasp is expressed in areas where βPS integrin is known to function (Hutson et al., 2003; Devenport and Brown, 2004; Narasimha and Brown, 2004), e.g., the leading edge during dorsal closure and the migdut during midgut fusion (Fig. 4, C–H). At late stages, Zasp expression is particularly strong in mesodermal tissues such as visceral, pharyngeal, and somatic muscles (Fig. 4, I and J).

D. melanogaster αPS2 and βPS integrin subunits are enriched at myotendinous junctions where they function in the adhesion of muscles to the tendon matrix (Leptin et al., 1989; Brabant and Brower, 1993; Brown, 1994). Strikingly, Zasp is also enriched at myotendinous junctions (Fig. 5 A). Coimmunostaining with anti-Zasp and anti–βPS integrin antibodies reveals that Zasp tightly colocalizes with βPS integrin at myotendinous junctions in the embryo (Fig. 5, A’–C’). In larvae, Zasp still localizes to myotendinous junctions but facing the cytoplasmic side of βPS integrin (Fig. 5, compare D and E). Finally, the diffuse muscle staining in embryos is refined into the specific localization of Zasp into a repetitive line pattern (Fig. 5 D). To see if these lines correspond to Z lines, we analyzed Zasp distribution in relation to α-actinin, a well-known marker of Z lines (Saide et al., 1989). Zasp and α-actinin tightly colocalize at myotendinous junctions from stage 16 onward and later in Z lines, confirming that Zasp localizes to Z lines (Fig. 5, G–I; and not depicted). We also analyzed expression and subcellular localization of Zasp with an endogenous homozygously viable GFP-Zasp fusion (G00189; Morin et al., 2001). We verified the fusion by RT-PCR and dsRNA injection into embryos. Live imaging of GFP-Zasp shows the same localization as antibody staining, confirming the specificity of our antibody (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200707045/DC1).
To determine if integrins recruit Zasp to myotendinous junctions, we examined Zasp distribution in zygotic mysXG43 mutant embryos lacking βPS integrin. Zasp and α-actinin no longer localized to the tips of detached muscles, or did so only in a weak gradient (Fig. 5, J–L). In a mysXG43 maternal and zygotic mutant, Zasp is completely unlocalized (unpublished data), indicating that Zasp and α-actinin are recruited to myotendinous junctions by integrins. The expression data suggest that Zasp functions together with integrins and α-actinin in several morphogenetic processes.

**zasp**<sup>4</sup> mutants die as first-instar larvae

To investigate if Zasp indeed functions in the formation of integrin adhesion sites, we made a zasp mutant by recombining two flippase recognition target–bearing piggyBac elements inserted in introns four (f04847) and nine (f04784) of zasp (Fig. 2 G). We recovered three identical lethal lines deleting exons 5–9 and resulting in a frameshift. The deletion lines, whose identity was verified by PCR and Southern blotting, remove all major splice variants (Figs. 2 G and 6 A, Table S3, and not depicted). They have no zasp mRNA expression as shown by RT-PCR and RNA in situ hybridization with a full-length probe (Fig. 6, B–D). In addition, Zasp protein is absent in stage-17 zygotic mutants (Fig. 6, E and F). Finally, zasp mutants that are transheterozygous over a large deficiency show the same phenotypes as zasp homozygotes even though we observed a higher embryonic lethality (not depicted and Fig. 6 G). The combined data show that we created a zasp hypomorph, which we refer to as zasp<sup>5</sup>. The majority of zasp<sup>5</sup> mutants die as first-instar larvae and 12% die as embryos. Most zasp<sup>5</sup> mutant larvae die within the first 24 h, coinciding with the onset of muscle contractility. Even though some larvae can live longer, they do not progress.

