The role of Integrin-dependent cell matrix adhesion in muscle development

Klodiana Jani

Doctor of Philosophy

Department of Biology, McGill University, 1205 Dr. Penfield Avenue, Montreal, Quebec H3A 1B1, Canada

January 2009

A thesis submitted to McGill University in partial fulfillment of the requirement of requirements of the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-66314-1
Our file Notre référence
ISBN: 978-0-494-66314-1

ABSTRACT

Cell adhesion is essential to cell motility and tissue integrity and is regulated by the Integrin family of transmembrane receptors. Integrin binds to ligand extracellularly and provide anchor to the intracellular cytoskeleton via adhesion scaffolding proteins. In order to link cell to the surrounding matrix Integrin needs to be activated. Intracellular activation signals induce perturbations in Integrin cytoplasmic domain that are translated into a conformational change in extracellular region for high affinity ligand binding. Integrin engagement by matrix, in turn, triggers the assembly of adhesion complexes. Such early adhesions promote cytoskeletal organization with subsequent contractile activity that exerts forces against initial Integrin-matrix adhesions. In response to force, Integrin strengthens the interaction with matrix through its clustering and successive recruitment of additional adhesion components. These bidirectional regulatory loops mediated by such interactions are largely dependent on the unique function of Integrin adhesion components.

We demonstrate a novel role for the PDZ/LIM domain protein Zasp as a core component of Integrin adhesions. Specifically, Zasp colocalizes with Integrins at focal adhesion in cultured cells and myotendinous junctions in *Drosophila* embryos. In both cases elimination of Zasp modifies Integrin function causing consequently defects in cell spreading and muscle attachment. Zasp supports Integrin adhesion to the extracellular matrix that is required to withstand tensile forces exerted during cell spreading and muscle contraction. Furthermore, we found that the distribution of Zasp in muscle Z-lines is essential to orchestrate the cross-linking of α-Actinin and Actin filaments. Disruption of Zasp leads to loss of muscle cytoarchitecture, pointing to a larger role for Zasp in sarcomere assembly. Finally, we demonstrate that Zasp, in addition to α-Actinin, physically interacts with the Integrin- and Actin-bound cytoskeletal protein Talin.

Collectively, our results point to a dual role for Zasp as a structural scaffold. First it regulates Integrin adhesion to the extracellular matrix by interacting with the head domain of Talin at the myotendinous junctions. Second, Zasp controls sarcomere assembly by tethering the presarcomeric α-Actinin component to the tail domain of Talin. Zasp finding as a crucial adhesion component provides further insights on the mechanism underlying Integrin-mediated adhesion.

ABRÉGÉ

L'adhérence cellulaire est essentielle pour la motilité cellulaire et l'intégrité tissulaire et est régulée par les membres de la famille des récepteurs membranaires de type Intégrines. Les Intégrines lient un ligand extracellulaire et permettent l'ancrage au cytosquelette intracellulaire par leur adhérence avec les protéines d'échafaudage. Pour relier la cellule à la matrice environnante Integrin doit être activé. Les signaux d'activation intracellulaire induisent des perturbations dans le domaine cytoplasmique des Intégrines qui entraîneront des changements dans la conformation dans la région extracellulaire enfin d'augmenter une plus grande affinité avec le ligand. L'engagement de l'Intégrine avec la matrice induit, subséquemment, l'assemblage des complexes d'adhésion. Ces complexes précoces promouvoient l'organisation du cytosquelette par leur activité contractile qui exerce des forces contre les interactions initiales Intégrine-matrice. En réponse à cette force, l'Intégrine renforce son interaction avec la matrice par l'agrégation et par le recrutement subséquent de components d'adhésion additionnels. Ces rétroactions bidirectionnelles médiées par ce type d'interactions sont largement dépendantes de la fonction unique des composantes d'adhésion dépendantes des Intégrines.

Nous démontrons une nouvelle fonction pour la protéine Zasp, contenant un domaine PDZ/LIM, comme composante essentielle des complexes d'adhésion Intégrines-dépendentes. Spécifiquement, Zasp co-localise avec les Intégrines aux points focaux d'adhésion en culture cellulaire et dans les jonctions musculotendineuses dans les embryons de la drosophile. Dans les deux cas, l'élimination de Zasp modifie la fonction des Intégrines causant des défauts dans l'étalement cellulaire et dans l'attachement des muscles. Zasp supporte l'adhésion des Intégrines à la matrice extracellulaire qui doit résister aux forces de tension exercées par l'étalement cellulaire et par la contraction musculaire. De plus, nous avons trouvé que la distribution de Zasp à l'interface des lignes Z des muscles est essentielle pour orchestrer la réticulation de α-Actinine et des filaments d'actines. La perte de Zasp amène la perte de la cyto-architecture des muscles, suggérant un rôle plus important de Zasp dans l'assemblage des sarcomères. Finalement, nous démontrons que Zasp, en plus de α-Actinine, interagit physiquement avec la Taline qui relie le cytosquelette d'actine aux Intégrines.

Collectivement, nos résultats suggèrent que Zasp joue un double rôle comme échafaud structural. Premièrement, Zasp régule l'adhésion des Intégrine à la matrice extracellulaire en interagissant avec le domaine de la tête de la Taline aux jonctions musculotendineuses. Deuxièmement, Zasp contrôle l'assemblage des sarcomères en amarrant la protéine présarcomérique α-Actinine au domaine de la queue de la Taline. La découverte du rôle crucial de Zasp dans le complexe d'adhésion cellulaire pourrait mener à une meilleure compréhension des mécanismes sous-adjacents à l'adhésion induite par les Intégrines.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter I

Chapter II

Chapter III

LIST OF SUPPLEMENTARY FIGURES

Chapter II

LIST OF TABLES

Chapter II

LIST OF ABBREVIATIONS

α-Actinin	Alpha-Actinin
α-Integrin	Alpha-Integrin
ALP	Alpha-actinin binding LIM domain protein
β-Integrin	Beta-Integrin
βPS	Beta-Position Specific
BCC	B-box zinc finger coil-coiled domain
C-terminus	Carboxy-Terminus
D-Titin	*Drosophila* Titin
DAG	Diacylglycerol
DlgA	*Drosophila* disc large tumor suppressor
dsRNA	Double Stranded RNA
ECM	Extracellular Matrix
FAK	Focal Adhesion Kinase
FAT	Focal Adhesion Targeting Domain
FERM	4.1, ezrin, radixin, and moesin domain
Fn	Fibronectin-like Domain
GST	Glutathione *S*-Transferase
GTPase	Guanosine triphosphate hydrolase enzymes
Ig	Immunoglobulin-like domain
ILK	Integrin Linked Kinase
LASP	LIM Domain and SH3 Domain Protein
LIM	Lin11, Isl-1, and Mec-3
MLP	Muscle LIM Domain Protein
NHL	Ncl-1, HT2A, and Lin-41 proteins
N-RAP	Nebulin-related anchoring protein
N-terminus	Amino-Terminus
PDZ	PSD95, DlgA, and Zo-1
PEVK	Proline, Glutamine, Valine, and Lysine motif

PINCH	Particularly Interesting New Cysteine- and Histidine-Rich Protein
PKC	Protein Kinase C
PSD95	Post Synaptic Density protein
RACK	Receptor for activated C Kinase
RET	Rearranged during Transfection
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH2	Src Homology 2 Domain
SH3	Src Homology 3 Domain
siRNA	Small Inhibitory RNA
Wech	A German Rhineland term for "detached"
ZASP	Z-band Alternatively Spliced PDZ domain Protein
ZO-1	Zonula Occludens-1 Protein

AUTHOR CONTRIBUTION

All research that I have conducted in Dr. Schöck's lab during my PhD is presented here in two manuscript-based chapters.

Chapter 1: Some sections of this chapter are part of a manuscript in preparation reviewing the molecular basis of Integrin-mediated adhesion in muscle. I performed the literature search for the review, I wrote all the sections, and prepared all the figures.

Dr. Schöck edited all sections of the review.

Chapter 2: This chapter has been published as follows:

Jani K., and Schöck F. (2007). "Zasp is required for the assembly of functional integrin adhesion sites". J. Cell. Biol. **179**(7): 1583-1597

The original genome-wide RNAi screen was conducted by Dr. Schöck in Norbert Perrimon's laboratory. I repeated such a screen with only 72 selected candidates, from which I chose to work with one of them. I performed all the experiments described within this Chapter; except for those illustrated in Figure 2.3.8 C and D. I wrote Materials and Methods Figure Legends as well, and I prepared all the figures. I also participated with Dr. Schöck in the writing of the Introduction, Results and the Discussion sections.

Dr. Schöck, performed the experiments described in Figure 2.3.8 C and D, wrote the Abstract, Introduction, Results, and Discussion sections as well as he edited my input.

Chapter 3: is a manuscript in preparation

I performed all the experiments, data analyses, and literature review described within this Chapter. I wrote the Abstract, Introduction, Results and Discussion sections, Materials and Methods, Conclusion, and Figure Legends. I also made all the figures in this Chapter.

Dr. Schöck edited all sections included in this Chapter.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Frieder Schöck for giving me the opportunity to accomplish the Ph.D thesis work in his lab as well as for his guidance and mentorship. Especially, I would like to acknowledge his contribution to the development of my scientific thinking and technical skills. Finally, I'm grateful to him for introducing me to the scientific community by offering me possibilities to participate in several conferences.

I am thankful to my supervisory committee, Dr. Jackie Vogel and Dr. Marc Therrien for their constructive feedback throughout my PhD program. I also thank Jan Michael Kugler and Dr. Chiara Gamberi for sharing their scientific and technical expertise with me. I thank past and present members of the Schöck Lab for their support, in particular, Judith Pandur for her ethical advices and Amir-Hossein Haghayeghi for his friendship.

I would like to acknowledge the invaluable support of my family, above all of my loving parents, for their gentile spirit, for always believing in me, for everything they have taught me, and for all the opportunities that they have given me. I am grateful to my sisters and brother for their incessant understanding and encouragement throughout the entire course of my Ph.D. To rest of my wonderful family, I remain thankful to my dearest nieces Emilia, Victoria, and Megi for the immeasurable joyous moments.

Finally, I would like to express my gratitude to my friends who have always been so understanding and forgiving, when I did not have enough time to spend with them. Especially, I am grateful to my dearest friends Sorana and Edor for their amazing friendship and advices during arduous times. Special thanks to my sharp-eyed friend Arsida, for editing this thesis. I wish to thank Sara, Ara and Amelia for the cheerful moments and companionships, and lastly, Max, Andi, Emma, Ernesto, Ilia, Horia, Sorin, Marc, Hulio, Carol, and Idlir for their incessant attentiveness and great Salsa partnership.

Chapter 1

Literature Review

1.1 OVERVIEW

During development, cell adhesion underlies the architecture of discrete tissues and organs in multicellular organisms. The Integrin family of cell adhesion receptors determines such tissue complexity by mediating cell-matrix and to a smaller degree cell-cell interactions. Investigations in tissue culture have demonstrated that Integrins contribute to many aspects of cellular behavior including adhesion, polarity, motility, proliferation, survival, and differentiation; whereas genetic studies, ranging from nematodes and insects to vertebrates, have specified the role of Integrins in maintaining proper tissue function and integrity during development and pathology. In humans, loss of Integrins leads to separation between epidermis and dermis, causing epidermolysis bullosa. In flies, loss of Integrins causes separation of the two epithelial layers that form the wing (Bökel and Brown, 2002; Devenport et al., 2007). Moreover, the attachment of muscle ends to the body wall requires Integrins (Mayer et al, 1997; Brown, 1994; Bloor and Brown, 1998), as shown by muscle detachment defects in the absence of Integrins.

The contractile apparatus of skeletal muscles provides a valuable system for studying the basic principles of Integrin-mediated adhesion. Skeletal muscles are composed of repetitive ultrastructural force-producing units called sarcomeres. Arrays of sarcomeres are then connected to the surrounding extracellular matrix through specialized sarcolemmal linkages. The molecular composition of these sarcolemmal networks, known also as Integrin-adhesion complexes, has been largely investigated in model organisms by immunofluorescence colocalization, coimmunoprecipitation, and *in vitro* binding assays. The functional significance of their localization has been uncovered in part by gene targeting analyses. They are envisaged as a means of transferring signals from the outside into the cell as well as maintaining the structural integrity of skeletal muscle upon repeated contraction.

The following literature review begins with a summary on the establishment of Integrin-adhesions in tissue culture. It then highlights the combined roles of cytoarchitecture and membrane linkages in force generation/transmission as well as in integration of signals needed to regulate a series of cellular behaviors. I also discuss the molecular components of the striated muscle cytoarchitecture and Integrin-linked adhesions with respect to their interactions and functions in *Drosophila* as well as in other vertebrate systems.

1.2 Assembly and dynamics of Integrin adhesions

1.2.1 Focal adhesion assembly

The regulatory effects of matrix-cell adhesion on cell behavior are established through interaction of the transmembrane Integrin receptors with the ECM components on the outside and protein complexes that connect Actin filaments to the Integrin tail on the inside (Burridge and Chrzanowska-Wodnicka, 1996). In culture cells, these tight Integrin adhesions known as focal adhesions change their dynamics in response to externally applied forces or contractile activity of the acto-myosin network within the cell (Reveline et al., 2001; Miyamoto et al., 1995a).

Maturation of focal contacts into focal adhesions correlates with Myosin activation through RhoGTPase-mediated light chain phosphorylation and with Actin filament alignment promoted by the bundling action of activated Myosin (Nobes and Hall, 1995; Chrzanowska-Wodnicka and Burridge, 1996). The assembled contractile machinery generates tension at Integrin adhesions, ultimately leading to homotypic oligomerization of Integrin transmembrane domain subunits (Li et al., 2003). Such Integrin clustering intensifies Integrin-ligand interactions followed by colonization of these Integrin adhesive contacts

with additional components (Balaban et al., 2001; Riveline et al., 2001). For instance, the signalling molecule FAK and the Actin-binding protein Vinculin are found to translocate to Integrin adhesions following Integrin clustering (Kornberg et al., 1991, Perez-Moreno et al., 1998; Balaban et al., 2001).

Alternatively, the recruitment of adhesion proteins may establish novel intermolecular interactions poised to regulate the Actin cytoskeleton reorganization (Delon and Brown, 2007). Indeed, tension-induced relocation of Zyxin from focal adhesions to stress fibres reinforces them through recruitment of the Actin-polymerization factor Ena/VASP by Zyxin (Yoshigi et al., 2005; Delon and Brown, 2007). Moreover, PKC relays the Integrin signals to the Actin cytoskeleton by phosphorylating WASP-interacting protein (WIP), leading to WASP-mediated activation of Arp2/3 Actin nucleating complex (Soriani et al., 2006). Collectively, these data substantiate the function of Integrin adhesions on the bidirectional transmission of mechanical forces across the plasma membrane.

Integrin binding to ligand establishes initial contacts with the cytoskeleton. Subsequently, the acto-myosin contractile forces exerted against these early cell-matrix interactions feeds back to strengthen such adhesions through Integrin clustering and consecutive recruitment of additional adhesion components, comprised of structural and signalling molecules. The signals generated in response to adhesion permit the cell to orchestrate several events varying from cytoskeleton organization to gene transcription. The translation of mechanical forces into chemical signalling that guides cell migration, matrix assembly, and tissue organization constitute the mechanism of mechanotransduction (Katsumi et al., 2004).

1.3 Muscle cytoarchitecture and mechanisms of contraction

Muscle contraction is accomplished through spatial organization of the cytosk-

eleton into repetitive contractile units of sarcomeres (Clark et al., 2002). This structure has been defined as a dynamic network of filament systems capable of generating force, of anchoring proteins capable of tethering such filament systems, and of signalling molecules capable of controlling many aspects of the striated muscle function (Boateng and Goldspink, 2008).

The following sections highlight the basis of striated muscle organization and function through a comprehensive review on sacomeric components.

1.3.1 Sarcomere structure

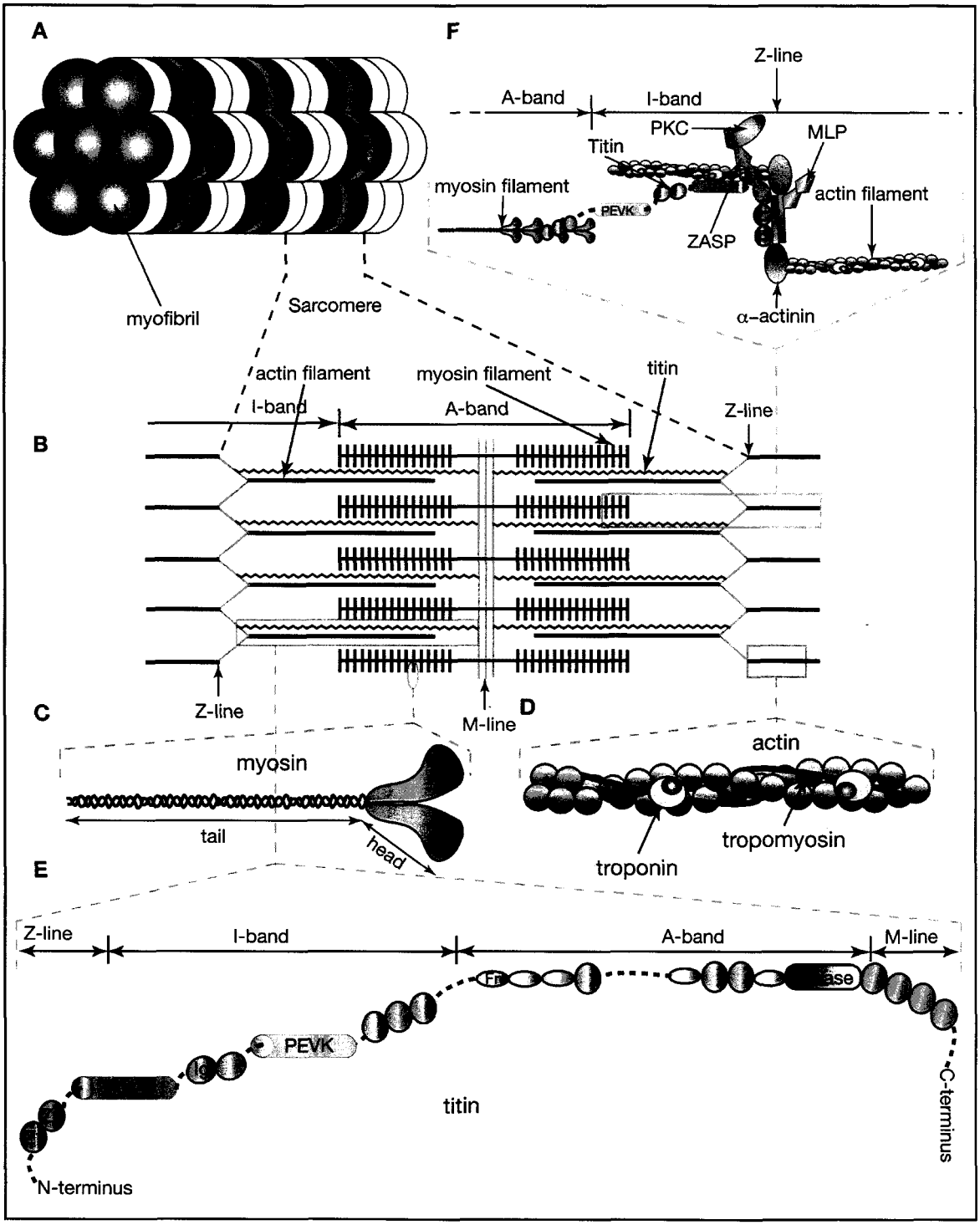
A sarcomere is defined as the segment between two neighbouring Z-lines (Clark et al., 2002; Boateng and Goldspink, 2008). The Actin filaments are anchored at the Z-lines via their barbed ends and run from I-band through the A-band to the H-band. Overlapping with the thin filaments at the A-band, the muscle specific Myosin thick filaments are cross-connected by a central structure known as the M-line, a Z-line analog (Figure 1.3.1). While the Z-line and the M-line arrange the sarcomere in the transverse plane, a third structural protein Titin connects these two components in the longitudinal plane (Boateng and Goldspink, 2008). Thus the sarcomere represents the smallest force-producing unit in a myofibril.

1.3.2 Sarcomere assembly (sarcomerogenesis)

Vast advancements have been made in understanding the mechanism underlying the organization of sarcomeres into highly ordered contractile units through studies conducted in primary myocytes cultures deriving mainly from chick and mouse embryonic muscles (Ojima et al., 1999; Dhume et al, 2006; Quach and Rando, 2006). Such investigations have demonstrated that sarco-

Figure 1.3.1: Structural organization of the sarcomere in striated muscle

(A) Striated muscle fiber comprising several myofibres. (B) Myofibres arranged into unit measured from two consecutive Z-lines. Schematic representation of the alignment of sarcomeric filaments into band and line structures (A-band, I-band, M-line, and Z-line). Actin filaments, which span the I-band and overlap with Myosin filaments in the A-band, are connected to the "zigzag" Z-line structure together with Myosin filaments by Titin filaments. Myosin and Titin filaments are cross-linked by M-band proteins. (C) Section of Actin filament, containing two helices connected by Tropomyosin molecules each containing one Troponin complex. (D) Diagram of single Myosin II protein with tail and heavy chain head regions. (E) Structure of Titin filament comprised of Ig-like domains as well as the unique PEVK sequence spanning the I-band region, Fn-like domains extending over the A-band region, and kinase domain positioned within the M-line region. (F) Diagram of Z-line components: an α-Actinin dimer cross-linking the Actin filament and individual Titin molecules at Z-line; Titin, α-Actinin, and ZASP forming a ternary complex at the Z-line (Au et al, 2004); LIM3 domain of ZASP binding PKC (Zhou et al., 1999), and MLP interacting with α-Actinin.