**Figure 5.** Zasp colocalizes with integrins at myotendinous junctions during embryonic development and with α-actinin at muscle Z lines. (A–C) Anti-Zasp antibody staining (A), anti–βPS integrin antibody staining (B), and merge of a stage-16 embryo (C). Zasp and βPS integrin colocalize at myotendinous junctions. Indicated areas are shown enlarged in A′–C′. (D–F) Anti-Zasp antibody staining (D), anti-βPS integrin antibody staining (E), and merge (F) of a first-instar larva. Note the slightly wider gap of Zasp staining compared with that of βPS integrin staining at the myotendinous junction. (G–I) Anti-Zasp antibody staining (G), anti–α-actinin antibody staining (H), and merge (I) of a first-instar larva. Note the tight colocalization of Zasp and α-actinin at myotendinous junctions and Z lines. (J–L) Anti-Zasp antibody staining (J), anti–α-actinin antibody staining (K), and merge (L) of a zygotic mysXG43 mutant embryo. Zasp and α-actinin no longer localize at the termini of detached muscles (J, arrow). Asterisks indicate myotendinous junctions. Arrowheads indicate Z lines. Bar, 50 μm.
Figure 6. *zasp* mutant embryos die as first-instar larvae. (A) Putative deletion lines were screened for the presence of residual piggyBac elements by means of PCR, using transposon-specific primers (RB(WH+)) reverse and forward in combination with genome-specific primers (exon 3 forward and 9 reverse). Amplification of both PCR products indicates the presence of both residual piggyBac elements and therefore a recombination and deletion event. Genomic DNA extracted from control flies (C, pBac(WH)f04847) is only amplified with the e3forward/RBreverse primers. 1- and 24 kb size markers are indicated. (B) Absence of *zasp* mRNA in two deletion lines was verified by RT-PCR using primers CACCAAGGCCA-ACCCACAGCTGCTG and GCCGGCGTGAT-1CTTGCAG. Amplification of a 2.1-kb band (asterisk) is detected only in wild-type embryos (wt). (C, D and E) RNA in situ hybridization with a full-length antisense probe demonstrates absence of *zasp* mRNA in *zasp* mutant embryos (D), (E and F) Anti-Zasp antibody staining reveals no obvious Zasp protein in mutant embryos (F). (G) Stage of death of *zasp* mutants. (H) Developmental stage of *zasp* mutant larvae was determined by the number of teeth on the mouth hooks, which increase with instar. Mouth hooks of *zasp* mutant larvae (iv) look like those of first-instar wild-type larvae (i). Bar, 50 μm.

Zasp is required for sarcomere assembly and recruits α-actinin to the Z line

As muscle contractions were slower in *zasp* mutant larvae than in wild-type larvae, we investigated sarcomere assembly in late-stage 17/first-instar larvae by comparing the actin organization in muscles of wild-type and *zasp* mutants. *zasp* mutant larvae have lost the typical striated muscle pattern, indicating a Z line defect (Fig. 7, A and B). In mammalians, the main interaction partner of Zasp at the Z line is α-actinin. We therefore wanted to know if *D. melanogaster* Zasp interacts biochemically with α-actinin. Immunoprecipitation of wild-type extracts with anti-Zasp antibody pulls down α-actinin (Fig. 7 C). In contrast, immunoprecipitation of extracts from *zasp* larvae or with preimmune serum does not pull down α-actinin, demonstrating the specificity of this interaction. Intriguingly, well-characterized α-actinin–null mutants, such as *Actn*Δ2, still develop a striated muscle pattern and Z lines (Fyrberg et al., 1990, 1998; Dubreuil and Wang, 2000), indicating that Zasp plays a more important role in Z line assembly than α-actinin. α-Actinin has no maternal contribution (Perrimon et al., 1985); therefore, *Actn*-null mutant larvae should correspond to a complete loss of function. To better characterize these differences, we investigated sarcomere and Z line ultrastructure of wild-type, *zasp*, and *Actn*Δ2 mutant larvae by transmission electron microscopy (Fig. 7, D–G). In freshly hatched wild-type larvae, Z lines can be readily observed and are spaced at regular intervals (Fig. 7 D). In 1-d-old α-actinin mutant larvae, we still observed Z lines spaced at regular, but wider intervals than in the wild type, possibly because of detachment of actin fibers from the Z line (Fig. 7 E). These phenotypes correspond to previous observations (Fyrberg et al., 1998). In contrast, in freshly hatched *zasp* mutant larvae, Z lines are either completely absent with only occasional accumulations of electron-dense material (Fig. 7 F) or Z line remnants are severely disorganized and irregularly spaced (Fig. 7 G). In addition, filaments are disorganized and no longer arranged in parallel arrays (Fig. 7, F and G).
To determine the mechanism of Zasp function at Z lines, we stained with anti–α-actinin antibody. zaspΔ mutant larvae lost α-actinin localization specifically at muscle Z lines, showing that Zasp is required to localize α-actinin to Z lines (Fig. 8, A and B). If Zasp is upstream of α-actinin, Zasp should still localize to Z lines in α-actinin mutants. In Actn1Δ larvae, which is a null allele (Fyrberg et al., 1990; Dubreuil and Wang, 2000), GFP-Zasp indeed still localizes to Z lines in a manner very similar to its wild-type distribution (Fig. 8, C and D). We finally tested the distribution of titin, which binds to α-actinin and has been proposed to form a ternary complex together with Zasp (Sorimachi et al., 1997; Young et al., 1998; Au et al., 2004). Titin no longer localizes to Z lines in Actn1 mutants (Fig. 8, E and F). All these phenotypes are completely penetrant and demonstrate that Zasp is required for Z line assembly.