A
F
Z-line
A-band
I-band
PKC
MLP
Titin
myosin filament
PEVK
ZASP
actin filament
α–actinin
myofibril
Sarcomere
actin filament
myosin filament
titin
I-band
A-band
Z-line
B
Z-line
M-line
C
D
myosin
actin
tail
head
tropomyosin
troponin
E
Z-line
I-band
A-band
M-line
PEVK
titin
N-terminus
C-terminus

mere assembly arises from a stepwise program that initiates at the basal surface of the sarcolemma (Dabiri et al., 1997; Rhee et al., 1994) with subsequent progression of newly formed sarcomere precursors to mature sarcomeres. Tethering of such precursors to sarcolemmal linkages is the first major task of sarcomerogenesis, as these connections will establish positional cues required for recruitment of the elements necessary to form a presarcomere. The transition of Z-line ancestors to fully developed cross-striations is accomplished through sequential addition of the structural components at different cell compartments, with the immature sarcomeres organized in the cell periphery and the most differentiated ones situated around the perinuclear region (Boateng and Goldspink, 2008).

Such series of events have been described in part by two models of sarcomerogenesis. The first proposes that Z-line precursors, comprised of α-Actinin and Actin filaments, assemble in a region separate from where the components of Myosin filaments gather (Holtzer et al., 1997; Schultheiss et al., 1990). Alternatively, the other model hypothesizes the simultaneous assembly of α-Actinin, Actin filaments, and nonmuscle Myosin-II filaments at the plasma membrane followed by addition of Titin that connects the thin and thick filament arrays and concomitant replacement of non-muscle Myosin-II by muscle Myosin-II filaments (Sanger et al., 2002). Although these two models are distinct, they unanimously emphasize the role of structural proteins in the proper assembly. Such assemblies are then released from the membrane attachments and fuse laterally with other developing sarcomeric structures to form mature sarcomeres.

1.3.3 Sarcomere maturation

In each mature sarcomere, the contractile force produced by the interaction of the Myosin head with the Actin filaments is transferred to both ends of the sar-

comere at the Z-lines. They mechanically connect the adjacent sarcomere units in sequence, thus establishing the repeating pattern appearance along the length of muscle. Next, the muscle fibres bundle together by keeping the sarcomeres precisely aligned in parallel longitudinally (Boateng and Goldspink, 2008). Then, the entire structure is anchored to the sarcolemma via specialized linkages that transmit forces across the sarcolemma to the extracellular matrix (Clark et al, 2002).

1.4 Molecular components of Z-line: the cytoskeletal anchor and transduction center

The Z-line that defines the lateral boundary of a single sarcomere unit constitutes the backbone for the insertion of Actin and Titin filaments (Clark et al., 2002; Frank et al., 2006) and the docking site for localization of signal transduction proteins (Lange et al., 2006). Molecularly, this sarcomeric borderline represents a multifunctional protein complex center that not only translates the strength and dynamic properties of the filamentous arrays into coordinated and efficient mechanical force but also activates intracellular signals leading to changes in gene transcription, cytoskeletal organization, and in protein-protein interactions. The following sections provide a general overview on contributions of Z-line structural and signalling proteins in coordinating the structural integrity along with the dynamics of sensing and signalling.

1.4.1 Structural components

α-Actinin

α-Actinin, which denotes the best characterized Z-line component (Robson et

al., 1970), is a member of the spectrin superfamily comprised of an amino-terminal Actin-binding domain, a central rod domain encompassing four spectrin domains, and a carboxyl terminal calmodulin-like domain (Djinovic-Carugo et al., 1999). The interaction between the rod domains of α-Actinin monomers establishes antiparallel dimers that are capable of cross-linking Actin and Titin filaments from adjacent sarcomeres. Although α-Actinin is a prominent component of the Z-lines and myotendinous junctions, results of molecular genetic studies in *Drosophila* suggest that α-Actinin may contribute to the stability of the sarcomere, rather than development of its architecture (Vigoreaux, 1994). Null mutations in the *Drosophila* α-Actinin gene are lethal (Fyrberg et al., 1990); however, mutants survive into larval development and retain some capacity to contract, albeit poorly (Fyrberg et al. 1998). They exhibit muscle attachment defects and sarcomeric abnormalities, but the organization of Actin into a striated pattern is fairly unaffected in such mutants (Dubreuil and Wang, 2000).

Analyses in live cultured myocytes implicate α-Actinin in the formation of the earliest sarcomere precursors at the cell membrane (Terai et al. 1989; Lu et al., 1992). Tracking the incorporation of GFP-tagged α-Actinin into the sarcomeric structures has shown that the fluorescently labeled protein along with Actin filaments cross-linked by α-Actinin emerges in small aggregates at the sarcolemma (Lu et al., 1992; Dabiri et al., 1997; Boateng and Goldspink, 2008). Disruption of the C-terminus of α-Actinin causes fragmentation of the sarcomeres and detachment of muscle fibers from the substrates (Schultheiss et al. 1992). Moreover, the insertion of nascent sarcomeres into sarcolemmal adhesion complexes invariably involves α-Actinin (Lu et al., 1992). Thus, α-Actinin portrays a structural scaffold required for organization of Actin filaments into cross-linked arrays and for their attachment to the membrane.

ALP-Enigma family of PDZ/LIM domains

Another important Z-line component is the ALP-Enigma family of cytoskeletal proteins, defined by a single N-terminal PDZ domain and one to three C-terminal LIM domains (Clark et al, 2002). Initially, thought to be exclusively required for Z-line maintenance, recent evidence, which will be discussed further in Chapter 2, points to a larger role of ALP-Enigma family in sarcomere organization.

In vertebrates, ALP was first identified as a binding partner of α-Actinin (Xia et al., 1997; Pomies et al., 1999) in intercalated discs (myotendinous junction homologues) and in Z-lines of cardiac muscle cells (Pashmforoush et al., 2001). Yet, the sarcomeric localization of ALP seems to not require α-Actinin binding (Henderson et al., 2003). *In vitro* investigations have mapped two interaction sites between human ALP and α-Actinin (Klaavuniemi et al., 2004), where the ALP PDZ domain is responsible for binding to the C-terminal of α-Actinin and the ZM motif is required for ALP interaction with the rod region of α-Actinin. The functional significance of such interaction is supported by finding that overexpression of ALP on rat neonatal cardiomyocytes potentiates sarcomere assembly. Moreover, addition of ALP to the α-Actinin-Actin mixture enhances significantly their co-sedimentation *in vitro* (Pashmforoush et al., 2001). Thus, a plausible function of ALP may be the cytoarchitectural organization by controlling α-Actinin-mediated cross-linking of the Actin filaments (Klaavuniemi et al., 2004). This hypothesis is also supported by *in vivo* observation in mice with target deletion of ALP that develop cardiomyopathy (Pashmforoush et al., 2001).

The Enigma subfamily members have been largely described in vertebrates to interact with a network of both structural and signalling components creating a complex involved in stabilization of Z-lines and signalling in response to contraction (Zhou et al., 1999; Kuroda et al., 1996;

Dyson et al., 2001; Guy et al., 1999). A yeast two-hybrid screen identified Enigma as the binding partner for receptor tyrosine kinase Ret (Wu et al., 1996). Such interaction, which is established via recognition of an unphosphorylated tyrosine-based motif of Ret by the second of three LIM domains of Enigma, seems to be required for Ret-mediated mitogenic signalling. Furthermore, the PDZ domain of Enigma binds to the C-terminal sequences of skeletal muscle β-Tropomyosin, which is a component of Actin filaments (Dyson et al., 2001; Guy et al., 1999). Together these observations support a model by which Enigma might link the LIM domain-binding partners to Actin filaments and Z-line.

ZASP/Cypher, the best characterized of the PDZ/LIM domain family of proteins in striated muscles, in vertebrates exists in six alternatively spliced isoforms, establishing two classes, cardiac and skeletal-muscle specific isoforms. The long isoforms contain three LIM domains while the short ones carry only one (Huang et al., 2003; Vatta et al., 2003; Arimura et al., 2004; Frank et al., 2006). The muscle-specific isoforms localize at Z-lines. Studies describing the molecular interactions of ZASP/Cypher have shown that the PDZ domain is responsible for α-Actinin binding, whereas the LIM domains are required for PKC binding (Zhou et al., 1999: Kuroda et al., 1996). Inactivation of ZASP/Cypher function in mice results in severe skeletal muscle defects with disrupted Z-lines and early postnatal death due to respiratory failure and cardiomyopathy (Zhou et al., 2001), thus suggesting for a ZASP/Cypher role in maintenaning the Z-line integrity during initial muscle contractions.

In *Drosophila*, there is a single gene representing exclusively the entire ALP-Enigma family of proteins. Its function is described thoroughly in the following chapters.

Muscle LIM Protein (MLP)

The MLP, a striated muscle-specific cysteine-rich protein has been also demonstrated to localize at the periphery of the Z-lines and at the sites of myotendinous junctions (Stronach, 1996; Clark et al., 2007). *Drosophila mlp* encodes two MLP transcripts: Mlp60 and Mlp84, both detected in striated muscle at different developmental stages (Stronach et al, 1996). In contrast to the diffuse appearance of Mlp60 protein observed throughout muscle fibers, Mlp84 concentrates at myotendinous junctions early on correlating with the development of functional myotendinous junctions and visible muscle contractions (Stronach et al, 1996). Interestingly, despite the early localization at myotendinous junction, Mlp84B depletion results in late muscle defects observed just before pupation (Clark et al, 2007). However, the onset and severity of phenotypes is enhanced when the activity of D-Titin, another Z-line structural component, is reduced in the *mlp84B* background, indicating that Mlp84B maintains the sarcomeric structural integrity in cooperation with D-Titin (Clark et al, 2007). Moreover, Mlp84B is also essential for normal cardiac function (Mary et al., 2008). Mlp84B-deficient and heart-specific *mlp84*B-RNAi-expressing flies exhibit diastolic interval prolongation, heart rhythm abnormalities and a reduced lifespan, while showing no obvious structural phenotype. Similarly, MLP-deficient mice develop severe dilated cardiomyopathy a few weeks after birth (Arber et al., 1997). Thus, within the myocardium, MLP appears to function as a sensor of the stretch-induced mechanical forces rather than a sarcomeric organizer.

MLPs have also been implicated in communication with the nuclear compartment (Arber et al, 1994). In contrast to what has been reported for vertebrate MLP/CRP3 distribution in tissue-culture cells, *Drosophila* Mlp(s) display some transient nuclear localization, but with no essential nuclear function (Stronach et al, 1996; Clark et al., 2007). Indeed, a Mlp84B transgene

carrying a nuclear localization signal (NLS) failes to rescue the pupal lethality in the *mlp84B* mutants (Clark et al., 2007). Although MLP may shuttle between the cytoplasm and nucleus, perhaps sending information concerning the developmental state of muscle, this function appears to be dispensable in *Drosophila* (Stronach et al, 1996).

LIM domain and SH3 domain protein (LASP)

The nebulin family of Actin-binding proteins, consisting of Lasp-1, Lasp-2, and N-RAP, plays important roles in Actin cytoskeleton organization. They function as cross-linking proteins binding to Actin via the nebulin repeats and to α-Actinin though the C-terminal Src homology 3 (SH3) domain (Lin et al., 2004; Zieseniss et al., 2008).

The exact role Lasp plays in the assembly of sarcomeres has recently been investigated in cultured cardiomyocytes. Immunolocalization places Lasp-2 in Z-lines associated with α-Actinin in the developing sarcomeres at the periphery of the cell (Zieseniss et al., 2008). Furthermore, disruption of its C-terminus inhibits Lasp-2 targeting to the Z-lines, although does not interfere with the sarcomere assembly. It appears that Lasp-2 serves as a reserve molecule poised to catalyze organization of Actin filament into bundles and stabilizing α-Actinin–mediated cross-linking of the Actin filaments. *Drosophila* Lasp, the single member of the nebulin family in flies also localizes to Z-lines (Zhou and Schöck, unpublished data).

Nonmuscle Myosin-II

A direct contribution of nonmuscle Myosin-II in sarcomere assembly has been proposed as a result of its presence in developing sarcomeres (Rhee et al., 94; Du et al., 2003). However, the presarcomere hypothesis seems not to be

applicable in chicken heart *in situ* (Ehler et al., 1999), an interpretation that is also supported by finding that homozygous null mice for nonmuscle Myosin-II are able to assemble sarcomeres (Tullio et al., 1997).

In flies, nonmuscle Myosin-II encoded by the *zipper* gene, is enriched at myotendinous junctions of the developing embryo, colocalizing with αPS2 Integrin, and in Z-lines soon after embryos emerge into larvae (Bloor and Keihart, 2001). Disruption of nonmuscle Myosin-II function leads to embryonic lethality and perturbs sarcomere assembly with Actin filaments unorganized along the muscle length rather than structured into discrete bands (Bloor and Keihart, 2000). Interestingly, although the absence of striated patterning observed in *Drosophila* embryos emphasizes its role in sarcomere assembly, evidence argues against nonmuscle Myosin-II mediating Integrin connections to the cytoskeleton. Indeed, in *zip* mutants the Actin filaments remain attached to the membrane, indicating that the primary function of nonmuscle Myosin-II is to support the assembly of the cytoskeleton.

Titin

Finally, Titin, the largest known protein and the third most abundant filament system of striated muscles after Actin and Myosin (Wang et al., 1979), is an additional sarcomeric structural component that is anchored via its N-terminus to the Z-line and the C-terminus to the M-line (Fürst et al., 1988).

Several lines of evidence indicate that the N-terminal region of Titin contributes to maintaining Z-line integrity in response to contraction (Gregorio et al., 1998; Ayoob et al., 2000). Expression of GFP coupled N-terminal Titin repeats, termed Z-repeats, in cultured cardiomyocytes demonstrates their crucial role in Titin targeting to Z-line. Moreover, overexpression of such repeats produce a dominant-negative effect associated with sarcomere disassembly (Ayoob et al., 2000). Titin might also influence sarcomere organi-

zation in cooperation with other Z-line components. Indeed, a direct interaction of Titin Z-repeats with α-Actinin has been reported (Sorimachi et al., 1997).

The I-band segmental elasticity of the sarcomere correlates closely with the extension of Titin's PEVK molecular motif and straightening of Ig/Fn3 domains (Wang et al., 2001). Interestingly, it is also proposed that the PEVK domain influences not only the extensibility of the sarcomere, but also the contractile properties. Consistent with this hypothesis, the PEVK motif associates with Actin filaments *in vitro* and this interaction seems to slow down the sliding velocity of Actin filaments over Myosin (Linke et al., 2002), thus opposing shortening during contraction (Kulke et al., 2001).

In vertebrates, the M-line Titin domain that contains a catalytically active serine/threonine (Ser/Thre)-kinase domain (Labeit and Kolmerer, 1995) appears to be involved in sarcomerogenesis. Conditional deletions of the kinase region result in sarcomere disassembly (Weinert et al., 2006; Musa et al., 2006). Moreover, removal of the entire M-region of Titin is frequently associated with diffuse distribution of muscle Myosin as well as with disrupted Z-lines, pointing to a role for Titin in the assembly of sarcomeres.

Surprisingly, *Drosophila* Titin (D-Titin) that contains most of the vertebrate Titin domains lacks the kinase domain (Zhang et al, 2000), suggesting that in flies, Titin has simply a structural function. Indeed, in the absence of D-Titin, Actin and Myosin fail to organize into a higher-level sarcomeric structure, thereby leading to embryonic lethality (Zhang et al, 2000).

Projectin, a D-Titin-like protein, may provide additional Titin functions in *Drosophila*, since it contains a functional kinase domain that is capable of autophosphorylation *in vitro* (Ayme-Southgate et al., 1991, 1995). A mutation in the kinase domain causes embryonic lethality in homozygotes, while in heterozogotes has no influence on muscle structure but rather on stretch activation in indirect flight muscle (IFM) (Moore et al., 1999). It is intriguingly po-

ssible that phosphorylation of the potential substrates, such as the Myosin light chain and Projectin itself, could affect Myosin filament geometry and/or axial displacement of Myosin head from the Actin filament, leading to an altered Actin-Myosin interaction (Moore et al., 1999). Altogether, these studies describe Titin as crucial sarcomeric component required for stabilization of Z-lines and signaling in response to stretch.

1.4.2 Signalling components

Protein Kinase C (PKC)

The sarcomere is not only the force-generating unit, but also a functional signalling organizing center (Lange et al., 2006; Pyle and Solaro, 2004). Signalling appears to play a role in sarcomere assembly, remodeling, as well as in mechanotransduction. Recruitment of signalling molecules to Z-line components represents the basic mechanism underlying these functions.

The interaction of the PKC family of serine/threonine kinases with Z-line modules appears to be an important link to signalling cascades. Mechanical stimuli induce PKC activation and its subsequent translocation in several cell compartments (Gu and Bishop, 1994). Many lines of evidence indicate that PKC targeting to the sites of its function requires PKC binding to anchoring proteins. The Z-line-associated protein ZASP/Cypher was identified as a possible Z-line anchor of PKC (Zhou et al., 1999; Kuroda et al. 1996). Receptor for activated C kinase (RACK) has also been implicated in PKC anchoring (Robia et al., 2005). In addition, Actin binds and preferentially anchors certain activated PKC isoforms under specific physiological conditions (Prekeris et al., 1998). Following its translocation to the Z-line, PKC is activated. Accordingly, overexpression of a PKC translocation inhibitor induced lethal cardiomyopathy (Chen et al., 1999), while overexpression of a

translocation stimulator led to improved protection against ischemic injury in mice (Dorn et al., 1999).

Once activated, PKC can phophorylate a number of membrane and Z-line anchored proteins. FAK, a primary mediator of Integrin signalling is activated via PKC-mediated phosphorylation (Heidkamp et al., 2003). Increased distribution of activated FAK in Z-lines, costameres and nuclei is also observed following PKC overexpression. In addition, Troponin, Vinculin, and Enigma have been recognized as phosphorylation substrates of PKC in sarcomeres (Kuroda et al., 1996; Kobayashi et al., 2004; Larsson, 2006).

A wealth of information has emerged regarding the indisputable role of PKC in costamere formation and sarcomeric assembly in response to mechanical stretch. In cultured cardiomyocytes, PKC has in fact been localized by immunocytochemical techniques to regions of the cell adjacent to the Z-line or within costameres (Borg et al., 2000; Heidkamp et al., 2003: Samarel, 2005). In addition, overexpression of constitutively active PKC in cultured neonatal cardiomyocytes increases focal adhesion formation (Strait et al., 2001). Finally, returning of the sarcomere to its optimal length after mechanical stretch requires addition of new sarcomeres. This sarcomerogenesis necessitates *de novo* protein synthesis of PKC (Mansour et al., 2004). Collectively, these findings suggest that translocation of PKC to costameric precursors in response to mechanical force may be required to regulate both costamerogenesis and sarcomerogenesis.

There are 6 PKC genes in *Drosophila* but their function in muscle development has not yet been analyzed (Shieh et al, 2002).

1.4.3 Conclusion

In summary, Z-lines represent an interface between the mechanical sensing and signalling. However, the molecular nature and the mechanisms that coor-

dinate their activities still remain elusive. Future work directed towards understanding their molecular specificity and regulation will enhance our understanding of how the force sensing is properly coupled with signalling for generation of meaningful contractions.

1.5 Membrane linkages to the cytoskeleton

The transmission of force to the external matrix requires tethering of the cytoarchitecture to the sarcolemma (Clark et al, 2002). Striated muscles have developed specialized membrane linkages that cooperate in maintaining the structural integrity of muscles during repeated contractions (Ross and Borg, 2001). They are considered as striated muscle specific elaborations of focal adhesions in cell culture (Ervasti, 2003) and are ideally suited as the point of conversion of the physical forces into intracellular chemical signals (Katsumi et al., 2004).

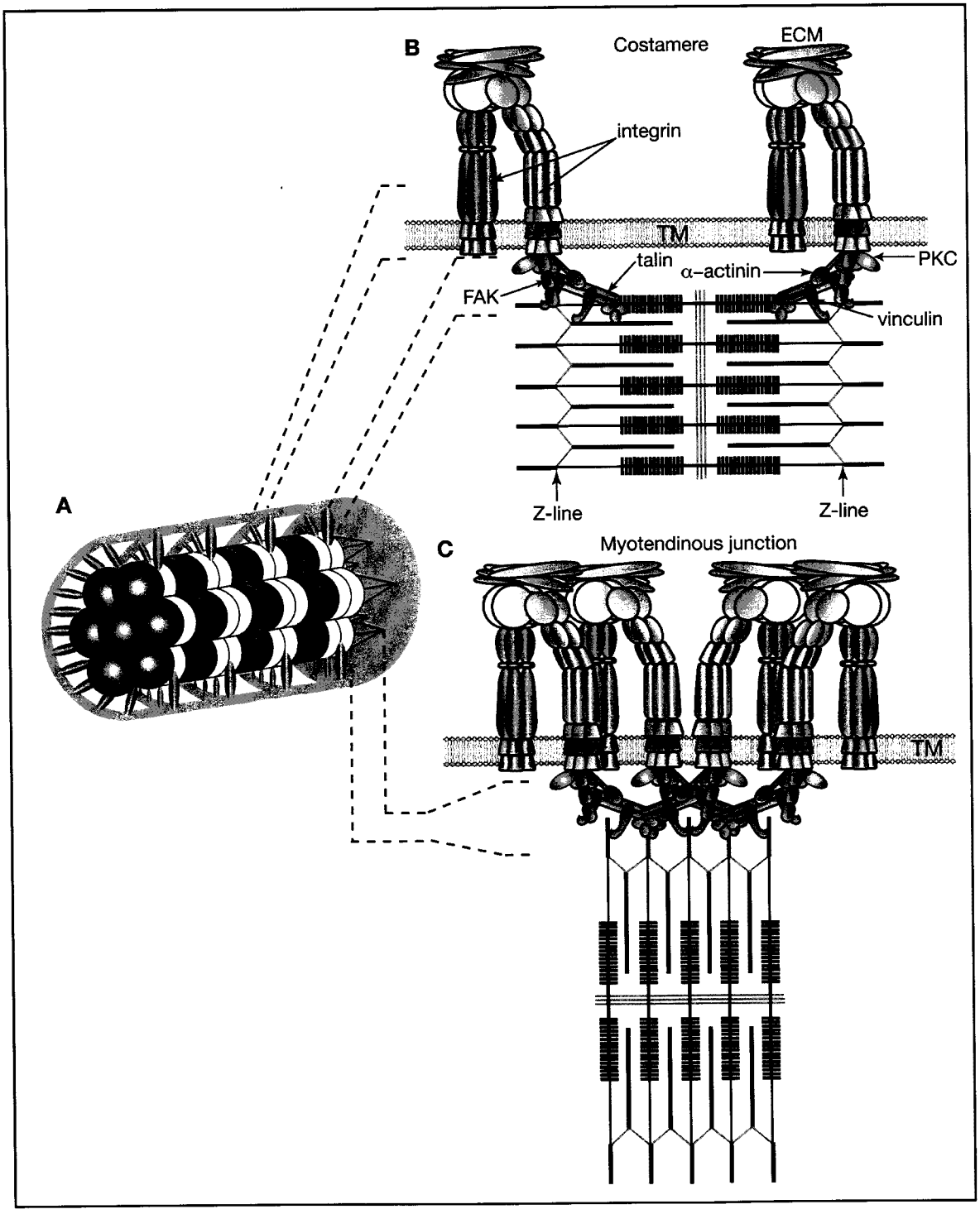
The assembly of each type of membrane linkages is described in more detail below, with an emphasis on their molecular components.

1.5.1 Costamere assembly in cultured skeletal muscles

The term "costamere" was first used to describe the Vinculin-containing, rib-like structure (named due to their morphological appearance in perpendicular view; Latin costa, rib; Greek meros, part) that encircles the muscle at the Z- and M-line perpendicular to its long axis (Pardo et al., 1983b). Costameres comprise peripheral and subsarcolemmal proteins that ensure the mechanical coupling between the sarcomere and sarcolemma (Frank et al., 2006) (Figure 1.5.1 B). Therefore, costameres transduce contractile forces from the Z-line to the membrane and maintain the structural integrity of the membrane linkages during contraction.

Figure 1.5.1: A schematic representation of cytoskeletal linkage complexes that connect the sarcomere to the sarcolemma.

The diagram (A) illustrates costameres as circumferential structures that couple the Z-lines to the sarcolemma and myotendinous junctions that attach the terminal Z-lines to the sarcolemma. Both linkage systems form Integrin-based adhesions (B and C) that link the ECM to the sarcomeric Actin cytoskeleton via association with Talin, α-Actinin, Vinculin and signal through interaction with FAK and PKC.


B
Costamere
ECM
integrin
TM
talin
α–actinin
PKC
FAK
vinculin
Z-line
Z-line
A
C
Myotendinous junction
TM

In cultured skeletal muscles cells costamerogenesis appears to proceed contemporaneously with sarcomerogenesis (Quach and Rando, 2006). The sarcomere represents the scaffolding structure for the progressive costamere alignment and maturation. Transition of sarcomere precursors into mature striations correlates with initiation of contractile activity that feeds back to establish clustering of costameric proteins into a transverse pattern over striations. Conversely, the costameric complexes signal to remodel the cytoskeleton and to cause further maturation of the sarcomere (Quach and Rando, 2006).

1.5.2 Myotendinous junction assembly in *Drosophila*

The myotendinous junction constitutes the termination point of muscle fibers (Clark et al., 2002). As in the costamere, the terminal myotendinous junction forms a sophisticated framework of membrane-associated adhesion molecules that anchor the sarcomeric Actin cytoskeleton to the ECM (Reedy and Beall, 1993) (Figure 1.5 C).

In *Drosophila,* the organization of myotendinous junctions relies on specific interactions of muscles with the ECM components and the epidermis (reviewed in Sink, 2006). The ECM connects muscles to the epidermis through a discrete thick structure known as tendon matrix (Brown, 2000). During embryonic development, muscles and epidermal cells develop from distinct germ layers, the mesoderm and ectoderm, respectively (reviewed in Sink, 2006). Muscle fibers enlarge through fusion and their leading edges extend towards the epidermal cells (Bate, 1990). Unlike in vertebrates, Integrins do not contribute to myoblast fusion in *Drosophila* (Schwander et al., 2003; Roote and Zusman, 1995; Gotwals et al., 1994). Exploration by the muscle fibers ceases when they contact a target epidermal attachment cell.

Once contacted, bidirectional communication occurs (Becker et al.,

1997). The muscles send signals to support the differentiation of epidermal attachment cells into specialized cells known as "tendon cells". The transmission of the signal requires Integrins, but this is due to their role in keeping the muscle in contact with the tendon cell, rather than being part of the signalling pathway (reviewed in Sink, 2006; Martin-Bermudo, 2000).

Furthermore, the tendon matrix builds up via ECM secretion at the sites where muscles make contact with the tendon cells and another muscle from the neighboring segment (Volk, 1999). Tiggrin and Laminin were identified as the major Integrin ligands at such junctions (Fogerty et al., 1994; Bunch et al., 1998; Gotwals et al., 1994). An additional ECM protein, Thrombospondin that is produced by tendon cells, was also discovered to work similarly in establishment of Integrin-ECM junction (Subramanian et al., 2007). Although it is not clear what triggers their initial deposition at the myotendinous junction, both muscle and tendon cell are shown to secrete such ECM components following the association between the muscle and the tendon cell (Fogerty et al., 1994; Bunch et al., 1998; Martin-Bermudo and Brown, 2000; Subramanian et al., 2007). Noteworthy, the absence of any of these ECM components causes the weakening of muscle attachment sites; although their defects do not fully mimic the Integrin mutant phenotype, suggesting overlapping functions (reviewed in Sink, 2006).

Following Integrin binding to their ligands, the migratory filopodia structures at the muscle leading edge arrest their growth to give rise to stable adhesions, referred to as myotendinous junctions (Frommer et al., 1996). To support such transition a number of structurally and functionally diverse proteins, some of which are discussed in further detail in later sections, commence to concentrate at the cytoplasmic face of the sarcolemma, establishing the Integrin-associated adhesions (Delon and Brown, 2007).

As the muscles start forming specialized attachments and the cytoskeleton becomes organized into sarcomeres, the embryonic developm-

ent of muscles terminates. Afterwards, the muscles are fully contractile and allow the embryo to hatch. Thus, the newly hatched larvae display several sophisticated structural cytoskeletal and sarcolemmal organizations required to maintain muscle integrity upon contraction: Z-lines that connect the Actin filaments from adjacent sarcomeres, costameres that mechanically couple the sarcomere to the sarcolemma via their interaction with the Z-line, and myotendinous junctions that anchor the muscle ends to the tendon matrix.

1.6 Molecular components of adhesion complexes

Cell adhesion involves the interplay between Integrins and the adhesion complexes comprised of Integrin-associated multidomain proteins that function as direct Integrin-Actin linkers, of adaptors that interact with the Actin-bound or Integrin-bound components all together forming the physical structure of the adhesion site, and of enzymes that modify such interactions and trigger signalling cascades (Geiger et al., 2001; Delon and Brown, 2008). Despite the vast molecular complexity, each of them has a unique function on Integrin-mediated adhesion.

1.6.1 Integrin

The Integrin family of heterodimeric transmembrane receptors represents the nodal point that links the Actin cytoskeleton to the ECM. While their globular extracellular domain contributes to binding the ECM, their short cytoplasmic tail organizes the Actin cytoskeleton through Integrin-associated protein complexes (Liu et al., 2000), whose molecular composition will be discussed in the following sections. As such, Integrins are positioned to transmit the externally and internally developed forces across the plasma membrane as well as to convert these forces into chemical signals that ultimately alter many

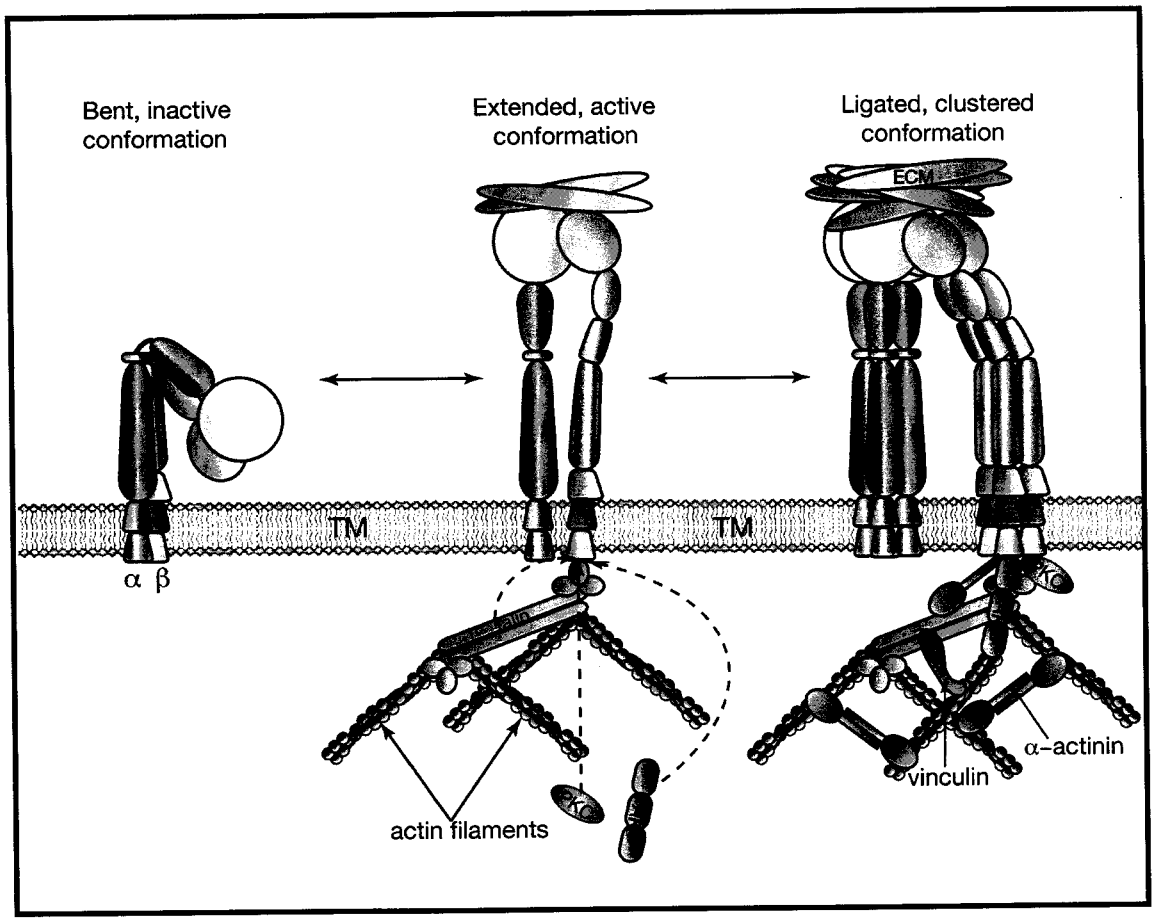
cellular behaviours (Katsumi et al., 2004; Delon and Brown, 2007).

The bidirectional aspect of Integrins controls Integrin function per se. Integrin adhesion to the ECM is accomplished through the action of intracellular signals (possibly generated by other cell receptors, such as G-protein coupled receptors) and mechanical forces that increase similarly the affinity of the receptor for ligand (Calderwood, 2004; Katsumi et al., 2005). Force-induced conformational changes in Integrin structure constitute the basis of mechanosensing.

In their resting state prior to contact with the ECM, Integrin heterodimers, composed of an α and a β subunit, are mostly in an inactive conformation, with their extracellular domain in a bent conformation and the two very short cytoplasmic domains held together by a transmembrane non-covalent salt bridge (Luo et al., 2007). Cellular stimulation induces disruption of the clasp between α and β cytoplasmic tails, followed by a conformational change in the extracellular domains from a bent to a more extended form for a high affinity ligand binding (Figure 1.6.1) (Liddington and Ginsberg, 2002). During this transition, the Integrin cytoplasmic tail also exposes the conserved regions for its binding partners that are required to establish contact with the Actin cytoskeleton. Following Actin organization, the cytoskeleton generates forces that are transmitted to Integrins, promoting their clustering (Figure 1.6.1). In turn, receptor aggregation increases Integrin avidity for ligand, thus strengthening Integrin adhesion (Katsumi et al., 2005; Delon and Brown, 2007). This model constitutes the mechanism of Integrin activation and clustering (Figure 1.6.1).

Functional analysis of Integrins in *Drosophila* has demonstrated their critical role in stable adhesion within muscles (reviewed in Brown et al., 2000). Integrins arise through combinations of two β subunits (termed βPS and βv) and five α subunits (termed αPS1 through 5). The attachment of embryonic muscles to the tendon matrix is accompanied by strong complem-

Figure 1.6.1: An illustration of Integrin activation model. See text for details


Bent, inactive conformation
Extended, active conformation
Ligated, clustered conformation
ECM
TM
TM
α β
talin
talin
actin filaments
vinculin
α–actinin

entary expression of two Integrins, αPS1βPS and αPS2βPS, at the sites of attachment (Bogaert et al., 1987; Leptin et al., 1989). αPS1 localizes at the ends of epidermal tendon cells, while αPS2 is restricted to the muscle ends (Fernandes et al., 1996). During embryogenesis, Integrins are not required to form the initial muscle-tendon contacts, which are presumably mediated by cell-cell adhesion molecules such as Cadherins, but to maintain the stable adhesions during muscle contractions (Martin-Bermudo and Brown, 2000; Leptin et al., 1989). Indeed, mutations in *myospheroid (mys)* as well as in *inflated (if)*, which encode βPS and αPS2, respectively, cause detachment of muscles from the tendon matrix soon after contraction begins (Wright, 1960; Devenport and Brown, 2004).

It is quite likely that sarcomerogenesis and costamerogenesis depends on Integrins. Immunostaining of Integrins in cultured mouse cardiomyocytes demonstrates their distribution in costameric structures (Imanaka-Yoshida et al., 1999). An early study with *Drosophila* myoblast cells in culture lacking βPS or αPS2 Integrin has also pointed to a role for Integrins in sarcomere assembly (Volk et al., 1990). Later work showed that blocking αPS2 Integrins inhibits muscle attachment to the epidermis and sarcomeric organization as well (Bloor and Brown, 1998). From these investigations, integrins appear to establish the cytoskeletal link to the sarcolemma.

A large body of evidence implicates Integrins in sensing mechanical forces (Katsumi et al., 2004). Analysis of mechanosensing *in vitro* generally relies on the culturing of cells on matrix coated silicone membranes (Katsumi et al., 2005). Mechanical force modifies affinity of Integrins for the ECM ligand (Katsumi et al., 2005) and these alterations can easily be observed by phase contrast microscopy as wrinkles in silicone rubber substrates (Harris et al., 1980). In addition, Integrins localized at costameres, mediate assembly of their ECM ligands in a costameric-like pattern (Imanaka-Yoshida et al., 1999). Collectively, these data indicate that mechanical stretch-induced conformatio-

nal activation of Integrins with subsequent clustering potentiates the cell-matrix adhesion (Schwartz and Ingber, 1994; Katsumi et al., 2005).

Other studies demonstrate the unquestionable role of Integrins in mechanotransduction. Integrins are also sites for localization of signalling molecules. Because the cytoplasmic tail of Integrins lacks endogenous catalytic activity, they signal by associating with protein kinases and GTPases such as focal adhesion kinase (FAK), Integrin linked kinase (ILK), and protein kinase C (PKC) (Giancotti and Tarone, 2003). Results obtained in mechanically stressed cultured cells and pressure-overload studies in intact myocardium have revealed rapid activation of downstream Integrin effectors in response to mechanical changes. Of note, FAK is rapidly phosphorylated/activated by mechanical stretch in rat cardiomyocytes (Kovacic-Milivojevic et al, 2001). Moreover, stretch-induced activation of PKC initiates a cascade of growth (hypertrophic) response in cardiomyocytes (Sadoshima and Izumo, 1993). These findings implicate FAK, PKC and Integrin in functioning as a sensory/signalling complex in muscle. Therefore, Integrins represent an interface between sensing and signaling.

1.6.2 Integrin-Actin linkers

Talin

One of the core components of Integrin adhesion complexes is the multidomain protein Talin that provides an essential link between Integrins and the Actin cytoskeleton (Horwitz et al., 1986; Brown et al., 2002). The FERM domain of its globular head region binds to Integrin, Actin, and FAK (Horwitz et al., 1986; Hemmings et al., 1996; Chen et al., 1995), whereas the tail region is responsible for binding Actin and Vinculin (Bass et al., 1999). In addition to its vastly described role as an essential component of focal adhesions, Talin is

also found in myotendinous junctions and costameres (Brown et al., 2002; Belkin et al., 1986; Imanaka-Yoshida et al., 1999). Its recruitment to the Integrin-adhesion complexes is dependent on Integrin activation/clustering (Miyamoto et al., 1995). Functional studies of Talin implicate it in maintaining the membrane linkage to the cytoskeleton in developing muscles. *In Drosophila*, loss of Talin causes a break between the Integrin cytoplasmic tails and the Actin cytoskeleton, but not between Integrins and the ECM (Brown et al., 2002). However, a later work in flies implicates Talin in strengthening the Integrin adhesion to the ECM (Tanentzapf and Brown, 2006). A mutation in the head region of Talin disturbs Integrin adhesion to the ECM, yet it does not disrupt Talin recruitment by Integrin at the myotendinous junctions nor its ability to connect Integrin to the cytoskeleton (Tanentzapf and Brown, 2006). Thus, it seems that the Talin head domain acts exclusively in strengthening the Integrin adhesion to the ECM.

Likewise, the vertebrate *Talin1* gene is required for maintaining Integrin attachment to myotendinous junctions (Conti et al., 2008). Mice with muscle-specific ablation of Talin1 develop progressive myopathy and display detachment of the sarcomeric cytoskeleton from myotendinous junctions. In addition, mechanical measurements on isolated muscles from Talin1-deficient mice show defects in their ability to generate and resist forces during contraction (Conti et al., 2008). Yet, Talin1 is not shown to play a role in sarcomero/costamerogenesis. However, the vertebrate Talin2 seems to contribute in such processes since it is upregulated during the sarcomere assembly (Senetar et al., 2007). In both cases, Talin functions to link Integrin to the sarcomeric cytoskeleton at the costamere and myotendinous junction.

Tensin

Tensin encoded by the *blistery* gene has also been reported as a myotendin-

ous junction component in skeletal muscles. The protein encompasses an N-terminal Actin-binding domain (Lo et al., 1994; Chuang et al., 1995), a *src* homology 2 domain (SH2), by which Tensin recruits proteins to activated receptor tyrosine kinases (Pawson and Nash, 2000), and C-terminal phosphotyrosine-binding domain that interacts with the β Integrin cytoplasmic tail (Gieger et al., 2001). Thus, Tensin appears to mediate the link of Integrins to the Actin cytoskeleton. Surprisingly, in contrast to other Integrin-Actin linkers, the absence of Tensin in *Drosophila* causes no obvious muscle phenotypes (Torgler et al., 2004). Flies are viable but develop wing blisters. Although Tensin appears to strengthen the Integrin link to the cytoskeleton during wing expansion, the lack of muscle defects suggest that Tensin may not contribute to Integrin function in muscles.

1.6.3 Adaptor proteins

Vinculin

Vinculin, an Actin-binding protein, orchestrates the reorganization of the cytoskeleton following the formation of the adhesion complexes (Ziegler et al., 2006). Vinculin is held in an inactive conformation due to an intramolecular interaction between its head and tail regions that masks the binding sites for Talin, α-Actinin, and Actin (Johnson and Craig, 1994; 1995; Bois et al., 2007). Both Talin and α-Actinin activate Vinculin by provoking structural changes that swings out the autoinhibitory head-tail interaction (Bois et al., 2006, 2007). Vinculin recruitment to adhesion sites followed by its activation may promote its attachments to the Actin filaments or stabilize the initial Talin-mediated Integrin linkage to the Actin cytoskeleton.

In *Drosophila*, Vinculin is enriched at the sites of Integrin junctions in developing embryos, thus nominating it as a plausible candidate in regulating

Integrin function in striated muscles (Alatortsev et al., 1997). Yet, deletion of the *vinc* gene results in viable and fertile flies with no obvious skeletal muscle defects (Alatortsev et al., 1997).

Even though many components of the Integrin-adhesion complex are encoded by unique genes in *Drosophila*, their function appears to be in part redundant. Alternatively, many components of Integrin adhesion complexes may function only in very specific circumstances or tissues.