**Zasp mediates muscle attachment together with integrins**

We next analyzed zaspΔ mutants for muscle detachment. We observed muscle-attachment defects from stage-16 embryos onward, as seen by detachment of muscle fibers from myotendinous junctions (Fig. 9, A and B). We also saw muscle patterning defects with muscles missing in some segments (unpublished data). Late–stage 17 embryos occasionally exhibit strong muscle detachment and rounding up of muscles (Fig. 9 C). This complete muscle detachment is progressive, as we observed a penetrance of 12% (n = 190) round muscles when we fixed embryos at stage 17, but almost complete penetrance when we fixed nonhatched embryos 24 h later (98%; n = 112). The phenotype is also progressive in larvae, where we observed 17% round muscle phenotypes (n = 174) when larvae were fixed at the end of first instar. This phenotype is similar to that of embryos lacking zygotic BPS integrin (mysG^{287}; Fig. 9 D), although it occurs later. BPS integrin still localizes to myotendinous junctions in zaspΔ mutants even when muscles round up, showing that Zasp is not required for integrin localization (Fig. 9 C’).

If Zasp is a crucial component of integrin adhesion sites, zaspΔ should also genetically interact with integrins. The αPS2 integrin subunit is only expressed in mesodermal cells, but not in epidermal cells of the myotendinous junction (Bogaert et al., 1987). To test for a genetic interaction, we used a hypomorphic mutation in αPS2 integrin (if^{287}) that shows weak muscle-attachment...
defects only at the last stage of embryogenesis. In ifSEF mutants, βPS integrin localization to myotendinous junctions is lost at stage 17, corresponding to the onset of the phenotype (Bloor and Brown, 1998; Devenport et al., 2007; Fig. 10, A and B). However, stage-16 ifSEF embryos show normal βPS integrin localization and no muscle phenotype (Fig. 10, C and D). Intriguingly, ifSEF mutants lacking one copy of zasp show a much more severe muscle-detachment phenotype and, additionally, the phenotype appears at an earlier stage when βPS integrin is still localized to myotendinous junctions (Fig. 10, E, F, and I). This genetic interaction is specific to zasp because we observed no enhancement with either of the two piggyBac elements used to generate the zasp mutation (Fig. 10, G-I; and not depicted). The strong genetic interaction we observed between Zasp and αPS2 integrin shows that they act together in maintaining muscle attachment.

**Discussion**

We identified a novel regulator of cell–matrix adhesion, Zasp, in an RNAi screen for integrin-dependent cell spreading. We propose that Zasp mediates two related functions, one upstream of α-actinin organizing the Z line and the other downstream of integrins regulating assembly of functional adhesion sites.