Paxillin

Paxillin serves as a scaffold and adaptor protein binding to Vinculin, FAK, and ILK through a series of N-terminal LDxxLLxxL (LD) motifs (Turner et al., 1990; Scheswohl et al., 2008; Nikolopoulos and Turner, 2001) and to Integrins, tyrosine phosphatases, and serine/threonine kinases via its four C-terminal LIM domains (Schaller et al., 1995; Liu et al., 1999; Turner, 2000). Association of Paxillin with Integrins and phosphorylation of its LIM domains are required for Paxillin localization at Integrin adhesions (Brown et al., 1998, Turner, 2000).

In *Drosophila*, the *paxillin* gene locus transcribes two transcripts (Yagi et al., 2001) that encode a protein homologous to the vertebrate Paxillin (DPxn37) and protein a with only three LIM domains, partly encoded by its own specific exon (PDLP). Both DPxn37 and PDLP are highly expressed at the myotendinous junctions of *Drosophila* embryos, but the biological significance of these localizations has not yet been analyzed.

However, studies in the vertebrate system have elucidated additional roles for Paxillin. Paxillin colocalizes in the sarcomeric Z-lines of neonatal cardiac myocytes with FAK (Kovacic-Milivojevic et al., 2001). Disruption of this interaction leads to sarcomere disassembly.

Wech

The newly discovered *wech* gene, which encodes a multidomain protein comprised of a B-box zinc-finger, a coiled-coil domain (BCC), and a C-terminal NHL domain (Löer et al., 2008), represents another missing link between Talin, βPS Integrin, and the ILK/PINCH complex. In flies, Wech colocalizes with Integrin, Talin and ILK at myotendinous junctions of developing embryos and in mice at Z-lines of adult muscles. Such distributions appear to involve Integrin and Talin but not ILK, suggesting that Wech may function downstream of Talin and upstream of ILK. Of note, co-immunoprecipitation experiments demonstrate that both BCC and NHL domains interact with the head domain of Talin, whereas the BCC domain is essential for binding to kinase domain of ILK (Löer et al., 2008). Moreover, flies deficient for *wech* exhibit phenotypes depicting Actin cytoskeleton detachment from the muscle membrane and similar in severity to Talin-null embryos but stronger than the *ilk* mutants. Together, these data suggest that Wech is a downstream Integrin adaptor that regulates Integrin-mediated adhesion by connecting Integrins to the cytoskeleton through direct interaction with Talin and ILK.

N-RAP

The muscle-specific N-RAP protein that concentrates at myotendinous junctions in skeletal muscle and at intercalated disks in cardiac muscles has been implicated in the initiation of sarcomerogenesis in cultured chick cardiomyocytes. Blocking N-RAP expression by siRNA resultes in impaired organization of striations throughout myocytes, as assessed by α-Actinin distribution with streaks and dots rather than mature striations (Dhume et al., 2006). Interestingly, it has been shown that the nebulin repeats of N-RAP pro-

mote Actin polymerization in vitro (Gonsior et al., 1998) and α-Actinin along with Talin have been identified as N-RAP binding partners (Lu et al., 2003; Luo et al., 1999). Thus, N-RAP may provide a structural link organizing α-Actinin and Actin assembly into nascent sarcomeres by establishing their attachment to the membrane (Carrol et al., 2004).

Particularly Interesting New Cysteine and Histidine-rich protein (PINCH)

Finally, the LIM domain-containing protein PINCH (Clark et al., 2003) represents an additional core component of the Integrin adhesion complexes. Immunostaining of *Drosophila* PINCH, encoded by the *steamer duck* (*stck*) gene, places it at myotendinous junctions in embryonic muscles and demonstrates that such distribution requires Integrins (Clark et al., 2003). Flies mutant for *stck* are lethal as embryos, which display detachment of Actin filaments from the sarcolemma. Interestingly, the PINCH binding partner ILK localized appropriately at the Integrin-rich junctions in such mutants, despite of the observation in mammalian cells suggesting that PINCH, ILK and Parvin proteins are recruited to ECM adhesion sites as pre-assembled protein complexes (Zhang et al., 2002). Mutations that ablate the formation of PINCH, ILK, Parvin ternary complex abolish the ability of each protein to localize to Integrin adhesions (Li et al., 1999; Zhang et al., 2002). Although PINCH role in relation to ILK remains elusive in flies, it could still be envisaged as a molecular scaffold supporting ILK assembly at Integrin junctions.

1.6.4 Signalling proteins

Integrin-Linked Kinase (ILK)

Several studies indicate that ILK is well positioned for a role in mechanotrans-

duction because it is involved in connecting Integrins to the cytoskeleton. Its three ankyrin-rich N-terminal repeats bind to the first LIM domain of PINCH (Tu et al. 1999; Clark et al., 2003), whereas its C-terminal region, comprised of a serine/threonine kinase domain, is required to interact with the cytoplasmic tail of β1 Integrin (Hannigan et al., 1996) and to the Actin-binding protein Parvin (Legate et al., 2006; Chen et al., 2005). Moreover, ILK is enriched at myotendinous junctions in skeletal muscles, where it colocalizes with Integrins (Zervas et al., 2001; Gheyara et al., 2007) and at costameres in cardiac muscles (Bendig et al., 2006). *ilk* deficient mice and flies die as embryos. They exhibit cytoskeleton detachment from the membrane, which in flies occur slightly later than in Integrin mutants (Gheyara et al., 2007; Zervas et al., 2001). In both cases ILK seems to mediate the Integrin link to the cytoskeleton. However, a recent study in zebrafish demonstrates a different role for ILK in adhesion (Postel et al., 2008). Inhibition of ILK expression by morpholino interference demonstrates the necessity of ILK in strengthening the adhesion of Integrins to ECM, thus suggesting for an ILK implication in mechanosensing. Interestingly, this novel role requires the presence of its functional kinase domain (Postel et al., 2008). Mutation on lysine 220 of zebrafish ILK can not rescue the skeletal muscle detachment phenotype. These results are in agreement with the previous observations that mutations affecting ILK kinase activity correlate with reduced contractility of cardiac muscles in zebrafish and human (Bendig et al., 2006; Postel et al., 2008). Yet, the genetic data in flies suggest that its function does not require an active kinase domain (Zervas et al., 2001). From these studies ILK is portrayed as a mechanosensor, required to potentiate the adhesion of Integrins to ECM and as a mechanotransducer required to establish and/or modify interactions with/of other adhesion components. In fact, phosphorylation of β-Parvin by ILK is required for binding of β-Parvin to α-Actinin *in vitro* (Yamaji et al., 2004).

Focal Adhesion Kinase (FAK)

FAK is a non-receptor tyrosine kinase that plays a central role in signalling through Integrins and a variety of other receptors (Parsons, 2003). It contains an N-terminal FERM domain that binds to the β Integrin tail (Schaller et al., 1995; Sieg et al., 2000), a central catalytic domain, and a C-terminal focal adhesion-targeting (FAT) domain that binds to Paxillin (Hayashi et al., 2002) and Talin (Chen et al., 1995). FAK folds into an autoinhibited conformation through interaction between the FERM and kinase domain region (Cooper et al., 2003). Integrin binding to amino terminus of FAK releases such autoinhibition and leads to FAK phosphorylation with activation (Cooper, et al., 2003).

The role of this kinase in mechanotransduction comes primarily from investigations in tissue culture. Mechanical stretch-induced FAK phosphorylation leads to its translocation from the perinuclear region and A-band (where it binds muscle Myosin) to the Z-lines, costameres and nuclei (Torsoni et al., 2003; Fonseca et al., 2005). Then, FAK phosphorylation supports recruitment of the Src family of protein tyrosine kinases (Li et al., 1997; Schaller et al., 1994), which in turn phosphorylate other proteins involved in adhesion and cytoskeleton organization (Schaller et al., 1994).

FAK is also crucial for sarcomere assembly and costamere alignment in cultured skeletal muscle cells (Quach and Rando, 2006). *In vivo* observation of striated muscles in adult mice demonstrate that FAK co-distributes with Integrins and Vinculin at costameres (Quach and Rando, 2006). In myoblasts undergoing differentiation FAK localizes at Integrin adhesion complexes and this enrichment is maintained throughout progressive maturation of sarcomeres and costameres. It appears that costameric FAK supports sarcomere assembly during skeletal muscle differ-

entiation. Indeed, expression of focal adhesion targeting domain (FAT) in myoblasts disrupts their differentiation into mature sarcomeres. Moreover, inhibiton of FAK signalling by siRNA results in impaired costameric and sarcomeric maturation (Quach and Rando, 2006). Collectively, these data describe FAK as key component of the mechanotransduction apparatus.

Although FAK appears to be a direct effector of Integrin signalling, yet in *Drosophila* it is not required for muscle attachment. Indeed, flies lacking Fak56 are viable and fertile (Grabbe et al., 2004).

1.7 Conclusion

The intrinsic sensitivity of Integrin to mechanical stimuli is essential for triggering cellular responses that actively regulate the organization and contractile activity of the cytoskeleton as well as gene expression. In this review, the bidirectionality of Integrin function was investigated in striated muscle using numerous examples from *Drosophila* and other model systems that demonstrated the reliance of mechanosensing and mechanotransduction on cytoskeletal and membrane-associated networks. Thus, the structural perception of these complexes, in this review, evolved to the concept of them being nodal points in striated muscle sensing and signalling.

The pivotal roles of Integrin and adhesion components in striated muscle have been largely gained by gene targeting approaches and analyses of their respective muscle diseases. In some cases, muscle phenotypes are end results of inability of the cytoskeleton to remain adequately connected to the membrane and in other cases they are outcomes of incapacity of the membrane to stay attached to the extracellular matrix (Figure 1.7). Examples of absence of phenotypes are also observed, indicating that some components of Integrin-adhesion may function in very specific circumstances

Figure 1.7: Schematic presentation of myotendinous junction phenotypes observed upon targeting of genes required for their maintenance.

In contrast to wild type shown in (A) in the absence of Integrin, the sarcolemma detaches from the ECM (B), while in the absence of Integrin-adhesion proteins such as Talin, ILK, Wech and PINCH, the sarcolemma remains attached to the ECM but the sarcomeric Actin detaches from the membrane (C). In other cases, lack of Talin and ILK cause detachment of Integrin from ECM (D).

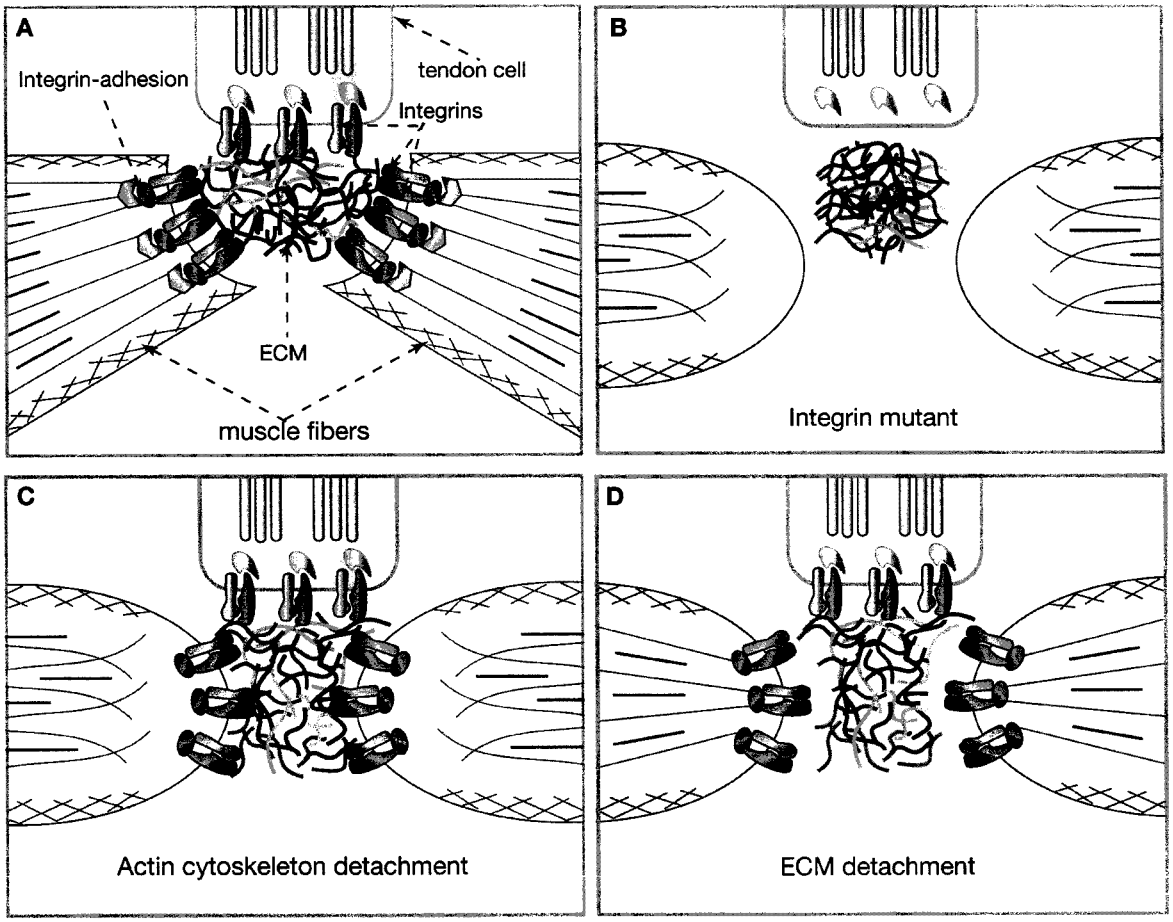

A
Integrin-adhesion
tendon cell
Integrins
ECM
muscle fibers
B
Integrin mutant
C
Actin cytoskeleton detachment
D
ECM detachment

FIGURE 1.8

or tissues.

Regardless, investigating the Integrin-mediated adhesion in striated muscles continues to demonstrate that this process depends on its interaction with the intracellular cytoskeletal networks and external loading of ligands for proper function.

Chapter 2

Zasp is required for the assembly of functional Integrin adhesion sites

2.1 Abstract

The Integrin family of heterodimeric transmembrane receptors mediates cell-matrix adhesion. Integrins often localize in highly organized structures such as Integrin adhesion sites in tissue culture and myotendinous junctions in muscles. Our RNAi screen for genes that prevent Integrin-dependent cell spreading identifies *zasp*, encoding the only known *Drosophila* 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 $zasp^{\Delta}$ 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 functional Integrin adhesion sites both in cell culture and in tissues.

2.2 Introduction

Integrin-mediated adhesion between the extracellular matrix (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 spreading 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 hemiadherens 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 (Ervasti, 2003; Garamvölgyi, 1965; Pardo et al., 1983). 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 (Ervasti, 2003; Pardo et al., 1983; Reedy and Beall, 1993; Volk et al., 1990). Both in mice, in *Drosophila*, and in *Caenorhabditis elegans*, Integrins are required for sarcomere assembly and Z-line formation (Bloor and Brown, 1998; Lecroisey et al., 2007; Schwander et al., 2003; Volk et al., 1990).

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 crosslinks antiparallel Actin filaments from opposite sarcomeres. Flies lacking α-Actinin die as first-instar larvae because of defects in Z-line maintenance, yet they form normal striated muscles initially (Dubreuil and Wang, 2000; Fyrberg et al., 1998). ALP/Enigma family proteins cooperate with α-Actinin in Z-line maintenance. ALP and Cypher/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 (Faulkner et al., 1999; Klaavuniemi et al., 2004; Pomies et al., 1999; Xia et al., 1997; Zhou et al., 1999). 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 ortholog, result in dilated cardiomyopathy (Arimura et al., 2004; Vatta et al., 2003).

In this study, we analyze Zasp, the only member of the ALP/Enigma family in *Drosophila*, 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 as well as Z-lines and functional myotendinous junctions in muscles.

2.3 Results

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

In order to identify novel genes involved in Integrin-mediated cell adhesion and spreading, we investigated two *Drosophila* cell lines, S2 and S2R+ cells, for their ability to uncover such genes using RNA-mediated interference (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 *mys* (encoding βPS Integrin) 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 differentia-

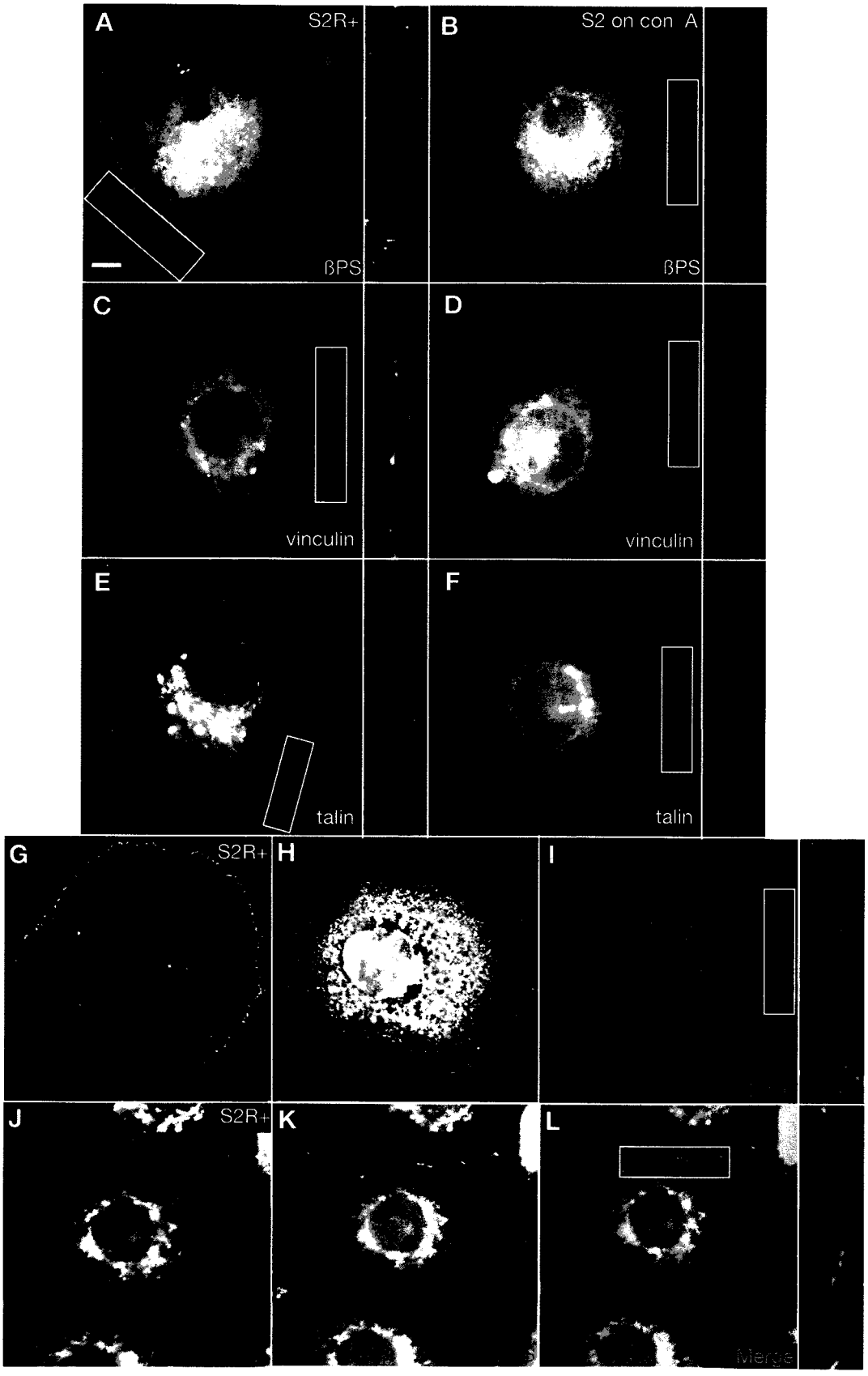
te Lectin-mediated cell spreading from Integrin-mediated cell spreading.

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 observe Integrin staining typical of Integrin adhesion sites, with bright foci along the cell edge and streaks in areas of potentially increased local forces (Figure 2.3.1 A). In contrast, S2 cells spread on concanavalin A do not exhibit these Integrin adhesion sites (Figure 2.3.1 B); instead of distinct foci and streaks, βPS Integrin is localized exclusively 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 observe 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 observe similar clusters at the cell edge in S2R+ cells, but not in S2 cells (Figure 2.3.1 C-F, and data not shown). We then determined if Talin, Vinculin and Integrin localize to the same clusters. We observe colocalization of Vinculin and αPS2 Integrin, as well as Talin and βPS Integrin (Figure 2.3.1 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; Ginsberg et al., 2005; Kunda et al., 2003; Rogers et al., 2003). While RNAi with *Abi* causes spreading defects characterized by a star-shaped morphology in both S2 and S2R+ cells, RNAi

Figure 2.3.1. *Drosophila* S2R+ cells exhibit Integrin adhesion sites.

Integrins cluster in adhesion sites in S2R+ cells. (A) Anti-βPS Integrin antibody staining of S2R+ cells and (B) S2 cells spread on concanavalin A. βPS Integrin localizes to bright foci and streaks at the cell edge of S2R+ cells. We observe similar clustering with anti-*C. elegans* Vinculin antibody staining (C), and anti-Talin antibody staining (E) in S2R+ cells, but not in S2 cells (D and F). (G-I) Colocalization of anti-*C, elegans* Vinculin (G), and anti-αPS2 Integrin antibody staining (H) at the cell edge of S2R+ cells. Merge is shown in (I). (J-L) Colocalization of anti-Talin (J) and anti-βPS Integrin antibody staining (K). Merge is shown in (L). Indicated areas are shown enlarged on the right. Scale bar, 15 μm.