In our screen, we compare cell spreading of S2 and S2R+ cells. S2R+ cells are S2 cells that have acquired novel traits over time, such as the ability to spread without externally added ECM ligands (Yanagawa et al., 1998). For our purposes, the only relevant difference between these two cell lines is the absence of αPS2 integrin from S2 cells. S2 cells transfected with αPS2 integrin spread and grow like S2R+ cells and, conversely, upon RNAi-mediated integrin depletion, S2R+ cells can be grown for weeks and look like S2 cells (Gotwals et al., 1994; unpublished data). S2R+ cells most likely secrete their own ECM ligand, which is similar to what has been reported for human fibroblasts (Grinnell and Feld, 1979). A good candidate may be Tenascin m, which is an αPS2βPS ligand (Graner et al., 1998) and which causes rounding up of S2R+ cells when depleted by RNAi (Kiger et al., 2003). We observed putative integrin adhesion sites in S2R+ cells that look like integrin adhesion sites and are composed of focal adhesion proteins like talin and vinculin. We were unable to observe actin fibers attached to these adhesion sites, probably because actin bundling in S2R+ cells is not sufficient to allow visualization by fluorescence microscopy. The colocalization of integrin with vinculin and talin, the disruption of these sites in mutants affecting cell spreading, and the absence of adhesion sites in S2 cells spread on concanavalin A strongly argue that we observed functional integrin adhesion sites.

Our pilot RNAi screen uncovered 12 genes that show exclusive phenotypes in S2R+ cells upon depletion. Five of these genes are known to function in cell–matrix adhesion either directly or by regulating mesodermal gene transcription or RNA processing, which validates our approach (Brown, 1994; Artero et al., 1998; Brown et al., 2002; Huelsmann et al., 2006). Several classical focal adhesion proteins, such as vinculin or FAK, were not included in the pilot screen because they did not show a phenotype in the genome-wide screen. This is not too
surprising given that some of them also do not have identifiable phenotypes in vivo (Alatortsev et al., 1997; Grabbe et al., 2004). Still, we have probably missed several genes because of the high variability of cell shapes in S2R+ cells, which is also evident in the high number of candidates in which we could not reproduce the originally observed cell shape change.

Integrin adhesion sites in cell culture are considered to be precursors of adhesion complexes found in tissues such as myotendinous junctions. In D. melanogaster S2R+ cells, Zasp localizes to integrin adhesion sites. The functional importance of this localization is shown by the loss of integrin adhesion sites upon Zasp depletion and the concomitant failure of cell spreading. Collectively, these results suggest that Zasp functions in organizing or maintaining integrin adhesion sites to allow cell spreading. Our fly data are in agreement with this conclusion, as we observed colocalization of Zasp and βPS integrin at myotendinous junctions and muscle detachment in zasp mutants, again demonstrating that Zasp plays a crucial role in integrin-mediated adhesion. The muscle-detachment phenotype is weaker than the tissue culture phenotype. This may be because of a maternal contribution of Zasp rescuing earlier muscle-attachment defects, or because the myotendinous junction contains more components and is therefore less easily disrupted. We also observed defects in other tissues that require integrin function, such as wing blisters in zasp clones and a genetic interaction of Zasp and integrin during wing formation, confirming the general role of Zasp in integrin adhesion (unpublished data). Vertebrate and C. elegans Alp/Enigma proteins have no reported function at integrin adhesion sites, but they are known to localize to focal adhesions and myotendinous junctions (Pomies et al., 1999; Pashmforouh et al., 2001; Henderson et al., 2003; McKeown et al., 2006). In two other studies, the authors observed no colocalization with vinculin at focal adhesions; however, the studies were not conducted in muscle cells but rather in CHO cells, endothelial cells, and blood platelets (Bauer et al., 2000; Klaavuniemi et al., 2004). Zasp is the only member of the Alp/Enigma family of PDZ-LIM domain proteins in D. melanogaster, whereas there are seven members of that family in vertebrates (Kadrmas and Beckerle, 2004). The lack of data for a function of vertebrate PDZ-LIM family members in integrin adhesion is therefore most likely owing to genetic redundancy.

Figure 9. zasp mutants have muscle-attachment defects. Stage-17 embryos are stained with an antibody against muscle myosin heavy chain (MHC; green) to visualize somatic muscles and an antibody against βPS integrin (red) to visualize myotendinous junctions. (A) Wild-type embryo. (B) zasp mutant embryo with mild muscle detachment. (C) zasp mutant embryo with severe muscle detachment. (D) mys mutant embryo shown for comparison. Indicated areas are shown enlarged in A’–D’. Arrows indicate detached muscles. Asterisks indicate myotendinous junctions. Bar, 50 μm.
Several lines of evidence argue that Zasp acts as a cytoskeletal adaptor downstream of integrins. First, Zasp is recruited to integrin adhesion sites in S2R+/cells and is recruited by βPS integrin to myotendinous junctions in the embryo. Second, even in zaspΔ embryos with strong muscle detachment, βPS integrin still localizes to myotendinous junctions or muscle tips. Third, during larval stages we observed Zasp on the cytoplasmic side of integrins.

We suggest that Zasp regulates or strengthens the link of integrins to the actin cytoskeleton after the initial attachment of integrins to actin via talin. Two lines of evidence support this proposal. First, the zasp muscle-attachment defect is weaker than a complete loss of function of βPS integrin or talin and is most similar to mutants in factors linking integrin to the cytoskeleton, like integrin-linked kinase (Zervas et al., 2001). Second, we observed muscle detachment after muscle contractions began in late-stage 17 embryos and larvae. The strongest evidence that Zasp plays a crucial role in the assembly of functional integrin adhesion sites is the strong genetic interaction between αPS2 integrin and Zasp.

Related to Zasp’s function at integrin adhesion sites, Zasp also organizes the Z line. There is ample evidence of α-actinin...
binding and cross-linking actin, and it has been established that Alp/Enigma proteins directly bind α-actinin (Faulkner et al., 1999; Zhou et al., 1999; Passamflowsh et al., 2001). We also observed a physical interaction of Zasp and α-actinin in D. melanogaster, but our data imply a considerably larger role for Zasp in establishing Z line structure and function than was previously appreciated. Our data indicate that Zasp acts upstream and recruits α-actinin because in the absence of Zasp, α-actinin no longer localizes to Z lines. In contrast, Zasp still localizes to Z lines in α-actinin–null mutants. Titin, which is anchored to Z lines by directly binding α-actinin (Ohtsuka et al., 1997; Sorimachi et al., 1997; Young et al., 1998), is not present at Z lines in Actn mutants, confirming the previously published biochemical data. Molecular modeling recently predicted a ternary complex of Zasp, α-actinin, and titin, with Zasp and titin binding to different surfaces of α-actinin (Au et al., 2004). Our data are in agreement with such a complex and indicate that Zasp is the most upstream component of the complex. Recruiting α-actinin to the Z line cannot be Zasp’s only function because a well-characterized α-actinin–null mutant still shows Z lines (Fyrey et al., 1998; Fig. 7 E), which are disrupted in zasp4 mutant larvae. We suggest that this additional function of Zasp is again dependent on integrins. Integrins connect Z lines laterally to the ECM surrounding muscle fibers and are required for sarcomere assembly (Volk et al., 1990; Schwander et al., 2003; Lecroisey et al., 2007).

Mutations in PDZ-LIM family members cause myopathies. Our work suggests that other mutations in members of the PDZ-LIM family should also be involved in integrin-related diseases in vertebrates.

In conclusion, we have identified a novel regulator of integrin function that plays a crucial role in assembling integrin adhesion sites.

Materials and methods

Tissue culture and RNAi

D. melanogaster S2 and S2R+ cells were treated with dsRNAs as previously described (Kiger et al., 2003; Rogers et al., 2003). For dsRNA synthesis, 17-flanked PCR products (provided by N. Perrimon, Harvard Medical School, Boston, MA) were used as templates for in vitro transcription with T7 RNA polymerase using the MEGAscript kit (Ambion). For zasp, the following additional primers flanked the T7 promoter (TAATACGACTATAGGAGA) were used for PCR followed by dsRNA synthesis: exon 3 (263 bp) CGCTGCACGTGAGGCAA and GCCCCAACCCACGCTGCA; and exon 5 (153 bp) CGCCGCACCGCCAGCCCAA and CACCGCGCGGCGGTTCTTTC. Cells were incubated with dsRNA for 5–7 d before being harvested for microscopy analysis, RTPCR, and Western blotting. Control experiments were performed in parallel without the addition of dsRNA.