A
S2R+
βPS
B
S2 on con A
βPS
C
vinculin
D
vinculin
E
talin
F
talin
G
S2R+
H
I
J
S2R+
K
L
Merge

with *rhea* results in cell spreading defects exclusively in S2R+ cells (Figure 2.3.2 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 S2R+ 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 involved specifically in Integrin-mediated processes and not in general cytoskeletal remodeling or cell viability (Table 2.3.1,

Supplementary Figure S1 (see Appendix)). 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.

Table 2.3.1. dsRNAs resulting in cell shape changes. Genes in bold show a phenotype only in S2R+ cells.

Gene name	common name or protein domains	Phenotype in S2R+ cells	Phenotype in S2 cells on con A
Abi	regulates SCAR	star-shaped	star-shaped
bhr	**novel**	**round**	**wild type**
BRWD3 (with 2 different dsRNAs)	**26 WD40**	**processes**	**wild type**
CG11063	**LIM**	**round/small**	**wild type**
CG30084	**PDZ/LIM**	**processes**	**wild type**
CG32138	**formin/WW**	**round/small**	**wild type**
CG3799	**ephexin RhoGEF**	**round**	**wild type**
CG8300	novel	round	round
dlp	**glypican**	**round**	**wild type**
Hem	regulates SCAR	star-shaped	star-shaped
if	**αPS2 Integrin**	**round or flaky**	**wild type**
Lasp	LIM/SH3	wild type	wild type
mbl	**muscleblind**	**round**	**wild type**
mys	**βPS Integrin**	**round**	**wild type**
R	**Rap1 GTPase**	**round**	**wild type**
rhea	**Talin**	**round/processes**	**wild type**
Sra-1	regulates SCAR	star-shaped	star-shaped

RNAi-mediated depletion of the other genes (listed in Table A1) did not result in reproducible cell shape changes (See Appendix).

2.3.2 The ALP/Enigma family has a single member in *Drosophila*

CG30084 represents the single member of the *Drosophila* ALP/Enigma family of PDZ-LIM domain proteins. We named CG30084 *zasp*, because the major predicted splice variant encodes a protein highly similar to human ZASP (Z-band alternatively spliced PDZ-motif protein) (Figure 2.3.2) (Faulkner et al., 1999). In *Caenorhabditis 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 Expressed Sequence Tags (ESTs, and Table 2.3.2). Together with data fromthe Berkeley *Drosophila* Genome Project we predict that *zasp* contains 20 exons and is transcribed into two major transcripts (Figure 2.3.2 G and H). The majority of ESTs (20 of 29) encode Enigma-like proteins (*zasp-RA*). We also found three ESTs corresponding to the ALP subfamily (*zasp-RB*, submitted to GenBank, accession number EF221635). LIM1 is truncated in the Enigma-like protein $Zasp^{Enigma}$, 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, while LIM2-4 are most closely related to the LIM domains of the Enigma subfamily (te Velthuis et al., 2007)

Treating S2R+ cells with *zasp* dsRNA targeting exon 5, which depletes $Zasp^{ALP}$ and $Zasp^{Enigma}$, results in severe spreading defects often associated with the formation of filopodia-like processes (Figure 2.3.2 I). S2R+ cells round up similar to *mys* or *rhea*-depleted cells. In contrast, we observe no phenotype in S2 cells (Figure 2.3.2 J). The absence of *zasp* mRNA after dsRNA treatment in both cell types was verified by RT-PCR (Figure 2.3.2 K). We observe an identical phenotype with dsRNA targeting exon 3, confirming the specificity of the spreading defect (data not shown).

Figure 2.3.2. Zasp is required for Integrin-dependent spreading of S2R+ cells.

(A-C) S2R+ cells spreading without addition of external ligand and (D-F) S2 cells spreading on concanavalin A. (A, D) No RNAi treatment, (B, E) with *Abi* RNAi, (C, F) 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, 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 $zasp^{\Delta}$ deletion, and dsRNAs used to target the *zasp* gene are indicated. Only the two major splice variants are shown. (H) The *Drosophila zasp* gene encodes two major proteins. $Zasp^{Enigma}$ is the Enigma-like protein and $Zasp^{ALP}$ 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 ortholog. (I, J) *zasp* e5 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 to untreated ones. *zasp* mRNA (153 bp e5 amplicon) is depleted in both S2R+ and S2 cells. Control PCR was done with primers against an untargeted gene. Scale bars, 15 µm.

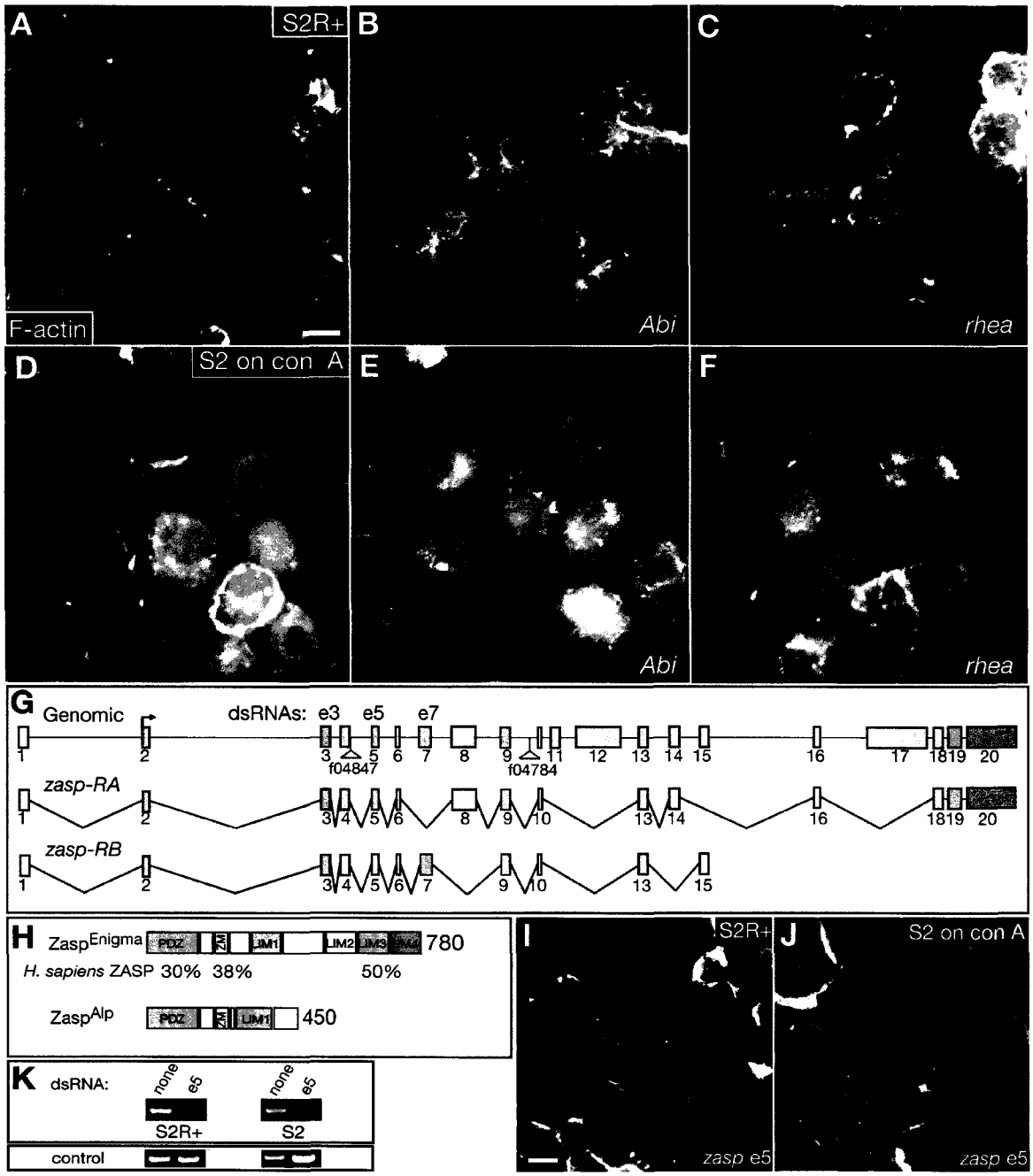

A
S2R+
F-actin
B
Abi
C
rhea
D
S2 on con A
E
Abi
F
rhea
G
Genomic
dsRNAs: e3 e5 e7
f04847
f04784
zasp-RA
zasp-RB
H
Zasp^Enigma
PDZ
ZM
LIM1
LIM2
LIM3
780
H. sapiens ZASP 30% 38% 50%
Zasp^Alp
450
I
S2R+
zasp e5
J
S2 on con A
zasp e5
K
dsRNA:
none
e5
S2R+
S2
control

Table 2.3.2. *zasp* splice variants

zasp-RA: **RH03424** exon structure (**IP01285** is identical except for the additional exon 12, 11662598 – 11661925)

exon	**mRNA**	**chromosome 2R (AE013599.4)**
1)	1 – 186	11702419 – 11702234 untranslated
2)	187 – 313	11690993 – 11690864 PDZ
3)	314 – 465	11670973 – 11670820 PDZ
4)	466 – 622	11670750 – 11670593
5)	623 – 756	11669411 – 11669278 ZM
6)	757 – 851	11668585 – 11668490
8)	852 – 1245	11667166 – 11666770
9)	1246 – 1442	11666051 – 11665857 LIM1 (70%)
10)	1443 – 1550	11664791 – 11664681
13)	1551 – 1702	11661472 – 11661319
14)	1703 – 1912	11660760 – 11660544
16)	1913 – 2040	11652759 – 11652628
18)	2041 – 2226	11651337 – 11651150 LIM2
19)	2227 – 2466	11651096 – 11650855 LIM3
20)	2467 – 3171	11650776 – 11650063 LIM4

zasp-RB (GenBank accession #EF221635): **LP01550, LP01361, RE06836** (starts at 11702419) exon structure

exon	**mRNA**	**chromosome 2R (AE013599.4)**
1)	1 – 169	11702403 – 11702234 untranslated
2)	170 – 296	11690993 – 11690864 PDZ
3)	297 – 448	11670973 – 11670820 PDZ
4)	449 – 605	11670750 – 11670593
5)	606 – 739	11669411 – 11669278 ZM
6)	740 – 834	11668585 – 11668490
7)	835 – 1055	11667785 – 11667563 LIM1 (30%)
9)	1056 – 1250	11666051 – 11665857 LIM1 (70%)
10)	1251 – 1359	11664791 – 11664681
13)	1360 – 1513	11661472 – 11661319
15)	1514 – 1700	11658654 – 11658459

GH15137 e16, 18-20;
HL08122 e14, 16, 18-20;
LP02021 e5-7, 9, 10, 11 (11663013 – 11662815), 12 (11662598 – 11661925), 13, 17 (11652342 – 11651431), 18-20;

ESTs with partial sequence information: We sequenced the 3′ ends of ESTs indicated in bold

GH16307 e1, …, e20; GH18981 e1, …, e20; GH22268 e1, …, e20;
GH25611 e1, …, e20; GH26874 e1, …, e20; GH28449 e1, …, e20;
GM32606 e1, …, e20; LP07307 e5, …; **LP11454 e1, …, e20;**
RE08540 e1, …, e20; RE19447 e1, …, e20; RE32053 e1, …, e20;
RE49166 e1, …, e20; RE53516 e1, …, e20; RE55390 e1, …, e20;
RE58207 e1, …, e20; RE73562 e1, …, e20; RH02578 e1, …, e20;
RH03452 e1, …, e20; SD03148 e7, 9, …; SD26735 e7, 9, …;

The deletion (*zasp*$^{\Delta}$) between f04847 (11670591) and f04784 (11664952) deletes exons 5 – 9 (5 exons including ZM and ALP-like LIM1 domain). It deletes all ESTs except GH15137 and HL08122, two unique ESTs. This indicates that we may have created a null for ALP- and Enigma-like splice variants, but a hypomorph for the entire *zasp* locus, because some splice variants may still be transcribed.

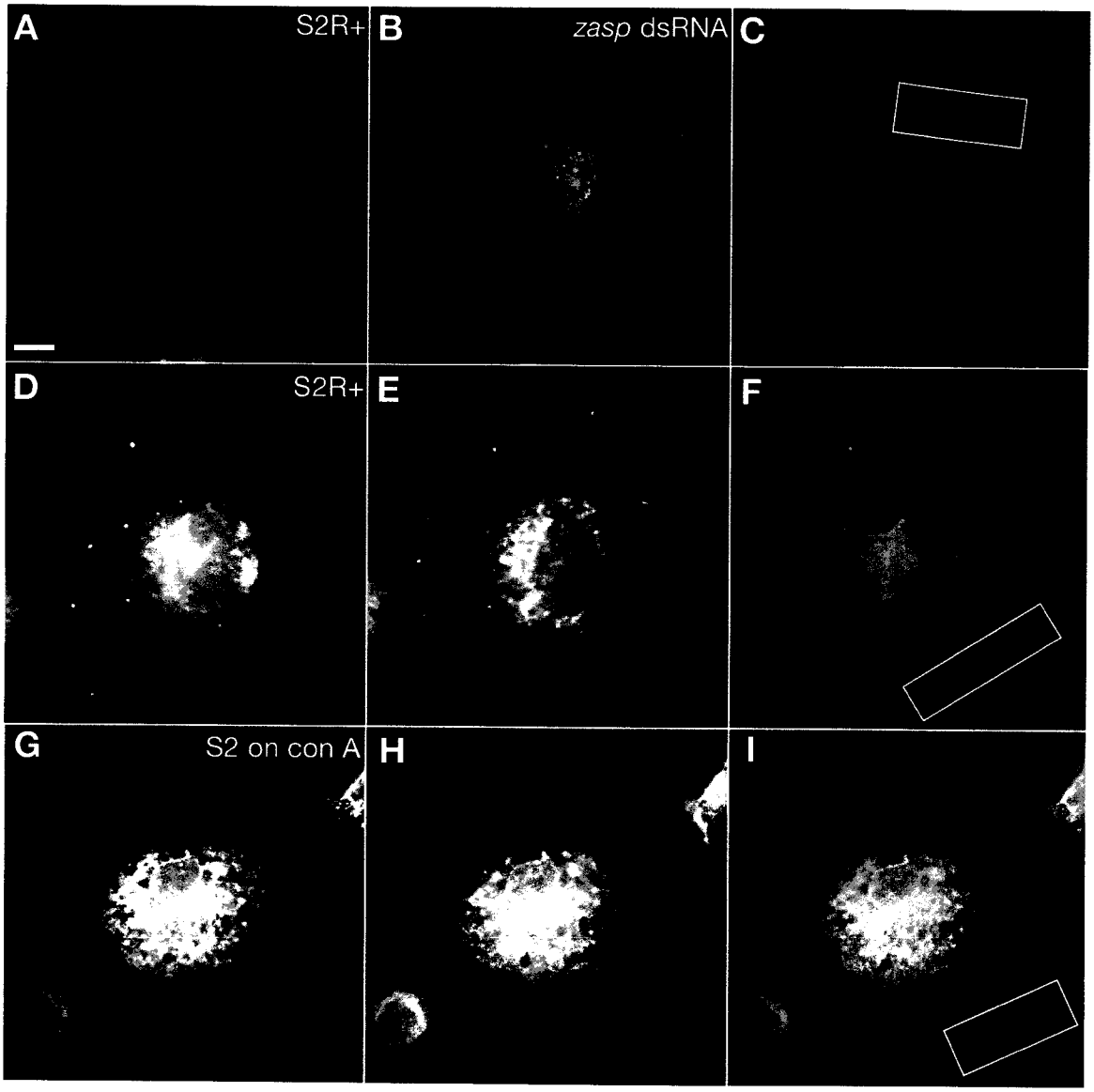
2.3.3 Loss of Zasp disrupts Integrin adhesion sites in S2R+ cells

We next determined if Integrin adhesion sites are affected in Zasp-depleted S2R+ cells. *zasp* e3 and e5 dsRNA-treated S2R+ cells show no Integrin adhesion sites (data not shown). Treating S2R+ cells with *zasp* e7 dsRNA, which only depletes $Zasp^{ALP}$, strongly impairs Integrin adhesion sites. In severe cases Integrin adhesion sites are either completely absent, or hardly visible (Figure 2.3.3 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 (Figure 2.3.3 C).

As loss of Zasp disrupts Integrin adhesion sites, we finally wanted to know if Zasp colocalizes with βPS Integrin in Integrin adhesion sites. For this purpose, we raised an antibody against $Zasp^{Enigma}$, which recognizes both $Zasp^{ALP}$ and $Zasp^{Enigma}$. Like βPS Integrin, Zasp localizes to the same bright foci and streaks at the cell edge (Figure 2.3.3 D-F). Furthermore, the absence of any localized Zasp staining in S2 cells spread on concanavalin A (Figure 2.3.3 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 2.3.3. Zasp localizes to Integrin adhesion sites and Zasp depletion disrupts Integrin adhesion sites.

(A) Anti-βPS Integrin antibody (green)/Alexa 594-phalloidin (red) co-staining of wild type S2R+ cells, or (B, C) cells treated with *zasp* e7 dsRNA. 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-F) Anti-βPS Integrin antibody (green)/anti-Zasp antibody (red) co-immunostaining of S2R+ cells and (G-I) S2 cells spread on concanavalin A. Zasp colocalizes with βPS Integrin in foci and streaks in S2R+ cells (D-F). Scale bar, 15 μm.

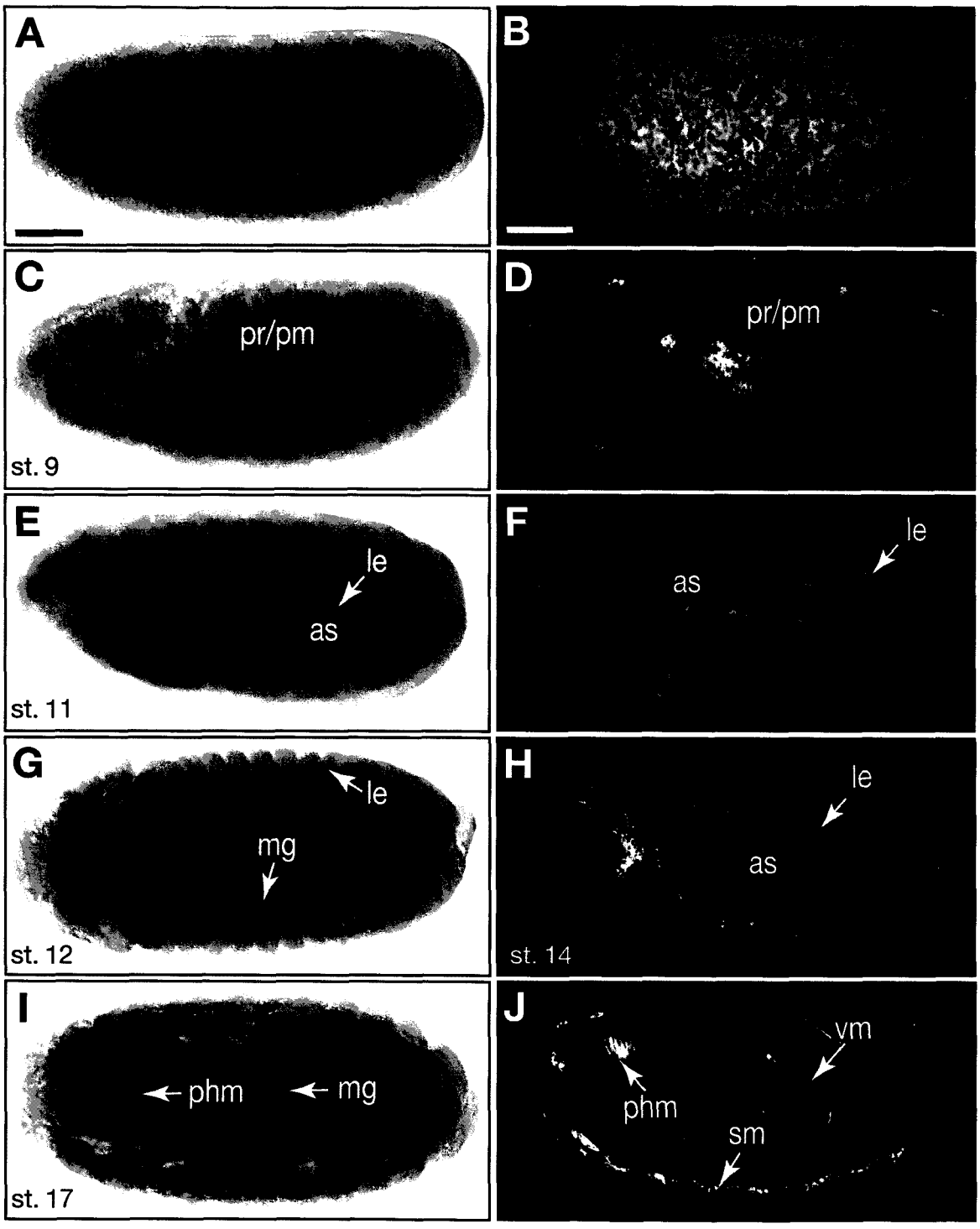

A
S2R+
B
zasp dsRNA
C
D
S2R+
E
F
G
S2 on con A
H
I

2.3.4 Zasp mRNA and protein expression is similar to that of βPS Integrin

In mammals, most ALP/Enigma family members function in Z-line maintenance (Faulkner et al., 1999; Pashmforoush et al., 2001; Pomies et al., 1999; Xia et al., 1997; Zhou et al., 2001; Zhou et al., 1999). To learn more about Zasp function and its potential involvement in the formation of Integrin adhesion sites, we investigated its expression profile during *Drosophila* embryogenesis. mRNA and protein expression largely overlap (Figure 2.3.4). Preblastoderm stage embryos show weak staining, indicating a maternal contribution (Figure 2.3.4, A and B). Zygotically, Zasp is expressed in areas where βPS Integrin is known to function (Devenport and Brown, 2004; Hutson et al., 2003; Narasimha and Brown, 2004), for example the leading edge during dorsal closure and the midgut during midgut fusion (Figure 2.3.4 C-H). At late stages, Zasp expression is particularly strong in mesodermal tissues such as visceral, pharyngeal, and somatic muscles (Figure 2.3.4, I and J).