Before fixation, dsRNA-treated S2R+ cells were replated on glass slides (VWR) and allowed to spread for 4 h, whereas dsRNA-treated S2 cells were replated on glass slides coated with concanavalin A and were allowed to spread for 2 h. Glass slides were coated for 30 min with 0.5 mg/ml concanavalin A, and then air dried for another 30 min before the addition of S2 cells. Cells were then fixed in 4% formaldehyde in PBS for 20 min.

After being permeabilized in 1 × PBS containing 0.1% Triton X-100 for 3 min, cells were washed twice in PBT (PBS containing 0.05% Tween 20), followed by incubation in blocking solution containing 1% BSA in 1 × PBS for at least 30 min at room temperature, and then stained with the primary antibody overnight at 4°C. After washing with PBT with several changes over 30 min, cells were incubated with the secondary antibody for 45 min at room temperature. After several washes, the glass slides were mounted in Prolong Gold antifade (Invitrogen). For RT-PCR, mRNA was isolated using Trizol (Invitrogen) according to the manufacturer’s instructions. Gene-specific cDNAs were reverse transcribed and amplified with the same set of primers used previously for dsRNA synthesis.

Generation of anti-Zasp antibody

We prepared a rabbit polyclonal antibody using a 6×His-tagged fusion protein corresponding to Zasp(38–233). cDNA was amplified from EST RH03424 as a template with CACCATGGCCCAACACGCTGTG and GCGGCCGCTAGTCATTGCG 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, which we transformed into TOP10 competent cells. Expression was induced in the presence of 0.2% IPTG. We purified the recombinant protein under denaturing conditions on Ni2+-affinity columns (QIAGEN) according to the manufacturer’s instructions. We tested antibody specificity by Western blotting and immunofluorescence detection comparing wild-type, G00189, and zasp+ embryos.

Immunoprecipitation assay

The indirect immunoprecipitation strategy was used by covalently binding the anti-Zasp antibody to AffiPrep resin (Bio-Rad Laboratories) using dimethylpimelimidate as a cross-linker. 10 μl of anti-Zasp serum or rabbit preimmune serum were incubated with 110 μl of beads in 1 ml of amylovulinate buffer (50 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM MgCl2, 300 mM KCl, 0.05% NP-40, 0.5 mM DTT, 10% glycerol, and one tablet of EDTA-free complete protease inhibitor cocktail). Beads were eluted by heating in 2× sample elution buffer (2% SDS, 62.5 mM Tris, pH 6.8, and 10% glycerol) without DTT for 10 min at 50°C to avoid IgG contamination. 1/10 of the supernatant was resolved by 8% SDS-PAGE and blotted on Hybond-C extra nitrocellulose membrane (GE Healthcare) for detection with anti-α-actinin monoclonal antibody (1:20), Anti-mouse IgG peroxidase-linked secondary antibody (1:2,500) was used together with the ECL detection kit for visualization (GE Healthcare).

Histochemistry and microscopy

RNA in situ hybridization of embryos was performed with digoxigenin-labeled RNA probes, made by in vitro transcription of RH03424 with Taq and T7 RNA polymerase yielding full-length antisense and sense probes, respectively. Embryos mutant for zasp were identified by the absence of staining with the antisense probe in ~25% of an unsorted collection. We also sorted homozygous zasp+ mutant embryos by the absence of the GFP balancer CyO, twiGal4 UAS-2xEGFP. In this case we hybridized green and non-green embryos in parallel and observed a signal only in green embryos. Images were obtained on a stereomicroscope (MZ16-FA; Leica) using a Plan Apo 2× objective with a digital camera (Qicam) and OpenLab software (Improvement). Embryos and larvae were fixed using heat fixation (Tepas, 1996). In brief, embryos were dechorionated in 50% bleach for 90 s, rinsed in water, immersed in boiling 1× embryonic wash buffer (70 mM NaCl and 0.65% Triton X-100) for 10 s, immediately cooled by adding 3 vol of ice-cold embryonic wash buffer, and placed on ice for 30 min. Embryos were deventilated in methanol/heptane.