Figure 2.3.4. Zasp protein and mRNA expression patterns overlap during embryogenesis.

RNA *in situ* hybridization with *Zasp* full-length antisense mRNA (left panels) and anti-Zasp antibody staining (right panels). (A) A low level of *zasp* mRNA, likely the maternal contribution, is visible in preblastoderm stage embryos. (B) Zasp protein is also detected in a preblastoderm stage embryo. (C, D) Zygotic mRNA and protein expression is first detected in the proctodeum (pr) and the midgut primordium (pm). (E, F) In stage 11 embryos *zasp* mRNA and Zasp protein expression is predominant in the leading edge (le) of epidermal cells adjacent to the amnioserosa (as). (G) Dorsal view of a stage 12 embryo reveals mRNA localization in the midgut (mg) and in the leading edge. (H) Zasp protein is expressed in several rows of germ band cells next to the leading edge at stage 14. (I) Strong mRNA expression is visible in the midgut and pharyngeal muscles (phm) in a dorsal view of a stage 17 embryo. (J) Zasp protein expression is additionally visible in somatic muscles (sm) and visceral mesoderm (vm). Scale bars, 50 μm.


A
B
C
pr/pm
st. 9
D
pr/pm
E
le
as
st. 11
F
le
as
G
le
mg
st. 12
H
le
as
st. 14
I
phm
mg
st. 17
J
vm
phm
sm

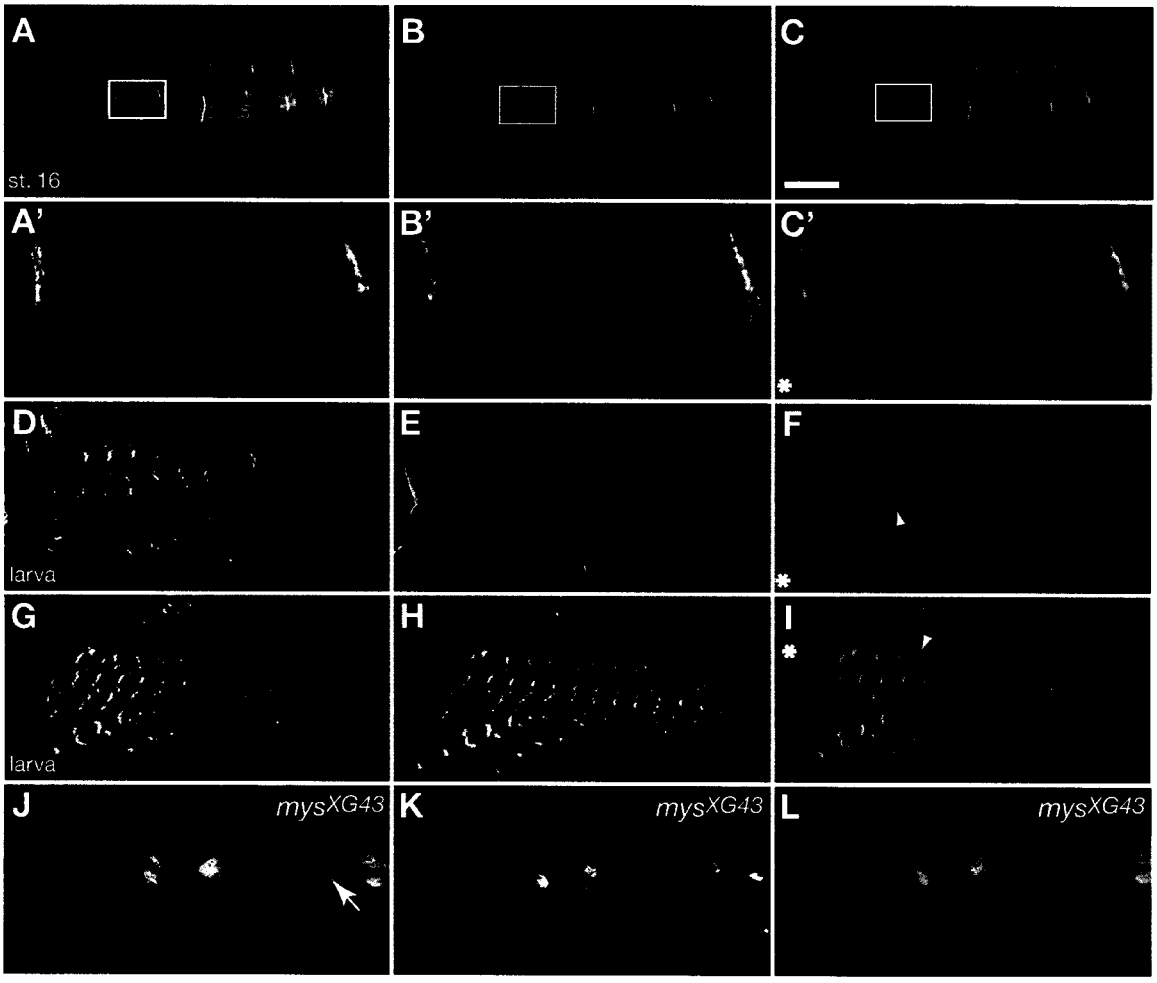
2.3.5 Zasp colocalizes with βPS Integrin at myotendinous junctions

Drosophila αPS2 and βPS Integrin subunits are enriched at myotendinous junctions, where they function in the adhesion of muscles to the tendon matrix (Brabant and Brower, 1993; Brown, 1994; Leptin et al., 1989). Strikingly, Zasp is also enriched at myotendinous junctions (Figure 2.3.5 A). Co-immunostainIng with anti-Zasp and anti-βPS Integrin antibodies reveals that Zasp tightly colocalizes with βPS Integrin at myotendinous junctions in the embryo (Figure 2.3.5 A'-C'). In larvae, Zasp still localizes to myotendinous junctions, but now facing the cytoplasmic side of βPS Integrin (compare Figure 2.3.5, D and E). Finally, the diffuse muscle staining in embryos is refined into the specific localization of Zasp into a repetitive line pattern (Figure 2.3.5 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 both at myotendinous junctions from stage 16 onward and later in Z-lines confirming that Zasp localizes to Z-lines (Figure 2.3.5 G-I, and data not shown). 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 by antibody staining, confirming the specificity of our antibody (Supplementary Figure S2 (see Appendix).

To determine if Integrins recruit Zasp to myotendinous junctions, we examined Zasp distribution in zygotic mys^{XG43} mutant embryos lacking βPS Integrin. Zasp and α-Actinin no longer localize to the tips of detached muscles, or only in a weak gradient (Figure 2.3.5 J-L). In a mys^{XG43} maternal and zygotic mutant, Zasp is completely unlocalized (data not shown), 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.

Figure 2.3.5. Zasp colocalizes with Integrins at myotendinous junctions during embryonic development and with α-Actinin at muscle Z-lines.

(A) Anti-Zasp antibody staining, (B) anti-βPS Integrin antibody staining and (C) merge of a stage 16 embryo. Zasp and βPS Integrin colocalize at myotendinous junctions. Indicated areas are shown enlarged in A', B', and C'. (D) Anti-Zasp antibody staining, (E) anti-βPS Integrin antibody staining and (F) merge of a first instar larva. Note the slightly wider gap of Zasp staining compared to that of βPS Integrin staining at the myotendinous junction. (G) Anti-Zasp antibody staining, (H) anti-α-Actinin antibody staining and (I) merge of a first instar larva. Note the tight colocalization of Zasp and α-Actinin at myotendinous junctions and Z-lines. (J) Anti-Zasp antibody staining, (K) anti-α-Actinin antibody staining, and (L) merge of a zygotic *mys*^{XG43} mutant embryo. Zasp and α-Actinin no longer localize at the termini of detached muscles (arrow in J). Asterisks indicate myotendinous junctions. Arrowheads indicate Z-lines. Scale bar, 50 μm.


A
B
C
st. 16
A'
B'
C'
D
E
F
larva
G
H
I
larva
J
mysXG43
K
mysXG43
L
mysXG43

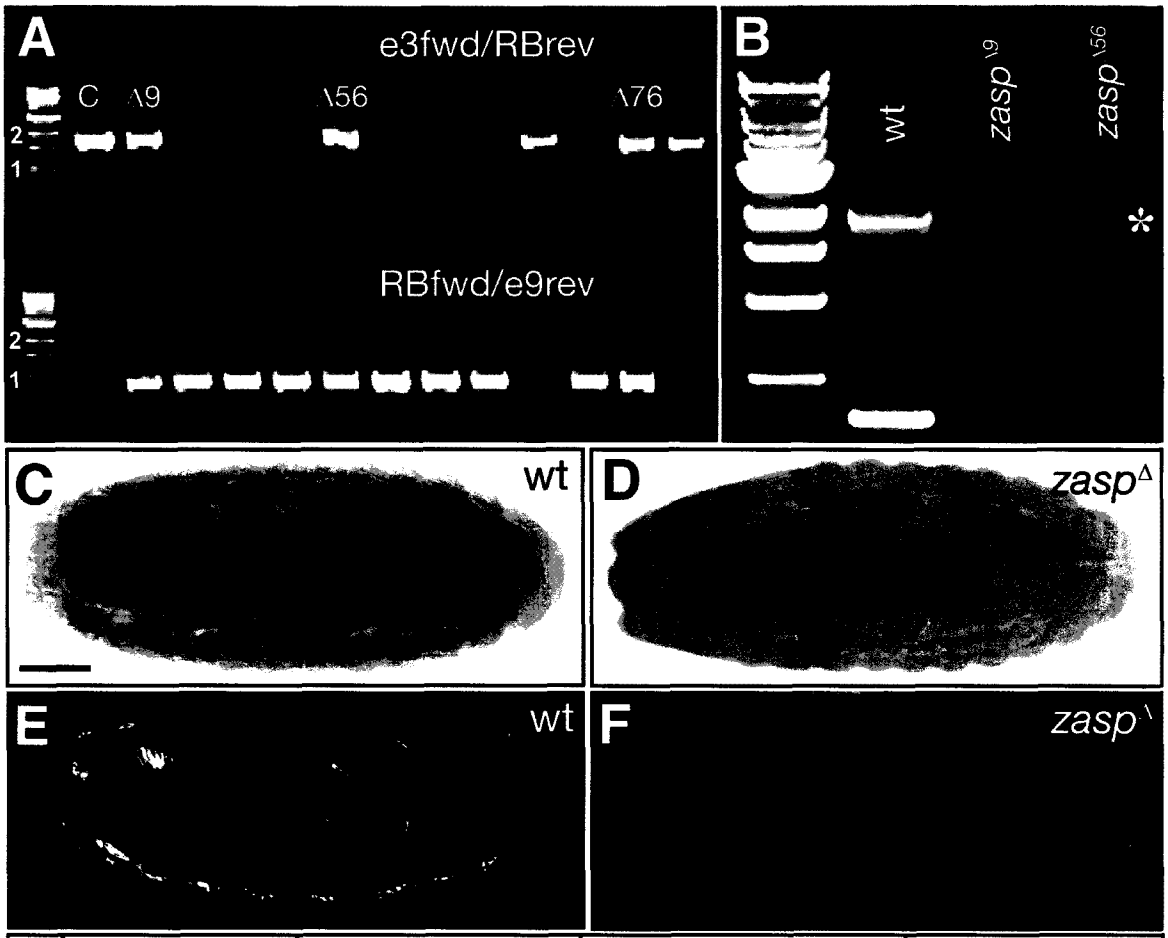
2.3.6 *zasp*$^{\Delta}$ mutants die as first instar larvae

In order to investigate if Zasp indeed functions in the formation of Integrin adhesion sites, we made a *zasp* mutant by recombining two FRT-bearing piggyBac elements inserted in intron four (f04847) and nine (f04784) of *zasp* (Figure 2.3.2 G). We recovered three identical lethal lines deleting exons five to nine and resulting in a frameshift. The deletion lines, whose identity was verified by PCR and Southern Blotting, remove all major splice variants (Figures 2.3.2 G and 2.3.6 A, Table 2.3.2 T1, and data not shown). They have no *zasp* mRNA expression as shown by RT-PCR and RNA in situ hybridization with a full-length probe (Figure 2.3.6 B-D). In addition, Zasp protein is absent in stage 17 zygotic mutants (Figure 2.3.6, E and F). Finally, *zasp* mutants transheterozygous over a large deficiency show the same phenotypes as *zasp* homozygotes, even though we observe a higher embryonic lethality (data not shown, and Figure 2.3.6 G). The combined data show that we created a *zasp* hypomorph, which we refer to as *zasp*$^{\Delta}$.

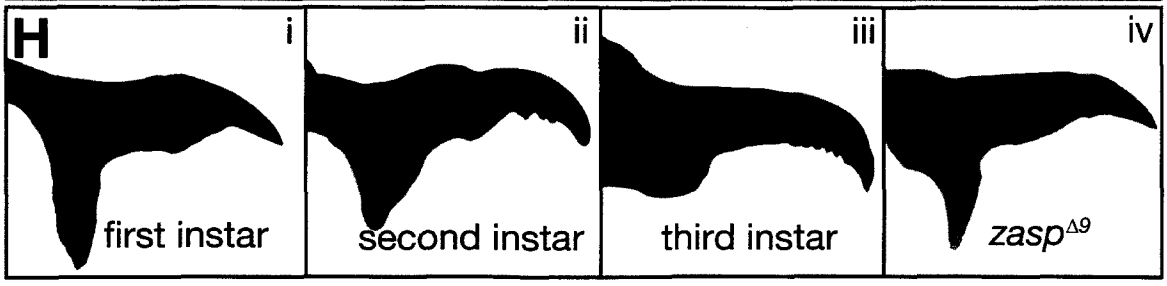
The majority of *zasp*$^{\Delta}$ mutants die as first instar larvae, 12% die as embryos. Most *zasp*$^{\Delta}$ mutant larvae die within the first 24 hours, coinciding with the onset of muscle contractility. Even though some larvae can live longer, they do not progress beyond the first instar stage as shown by their small size and mouth hook morphology (Figure 2.3.6 H).

Figure 2.3.6. *zasp*$^{\Delta}$ 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 exon 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 e3fwd/Rbrev primers. 1 and 2 kb size markers are indicated. (B) Absence of *zasp* mRNA in two deletion lines was verified by RT-PCR using primers CACCATGGCCCAACCACAGCTGCTG and GCGCGCGTGATTCTTGCAG. Amplification of a 2.1 kb band (asterisk) is detected only in wild-type embryos (wt). (C, D) RNA in situ hybridization with a full-length antisense probe demonstrates absence of *zasp* mRNA in *zasp*$^{\Delta}$ mutant embryos (D). (E, F) Anti-Zasp antibody staining reveals no obvious Zasp protein in *zasp*$^{\Delta}$ mutant embryos (F). (G) Stage of death of *zasp*$^{\Delta}$ mutants. (H) Developmental stage of *zasp*$^{\Delta}$ mutant larvae was determined by the number of teeth on the mouth hooks, which increase with instar. Mouth hooks of *zasp*$^{\Delta 9}$ mutant larvae (H_{iv}) look like those of first instar wild type larvae (H_i). Scale bar, 50 µm.



G genotype	stage of death	unhatched embryos	total embryos
Oregon R	–	0.6%	224
$zasp^{\Delta 9}$	first instar	11%	198
$zasp^{\Delta 56}$	first instar	12%	190
$zasp^{\Delta 9}/zasp^{\Delta 56}$	first instar	12%	206
$zasp^{\Delta 56}/Df(2R)Jp1$	first instar	43%	143

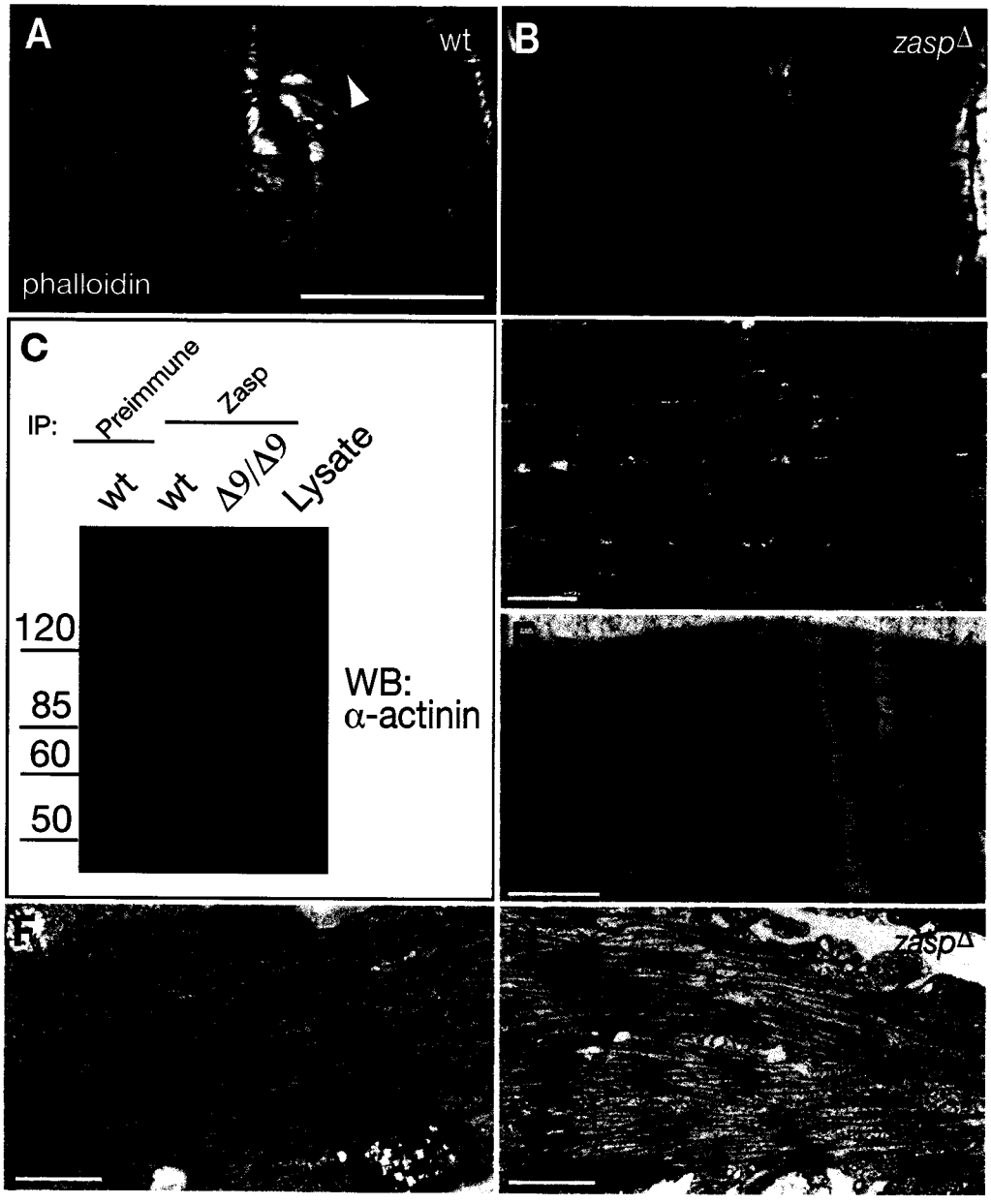


2.3.7 Zasp is required for sarcomere assembly

As muscle contractions were slower in $zasp^{\Delta}$ 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 $zasp^{\Delta}$ mutants. $zasp^{\Delta}$ mutants have lost the typical striated muscle pattern, indicating a Z-line defect (Figure 2.3.7, A and B). In mammals, the main interaction partner of Zasp at the Z-line is α-Actinin. We therefore wanted to know if *Drosophila* Zasp interacts biochemically with α-Actinin. Immunoprecipitation of wild-type extracts with anti-Zasp antibody pulls down α-Actinin (Figure 2.3.7 C). In contrast, immunoprecipitation of extracts from $zasp^{\Delta}$ 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 $Actn^{14}$ still develop a striated muscle pattern and Z-lines (Dubreuil and Wang, 2000; Fyrberg et al., 1998; Fyrberg et al., 1990), implicating 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, $zasp^{\Delta}$ and $Actn^{14}$ mutant larvae by transmission electron microscopy (Figure 2.3.7 D-G). In freshly hatched wild-type larvae Z-lines can readily be observed and are spaced at regular intervals (Figure 2.3.7 D). In one-day old α-Actinin mutant larvae we still observe Z-lines spaced at regular, but wider intervals than in the wild type possibly because of detachment of Actin fibers from the Z-line (Figure 2.3.7 E). These phenotypes correspond to previous observations (Fyrberg et al., 1998). In contrast, in freshly hatched $zasp^{\Delta}$ mutant larvae Z-lines are either completely absent with only occasional accumulations of electron-dense material (Figure 2.3.7 F), or Z-line remnants are severely disorganized and irregularly spaced (Figure 2.3.7 G).

Figure 2.3.7. *zasp*$^{\Delta}$ mutants develop no striated muscles and Z-lines.