Primary antibodies used were the following: mouse anti-α-actinin (1:10; provided by J. Saide, Boston University School of Medicine, Boston, MA; Sahai et al., 1989), rat actin (1:20; Clone A-7, 10A10 provided by N.H. Brown and G. Tanentzapf, Gurdon Institute, University of Cambridge, Cambridge, UK; Bogaert et al., 1987), mouse anti-β5 integrin (1:10; CF6G11; obtained from Developmental Studies Hybridoma Bank; Brower et al., 1984), mouse anti-paxillin (1:50; 165; obtained from BD Biosciences), rabbit anti-Italin (1:100; provided by N.H. Brown; Brown et al., 2002), rat anti–D-Trin-ZK (1:500; provided by D. Andrew, Johns Hopkins University School of Medicine, Baltimore, MD; Machado et al., 1998), rabbit antimuscle myosin heavy chain (1:400; provided by D. Kiehart, Duke University, Durham, NC; Kiehart and Feghali, 1986), mouse anti-phosphoryrinosine (1:200; 4G10; obtained from Millipore), mouse anti-C. elegans vinculin (1:10; M0H24; obtained from Developmental Studies Hybridoma Bank; Francis and Waterston, 1985), and rabbit anti-ZASP (1:300 for cells; 1:400 for embryos). Fluorescently labeled secondary antibodies of the Alexa series (Invitrogen) were used at a 1:300 dilution.
Filamentous actin was visualized with Alexa 488– or Alexa 594–labeled phalloidin (1:50 for tissue culture and 1:200 for embryos; Invitrogen). Embryos were devitellinized by hand.

After washing for 1 h in PBT, embryos were preincubated for 1 h in PBT containing 5% normal goat serum (PBNT), followed by an overnight incubation at 4°C with primary antibody, which was diluted in PBNT containing 0.1% BSA. After a 1-h wash in PBT and preincubation in PBNT for 1 h, embryos were incubated in secondary antibody for 2 h at room temperature and embedded in Prolong Gold antifade solution after several washes in PBT.

Tissue culture images were obtained on an upright microscope (DM6000B; Leica) using a 63×1.4 NA oil objective (HCX PL APO CS) with a digital camera (Orca-ER; Hamamatsu) and OpenLab software. Embryo images were obtained on a confocal microscope (LSM510 Meta; Carl Zeiss, Inc.) using a 40×1.3 NA (Plan-Neofluar) or a 63×1.4 NA (Plan Apo) oil objective and processed using ImageJ (National Institutes of Health and Photoshop (Adobe)). Live imaging was done as previously described (Schöck and Perrimon, 2003). All images were obtained at room temperature.

Electron microscopy
First-instar larvae were fixed in 5% glutaraldehyde and 0.1 M cyscodylate buffer, pH 7.4, for 1 h at room temperature. After cutting off the extremities, the specimens were transferred into fresh 2.5% glutaraldehyde and 0.1 M cyscodylate buffer and fixed for another 5 h at 4°C. Larvae were postfixed in 1% osmium tetroxide for 2 h at 4°C. The samples were washed with several changes of 0.1 M cyscodylate buffer for 40 min at 4°C, followed by staining with 2% tannic acid in 0.1 M cyscodylate buffer for 1 h at 4°C. After several washes in distilled water, the specimens were resteinated with 2% uranyl acetate for 1 h at 4°C and washed in distilled water for 45 min at 4°C, followed by dehydration in acetone at room temperature. Samples were embedded in epoxy resin (EPON-815; Electron Microscopy Sciences) and cut with an Ultracut A (Reichert). The muscleblind gene participates in the organization of Z-bands and epidermal attachments of Drosophila muscles and is regulated by Dm2e. Dev. Biol. 195:131–143.