(A) Late stage 17 wild type embryo stained with phalloidin to visualize Actin fibers. The striated muscle pattern, which indicates sarcomere differentiation, is clearly visible (arrowhead). A sarcomere extends from the center of one block of Actin staining to the next. (B) Late stage 17 *zasp*$^{\Delta}$ mutant embryo stained with phalloidin. No striation is evident. (C) Zasp physically interacts with α-Actinin in larvae. Immunoprecipitation (IP) was conducted with preimmune serum or anti-Zasp antibody using wild-type and *zasp*$^{\Delta 9}$ mutant larvae. Detection was performed by Western Blotting (WB) with anti-α-Actinin antibody. Only IP with anti-Zasp antibody with wild-type larvae co-precipitates α-Actinin. Molecular mass is indicated on the left in kDa. (D-G) Ultrastructural analysis of first-instar larval sarcomeres and Z-lines by electron microscopy. (D) Newly hatched wild type. (E) One-day old first instar *Actn*^{14} mutant. (F, G) Newly hatched *zasp*$^{\Delta}$ mutant. Black arrowheads indicate Z-lines, Z-line remnants or electron-dense material. Scale bar in A and B 50 µm, in D-G 1 µm.


A
wt
phalloidin
B
zaspΔ
C
IP:
Preimmune
Zasp
wt
wt
Δ9/Δ9
Lysate
120
85
60
50
WB:
α-actinin
zaspΔ

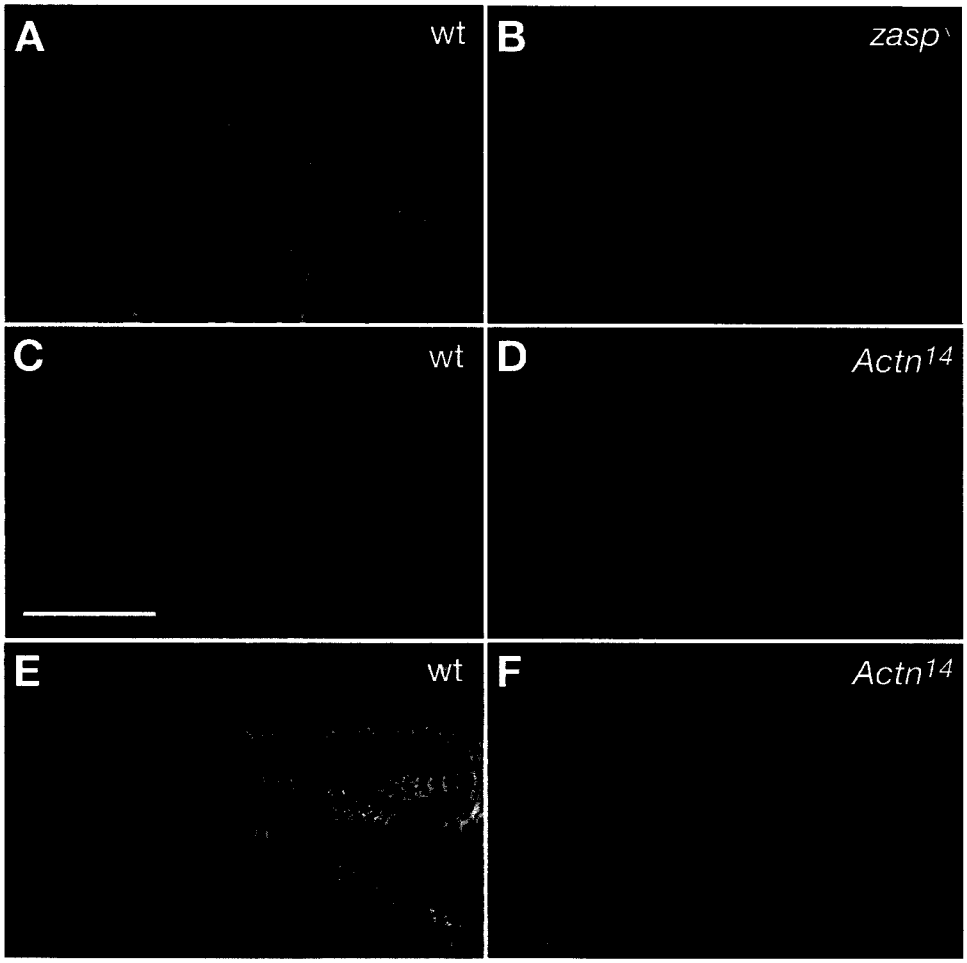
In addition, filaments are disorganized and no longer arranged in parallel arrays (Figure 2.3.7, F and G).

2.3.8. *zasp*$^{\Delta}$ mutants fail to recruit α-Actinin to Z-lines.

To determine the mechanism of Zasp function at Z-lines, we stained with anti-α-Actinin antibody. *zasp*$^{\Delta}$ mutant larvae have lost α-Actinin localization specifically at muscle Z-lines, showing that Zasp is required to localize α-Actinin to Z-lines (Figure 2.3.8, A and B). If Zasp is upstream of α-Actinin, Zasp should still localize to Z-lines in α-Actinin mutants. In *Actn*^{14} larvae, which is a null allele (Dubreuil and Wang, 2000; Fyrberg et al., 1990), GFP::Zasp indeed still localizes to Z-lines very similar to its wild type distribution (Figure 2.3.8, 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 (Au et al., 2004; Sorimachi et al., 1997; Young et al., 1998). Titin no longer localizes to Z-lines in *Actn* mutants (Figure 2.3.8, E and F). All these phenotypes are completely penetrant and demonstrate that Zasp is required for Z-line assembly.

Figure 2.3.8. *zasp*$^{\Delta}$ mutants do not recruit α-Actinin to Z-lines.

(A) Wild type larva stained with anti-Zasp antibody (in green) and anti-α-Actinin antibody (in red). (B) In *zasp*$^{\Delta}$ mutant larvae α-Actinin fails to localize to Z-lines, and instead appears to localize along the length of Actin filaments. (C) Localization of endogenous GFP::Zasp (*G00189*) in fixed wild type first-instar larva. (D) GFP::Zasp still localizes to Z-lines in an *Actn*^{14} null mutant larva. (E) Anti-Titin-KZ/anti-α-Actinin co-immunostaining of wild type, and (F) *Actn*^{14} mutant first-instar larva. Titin does not localize to Z-lines in the *Actn* mutant. Scale bar, 50 μm.

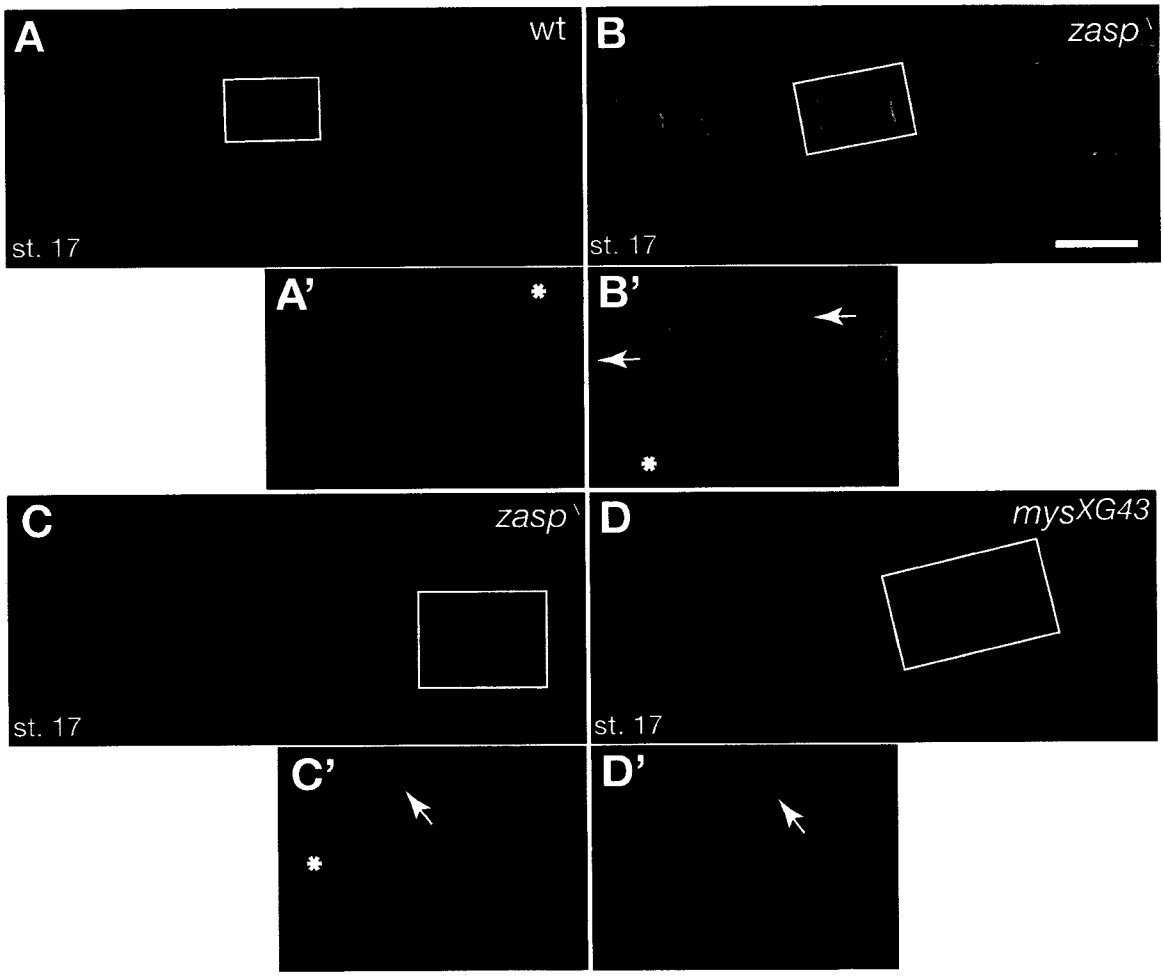

A
wt
B
zasp
C
wt
D
Actn14
E
wt
F
Actn14

2.3.9 Zasp mediates muscle attachment together with Integrins

We next analyzed *zasp*$^{\Delta}$ mutants for muscle detachment. We observe muscle attachment defects from stage 16 embryos onward, as seen by detachment of muscle fibers from attachment sites (Figure 2.3.9, A and B). We also see muscle patterning defects with muscles missing in some segments (data not shown). Late stage 17 embryos occasionally exhibit strong muscle detachment and rounding up of muscles (Figure 2.3.9 C). This complete muscle detachment is progressive, as we observe a penetrance of 12% (n = 190) round muscles, when we fix embryos at stage 17, but almost complete penetrance when we fix non-hatched embryos 24 hours later (98%, n = 112). The phenotype is also progressive in larvae, where we observe 17% round muscle phenotypes (n = 174), when larvae are fixed at the end of first instar. This phenotype is similar to that of embryos lacking zygotic βPS integrin (*mys*^{XG43}; Fig. 9 D), although it occurs later. βPS integrin still localizes to myotendinous junctions in *zasp*^{D} mutants, even when muscles round up, showing that Zasp is not required for integrin localization (Fig. 9 C').

Figure 2.3.9. *zasp*$^{\Delta}$ 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*$^{\Delta}$ mutant embryo with mild muscle detachment. (C) *zasp*$^{\Delta}$ mutant embryo with severe muscle detachment. (D) *mys*^{XG43} mutant embryo shown for comparison. Indicated areas are shown enlarged in A', B', C', and D'. Arrows indicate detached muscles. Asterisks indicate myotendinous junctions. Scale bar, 50 μm.


A
wt
st. 17
B
zasp
st. 17
A'
B'
C
zasp
st. 17
D
mysXG43
st. 17
C'
D'

2.3.10. Zasp genetically interacts with Integrins

If Zasp is a crucial component of Integrin adhesion sites, $zasp^{\Delta}$ 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^{SEF}) that shows weak muscle attachment defects only at the last stage of embryogenesis. In if^{SEF} 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) (Figure 2.3.10, A and B). However, stage 16 if^{SEF} embryos show normal βPS Integrin localization and no muscle phenotype (Figure 2.3.10, C and D). Intriguingly, if^{SEF} mutants lacking one copy of $zasp^{\Delta}$ display 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 (Figure 2.3.10, E, F, and I). This genetic interaction is specific to $zasp^{\Delta}$ since we observe no enhancement with either of the two piggyBac elements used to generate the $zasp^{\Delta}$ mutation (Figure 2.3.10, G, H, and I, and data not shown). The strong genetic interaction we observe between Zasp and αPS2 Integrin shows that they act together in maintaining muscle attachment.

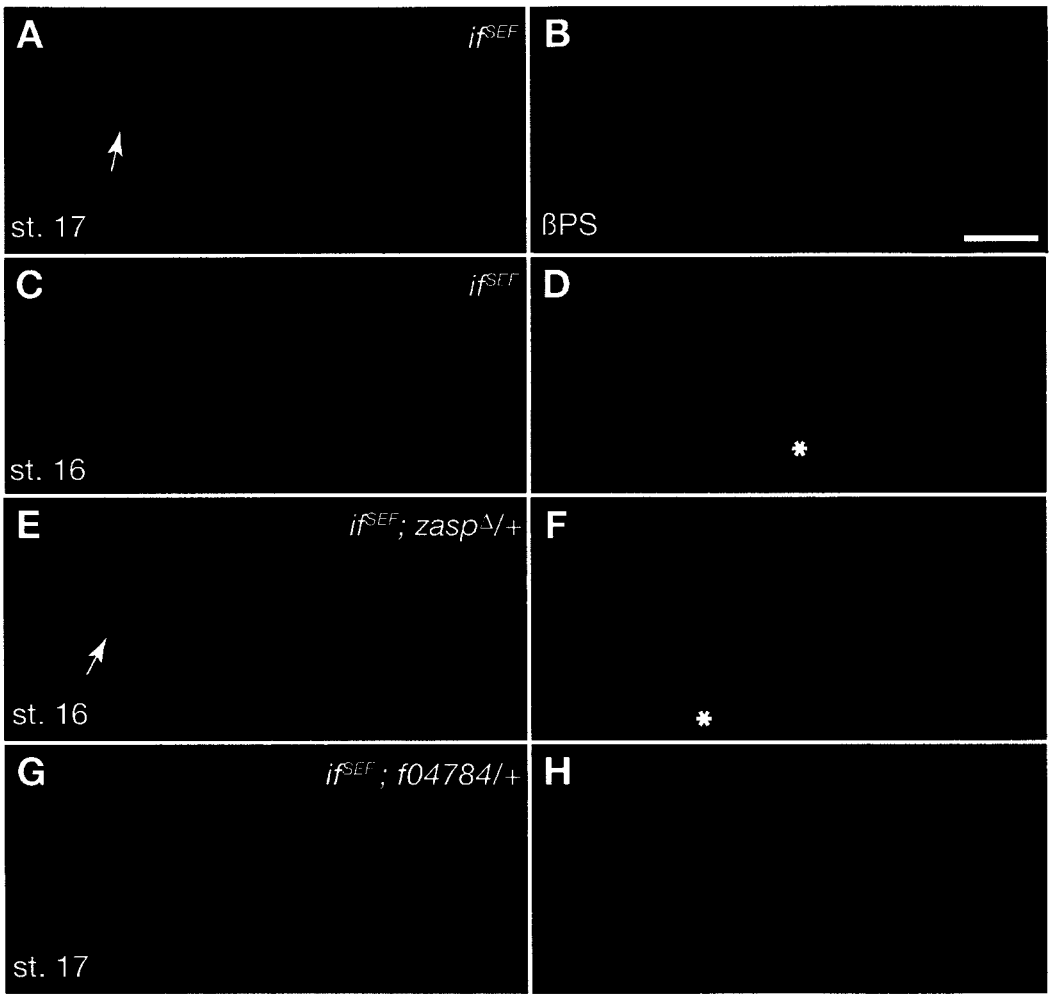
2.4 Discussion

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

In our screen we compare cell spreading of S2 and S2R+ cells. S2R+

Figure 2.3.10. Zasp genetically interacts with Integrins.

To determine the stage at which muscle attachment fails, embryos were labelled with antibodies against muscle Myosin heavy chain (MHC, green) and βPS Integrin (red, right panels). Merge is shown in the left panels. (A, B) Stage 17 if^{SEF} embryo showing mild muscle detachment. (C, D) Stage 16 if^{SEF} embryo showing wild type muscle organization. (E, F) Stage 16 if^{SEF}; $zasp^{\Delta 9}$/+ embryo showing severe muscle detachment. (G, H) Stage 17 if^{SEF}; *f04784/+* embryos showing mild muscle detachment identical to if^{SEF} embryos. (I) Percentage of embryos showing muscle detachment. ND, not determined. Arrows indicate detached muscles. Asterisks indicate myotendinous junctions. Scale bar, 50 μm.



I

Percentage of embryos with muscle detachment

Genotype	Stage 16	Stage 17	N*
if^{SEF}/Y	0% (14)	100% (18)	32
if^{SEF}/Y; +/f04784 and if^{SEF}/+; +/f04784	0% (17)	53% (21)	38
if^{SEF}/Y; +/ $zasp^{\Delta}$ and if^{SEF}/+; +/ $zasp^{\Delta}$	44%	ND	34

*Total number of observed embryos of indicated genotype(s)

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; and unpublished observations). S2R+ cell most likely secrete their own ECM ligand, 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 observe 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 are unable to observe Actin fibers attached to these adhesion sites, probably because Actin bundling in S2R+ cells is not sufficient to allow visualisation 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 observe functional Integrin adhesion sites.

Our pilot RNAi screen uncovered twelve 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 (Artero et al., 1998; Brown, 1994; Brown et al., 2002; Huelsmann et al., 2006). Several classical focal adhesion proteins such as Vinculin or focal adhesion kinase 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 where 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 *Drosophila* 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. Taken together, 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 observe colocalization of Zasp and βPS Integrin at myotendinous junctions and muscle detachment in $zasp^{\Delta}$ mutant embryos, again demonstrating that Zasp plays a crucial role in Integrin-mediated adhesion. The muscle detachment phenotype is weaker than the tissue culture phenotype, possibly because of the Z-line defect, which precludes normal muscle contractility. Alternatively, the weaker detachment phenotype may be due to a maternal contribution of Zasp rescuing earlier muscle attachment defects, or the myotendinous junction contains more components and is therefore less easily disrupted. We also observe defects in other tissues that require Integrin function, such as wing blisters in $zasp^{\Delta}$ clones and a genetic interaction of Zasp and Integrin during wing formation, confirming the general role of Zasp in Integrin adhesion (K. J. and F. S., unpublished). Vertebrate and *C. elegans* ALP/Enigma proteins have no reported function at Integrin adhesion sites, but they are known to localize to Integrin adhesion sites and muscle attachment sites (Henderson et al., 2003; McKeown et al., 2006; Pashmforoush et al., 2001; Pomies et al., 1999). In two other studies, the authors observed no colocalization with Vinculin at Integrin adhesion sites, however, they were not conducted in muscle cells, but rather

in Chinese hamster ovary 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 *Drosophila*, while 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.

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. Conversely, even in $zasp^{\Delta}$ embryos with strong muscle detachment, βPS Integrin still localizes to myotendinous junctions or muscle tips. Lastly, during larval stages we observe 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: 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). Secondly, we observe muscle detachment after muscle contractions begin 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; Pashmforoush et al., 2001; Zhou et al., 1999). We also observe a physical interaction of Zasp and α-Actinin in *Drosophila*, but our data imply a considerably larger role for Zasp in establishing Z-line structure and function than 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 (Figure 2.3.7 E and Fyrberg et al., 1998), which are disrupted in $zasp^{\Delta}$ mutant larvae. We suggest that this additional function of Zasp is again dependent on Integrins. Integrins connect Z-lines laterally to the extracellular matrix surrounding muscle fibers and are required for sarcomere assembly (Lecroisey et al., 2007; Schwander et al., 2003; Volk et al., 1990).

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.

2.5 Materials and methods

2.5.1 Tissue culture and RNAi

Drosophila S2 and S2R+ cells were treated with dsRNAs as previously described (Kiger et al., 2003; Rogers et al., 2003). For dsRNA synthesis, T7-flanked PCR products, generously provided by Norbert Perrimon (Department

of Genetics, 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 by the T7 promoter (TAATACGACTACTATAGGGAGA) were used for PCR followed by dsRNA synthesis: e3 (263 bp) CGCTGCACTGTGATGACAAA and GCCCAACCACAGCTGCTGCAA; e5 (153 bp) CCGAGCACACCGCCAGCCAA and CAACGCGGCCCGTCCCTTCTC. Cells were incubated with dsRNA for 5-7 days before being harvested for microscopy analysis, RT-PCR, and Western blotting. Control experiments were carried out in parallel without addition of dsRNA.

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

After being permeabilized in 1x PBS containing 0.1% Triton X-100 for 3 minutes, cells were washed twice in PBT (PBS containing 0.05% Tween-20), followed by incubation in blocking solution, containing 1% BSA in 1x PBS for at least 30 minutes at room temperature and stained with the primary antibody over night at 4° C. After washing with PBT with several changes over 30 minutes, cells were incubated with the secondary antibody for 45 minutes at room temperature. After several washes, the glass slides were mounted in Prolong Gold antifade (Molecular Probes). 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.

2.5.2 Generation of anti-Zasp antibody

We prepared a rabbit polyclonal antibody using a 6x His-tagged fusion protein corresponding to $Zasp^{Enigma}$. cDNA was amplified from EST RH03424 as template with CACCATGGCCCAACCACAGCTGCTG and GCGCGCGTGATTCTTGCAG 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% L-arabinose. We purified the recombinant protein under denaturing conditions on Ni^{2+}-affinity columns (Qiagen) according to the manufacturer's instructions. We tested antibody specificity by Western Blotting and immunofluorescence detection comparing wild type, *G00189*, and $zasp^{\Delta}$ embryos.

2.5.3 Immunoprecipitation assay

The indirect immunoprecipitation strategy was used by covalently binding the anti-Zasp antibody to AffiPrep Protein A-Sepharose beads (Biorad) using dimethylpimelimidate (DMP) as a crosslinker. 10 μl of anti-Zasp serum or rabbit preimmune serum were incubated with 110 μl of beads for 2 hours at room temperature. Previous to treatment with DMP, the beads were washed in 0.2 M sodium borate pH 9.0. The crosslinking reaction was stopped by washing the beads with 0.2 M ethanolamine/0.2 M NaCl pH 8.5 for 1 hour at room temperature. First instar larvae extracts were incubated with 50 μl of conjugated beads for 4 hours at 4°C. They were rinsed followed by three washes with the Lysis Buffer (50 mM HEPES pH 7.4, 1 mM EGTA, 1 mM $MgCl_2$, 300 mM KCl, 0.05% NP-40, 0.5 mM DTT, 10% glycerol, and one tablet

EDTA-free complete Protease Inhibitor cocktail). Beads were eluted by heating in 2x Sample Elution Buffer (2% SDS, 62.5 mM Tris pH 6.8, 10% Glycerol) without DTT for 10 minutes 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 (Amersham) for detection with anti-α-Actinin monoclonal antibody (1:20). Anti-mouse IgG, horseradish peroxidase-linked secondary antibody (1:2500) was used together with the ECL detection kit for visualization (GE Healthcare).

2.5.4 Histochemistry and microscopy

RNA in situ hybridization of embryos was performed with digoxigenin-labeled RNA probes, made by in vitro transcription of RH03424 with T3 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 approximately 25% of an unsorted collection. We also sorted homozygous $zasp^{\Delta}$ mutant embryos by the absence of the GFP balancer *CyO, twi-Gal4 UAS-2xEGFP*. In this case we hybridized green and non-green embryos in parallel, and observed signal only in green embryos. Images were obtained on a Leica MZ16-FA stereomicroscope using a PLANAPO 2.0x objective with a Qicam digital camera and OpenLab software (Improvision).

Embryos and larvae were fixed using heat fixation (Tepass, 1996). Briefly, embryos were dechorionated in 50% bleach for 90 seconds, rinsed in water, immersed in boiling 1x Embryonic Wash buffer (70 mM NaCl, 0.05% Triton X-100) for 10 seconds, immediately cooled by adding three volumes of ice-cold Embryonic wash buffer, and placed on ice for 30 minutes. Embryos were devitellinized in methanol/heptane.

Primary antibodies used: mouse anti-α-Actinin (1:10, kindly provided by

Judith Saide, Department of Physiology and Biophysics, Boston University School of Medicine, Boston, MA) (Saide et al., 1989), rat anti-αPS2 Integrin (1:10, 7A10 kindly provided by Nick Brown and Guy Tanentzapf, Gurdon Institute, University of Cambridge, Cambridge, UK) (Bogaert et al., 1987), mouse anti-βPS Integrin (1:10, CF.6G11 from DSHB, University of Iowa, Iowa City, IA) (Brower et al., 1984), mouse anti-Paxillin (1:50, 165 from BD Biosciences Pharmingen), rabbit anti-Talin (1:100, kindly provided by Nick Brown) (Brown et al., 2002), rat anti-D-Titin-KZ (1:500, kindly provided by Deborah Andrew, Johns Hopkins University School of Medicine, Baltimore, MD) (Machado et al., 1998), rabbit anti-muscle Myosin heavy chain (1:400, kindly provided by Dan Kiehart, Department of Biology, Duke University, Durham, NC) (Kiehart and Feghali, 1986), mouse anti-phosphotyrosine (1:200, 4G10 from Upstate/Millipore), mouse anti-*C. elegans* Vinculin (1:10, MH24 from DSHB) (Francis and Waterston, 1985), rabbit anti-Zasp (1:300 for cells, 1:400 for embryos). Fluorescently labeled secondary antibodies of the Alexa series (Molecular Probes) 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, Molecular Probes). Embryos were devitellinized by hand.

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

Tissue culture images were obtained on a Leica DM6000B upright microscope using a 63x, 1.4 numerical aperture HCX PL APO CS oil objective with a Hamamatsu Orca-ER digital camera and OpenLab software. Embryo

images were obtained on a Zeiss LSM510 Meta confocal microscope using a 40x PLAN-NEOFLUAR, 1.3 numerical aperture or a 63x PLAN APO, 1.4 numerical aperture oil objective and processed using ImageJ and Adobe Photoshop. Live imaging was done as described (Schöck and Perrimon, 2003). All images were obtained at room temperature.

Electron microscopy

First instar larvae were fixed in 5% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.4) for 1 hour at room temperature. After cutting off the extremities, the specimens were transferred into fresh 2.5% glutaraldehyde, 0.1 M cacodylate buffer and fixed for another five hours at 4° C. Larvae were postfixed in 1% osmium tetroxide for 2 hours at 4° C. Then the samples were washed with several changes in 0.1 M cacodylate buffer for 40 minutes at 4° C, followed by staining with 2% tannic acid in 0.1 M cacodylate buffer for 1 hour at 4° C. After several washes in distilled water, the specimens were restained with 2% uranyl acetate for 1 hour at 4° C and washed in distilled water for 45 minutes 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 a Reichert Ultracut AV. Then sections were stained in 7% uranyl acetate in absolute methanol for 4 minutes followed by incubation in Reynolds lead citrate for 4 minutes and examined on a FEI Tecnai 12 transmission electron microscope.

2.5.5 Fly stocks and genetics

We used the following stocks: mys^{XG43} and if^{SEF} from Nick Brown, if^{3}, $Actn^{8}$, $Actn^{14}$, *G00189*, and *Df(2R)Jp1* from the Bloomington *Drosophila* stock center, and *f04847* and *f04784* from the Exelixis *Drosophila* stock collection at Harvard Medical School.

We generated a *zasp* mutant by recombining two FRT-bearing piggy-

Bac elements as previously described (Parks et al., 2004). To verify the presence and location of piggyBac elements we conducted genomic PCRs using genome-specific primers (CAATCTGCGTCACAAGGATG for f04847 and TTTGCGTTTTCATTTCATCG for f04784) and the piggyBac-specific primers: RB(WH+)fwd: TCCAAGCGGCGACTGAGATG and RB(WH+)rev: CCTCGATATACAGACCGATAAAAC. We used a GFP balancer (CyO, *twi-Gal4 UAS-2xEGFP*) to identify homozygous $zasp^{\Delta}$ mutant embryos.

To image Z-lines in *Actn* mutant larvae, we crossed $Actn^{14}$/FM7c, *twi-Gal4 UAS-2xEGFP* females to FM7c, *twi-Gal4 UAS-2xEGFP*; *G00189*/+ males. To test for genetic interaction between αPS2 Integrin and Zasp, we crossed *y w* if^{SEF}/FM7, *eve-lacZ* females to $zasp^{\Delta}$/CyO, *twiGal4 UAS2EGFP* males. Embryos were distinguished by detecting β-galactosidase activity with X-Gal staining. Briefly, non-green embryos were fixed for 7 minutes in n-heptane saturated with 2.5% glutaraldehyde in PBS at room temperature. After washing and permeabilization for another 2 hours with several changes of PBT, we stained the embryos with staining solution (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, 5 M NaCl, 1 M $MgCl_2$, 50 mM $K_3[Fe(CN)_6]$, 50 mM $K_4[Fe(CN)_6]$) containing 1/50 volume of 10% X-gal solution in dimethyl sulfoxide for 1 hour at 37° C. After stopping the reaction with several washes in PBT, we proceeded with the standard antibody staining procedure.

2.6 Acknowledgments

We thank E. C. Davis and J. Mui for help with electron microscopy and N. Perrimon for providing dsDNAs with attached T7 promoter sequence for the candidate genes tested in the pilot screen. We thank N. H. Brown for flies, and D. J. Andrew, N. H. Brown, D. P. Kiehart, J. Saide, G. Tanentzapf, and the Developmental Studies Hybridoma Bank for antibodies and F. Fagotto, L. Nilson, J. Vogel, C. Gamberi, and J.-M. Kugler for comments on the manuscr-

ipt. This work was supported by the New Opportunities grant 9607 (F.S.) from the Canada Foundation for Innovation, and by operating grants IC1-70768 and MOP-74716 (F.S.) from the Canadian Institutes of Health Research. F. S. is a CIHR New Investigator (MSH-76596).

Connecting text

In the research presented in Chapter 2, we analyzed Zasp, the only member of the Alp/Enigma family in *Drosophila*, and identified novel roles for Alp/Enigma family proteins both in tissue culture cells and flies. Subsequent analysis of Zasp expression reveals its localization at Integrin adhesions such as focal adhesions in tissue culture and myotendinous junctions in *Drosophila* embryos. Accordingly, depletion of Zasp disrupts cell adhesions and leads to a range of defects in embryogenesis including muscle detachment from myotendinous junctions as well as sarcomere disassembly. Our data thus indicate that Zasp is required for the formation of three different Integrin adhesions: focal adhesions in tissue culture, functional myotendinous junctions, and sarcomeric Z-lines in muscles. The notion of a role for Zasp in Integrin function is further supported by the strong genetic interaction observed between Zasp and Integrin.

More recently, we inquired whether the observed muscle detachment phenotype in $zasp^{\Delta}$ mutants is due to detachment of Actin fibres from the sarcolemma, where Integrins are anchored, or due to Integrin detachment from the ECM. The investigation presented in Chapter 3 describes our resent findings on the mechanisms by which Zasp regulates Integrin-mediated adhesion.

Chapter 3

Zasp is required to strengthen the Integrin link to the extracellular matrix

3.1 Abstract

The transmembrane Integrin receptor links the extracellular environments to the cytoskeletal signaling networks. Integrin needs to be activated in order to bind to the appropriate ligands. Binding of the adaptor protein Talin to the β Integrin cytoplasmic tail increases Integrin affinity for ligand, by inducing a conformational change in the Integrin cytoplasmic domain that propagates to the extracellular ligand binding region. Here we show that PDZ/LIM domain protein Zasp supports Integrin activation by interacting with Talin. In *Drosophila*, Zasp colocalizes with Talin in myotendinous junctions of developing embryos. Intriguingly, in $zasp^{\Delta}$ embryos Talin is still found at the ends of the detached muscles along with αPS2 Integrin but separated from the ECM. Moreover, co-immunoprecipitation assay demonstrates that the two proteins are present in a common molecular complex *in vivo*. Finally, the LIM and PDZ domain of Zasp physically interacts with Talin head and tail region, respectively. Collectively, our results anticipate a role for Zasp in strengthening Integrin adhesion to the ECM, possibly by enhancing the Integrin activating function of Talin.

3.2 Introduction

The attachment of muscle fibres to the extracellular matrix is crucial for muscle development and integrity and is mediated to a large extent by the Integrin family of heterodimeric transmembrane receptors (Bloor and Brown, 1998; Mayer et al., 1997). Integrin binds to ligand extracellularly and provides anchor to the intracellular cytoskeleton via cytoplasmic scaffolding proteins (Delon and Brown, 2008). In muscles, the cytoskeletal networks assemble at myotendinous junctions and costameres, which tether the terminal myofibres and sarcomeric Z-lines to the sarcolemma, respectively (Reedy and Beall, 1993; Pardo et al., 1983). This architecture provides the mechanical force that is required to resist repetitive muscle contraction during larval locomotion (Löer et al., 2008).

In response to force, these anchoring structures, also known as Integrin-adhesions, increase in size linearly to stabilize and strengthen their coupling to the external environment (Choquet et al., 1997; Chrzanowska-Wodnicka and Burridge, 1996; Katsumi et al., 2005; Quach and Rando, 2006). Fundamental to this process is the ability of Integrin to alter its binding strength for extracellular matrix components. The adhesiveness can be modulated by changes in either affinity or density (Kinashi, 2005). Intracellular activation signals induce perturbations in Integrin cytoplasmic domain that are translated into a conformational change in extracellular region for high affinity ligand binding. The affinity modulation of individual Integrin molecules is referred to as Integrin activation (Han et al., 2006; Caldewood, 2004; Qin et al., 2004). Clustering of activated Integrin can also intensify the adhesion strength (Li et al., 2003). Local concentration of activated Integrin guides the recruitment of ECM ligands, thus increasing Integrin's binding avidity for ligand without requiring a change in affinity of individual Integrin (Imanaka-Yoshida et al., 1999; Diamond and Springer, 1994).

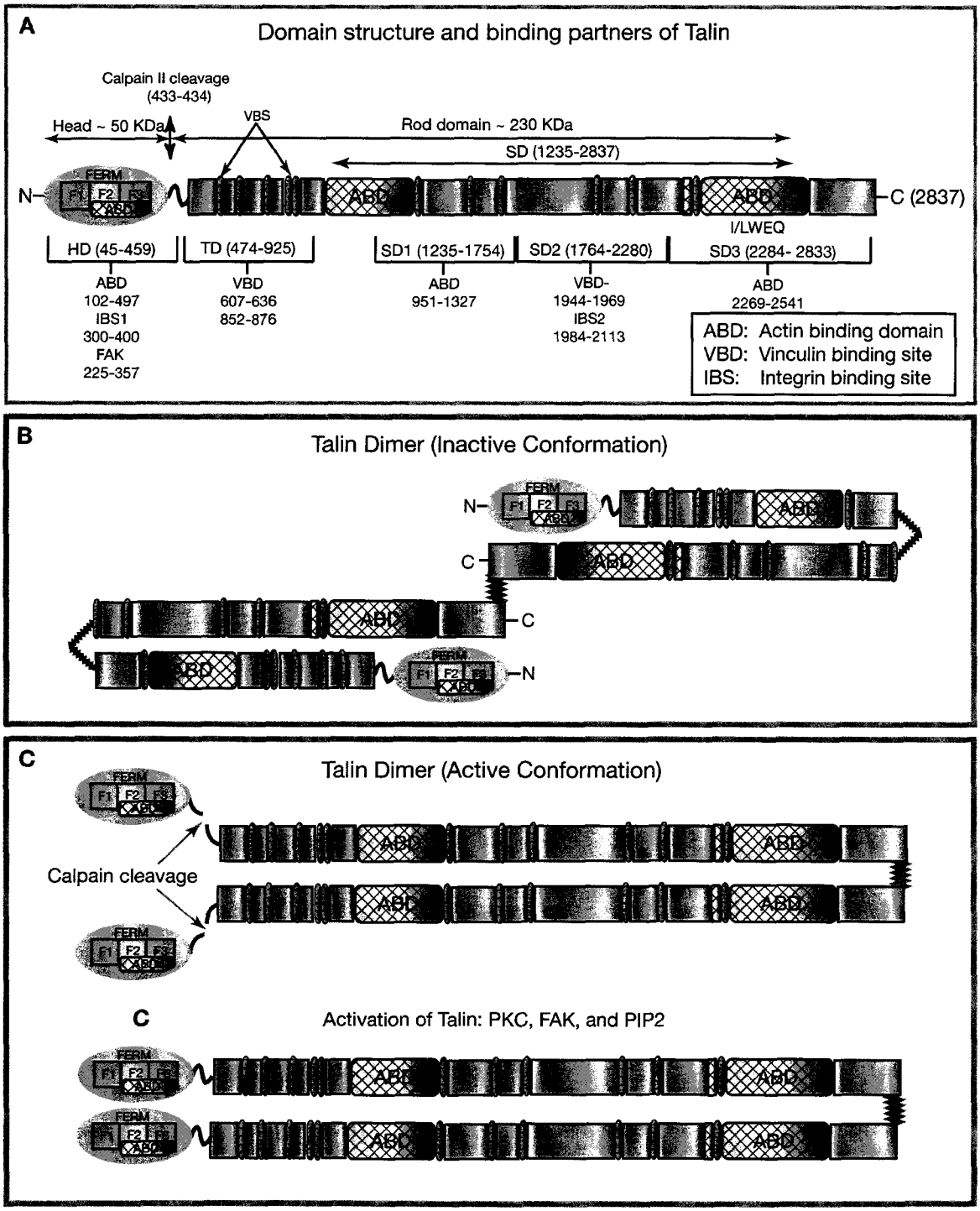
Binding of the adhesion protein Talin to Integrin switches Integrin's affinity for matrix components (Tadokoro et al., 2003; Caldewood, 2004). The major Integrin binding site of Talin lies in the amino-terminal head region (Tadokoro et al., 2003), which contains a FERM (4.1/ezrin/radixin/moesin) domain subdivided into F1, F2 and F3 subdomains (Figure 3.2 A). The F3 subdomain docks at one of the two tyrosine NPXY tight motifs of β Integrin cytoplasmic tail (Vinogradova et al., 2002; Tadokoro et al., 2003). This interaction induces the separation of the transmembrane bridge between α and β tails and subsequent conformational alterations in Integrin extracellular domains, thus leading to Integrin activation. Other Integrin binding sites have been found within the rod domain of Talin (Xing et al., 2001; Moes et al., 2007). This region contains also at least two Actin-binding sites and several binding sites for Vinculin (Hemmings, et al., 1996; Bass et al., 1999). The main function of Talin's rod is to link Integrin to the cytoskeleton and to bind high affinity Integrin, ultimately leading to its clustering (Moes et al., 2007).

The *in vivo* observation for a Talin role in Integrin activation comes from a study investigating Integrin-mediated muscle attachment in *Drosophila* embryos. Embryos harboring a single amino acid mutation in the Talin head, which perturbs Talin binding to the Integrin cytoplasmic tail, exhibit defective attachment of Integrins to their matrix ligand (Tanentzapf and Brown, 2006). However, this mutation does not impair the ability of Talin to increase the avidity of Integrin adhesion by clustering and to carry out its function as a cytoplasmic linker (Tanentzapf and Brown, 2006).

On the other hand, little is known about Talin activation per se. The Integrin-binding site (Yan et al., 2001; Martel et al., 2001) and at least one Vinculin-binding site (Papagrigoriou et al., 2004) in Talin are masked, proposing that Talin function is itself regulated (Cram and Schwarzbauer, 2004). Therefore, full-length Talin needs to be activated in order to release the head domain for binding to the Integrin β cytoplasmic tail (Figure 3.2 B).

Figure 3.2 Domain structure and binding partners of Talin.

Schematic presentation of Talin domains (A). Inactive Talin folds into a head-tail autoinhibitory conformation (B). Calpain cleavage and binding of other Talin partners induce Talin activation (C). Note, the C-terminal helices of two Talin monomers form a dimer (Critchley and Gingras, 2008).


A
Domain structure and binding partners of Talin
Calpain II cleavage
(433-434)
Head ~ 50 KDa
VBS
Rod domain ~ 230 KDa
SD (1235-2837)
FERM
F1
F2
F3
ABD
N
C (2837)
I/LWEQ
HD (45-459)
TD (474-925)
SD1 (1235-1754)
SD2 (1764-2280)
SD3 (2284- 2833)
ABD
102-497
IBS1
300-400
FAK
225-357
VBD
607-636
852-876
ABD
951-1327
VBD-
1944-1969
IBS2
1984-2113
ABD
2269-2541
ABD: Actin binding domain
VBD: Vinculin binding site
IBS: Integrin binding site
B
Talin Dimer (Inactive Conformation)
C
Talin Dimer (Active Conformation)
Calpain cleavage
C
Activation of Talin: PKC, FAK, and PIP2

Talin is unfolded by Calpain (Figure 3.2 C), which cleaves off the amino-terminal head domain involved in β Integrin and Actin binding (Franco et al., 2004; Yan et al., 2001). However, this mechanism is unlikely to play a role in muscle attachment, since Calpain cleavage removes Talin's main function, connection of Integrins to the Actin cytoskeleton. Talin is also activated by phosphoinositides (Martel et al., 2001), which are produced by phophatidylinositol phosphate kinase I (PIPKI) that, in turn, binds the Talin head domain ensuring local PIP2 production (Ling et al., 2002). PIPKI-mediated Talin activation is unlikely to explain Talin-mediated Integrin inside-out activation, since both PIPKI and Integrin compete for Talin head domain binding.

It is also believed that phosphorylation regulates Talin function (Tidball and Spencer, 1993; Ratnikov et al., 2005). In platelets, translocation of Talin from the cytosol to the membrane is associated with phosphorylation of Talin's head domain on serine/threonine residues (Bertagnolli et al., 1993). Moreover, mapping of the PKC sites in Talin *in vitro* have demonstrated that Talin head domain is the only phosphorylation target of PKC (Critchley, 2004). Yet, the biological relevance of PKC-mediated Talin phosphorylation in Integrin activation has not been established *in vivo* (Han et al., 2006).

Recently the protein Kindlin-2 was found as a co-activator of Talin, supporting Integrin activation (Ma et al., 2008). Expression of Kindlin-2 in tissue culture induces statistically significant but very weak Integrin activation compared with Talin. However, when co-expressed with Talin a dramatic enhancement of such activation is observed. The synergism encountered between Talin and Kindlin-2 is a good example demonstrating that other factors may collaborate with Talin for Integrin activation.

We have previously established that Zasp is a downstream target of Integrin function (Jani and Schöck, 2007). What still remains to be determined is how Zasp influences Integrin-mediated adhesion. Chapter 3 discusses the results and progress I have made in answering some of these questions. The

results will be elaborated further in Chapter 4.

In summary, we have evidence suggesting that the inability of Integrins to promote proper matrix adhesion is a consequence of disrupted Zasp function. Our data imply that Integrin affinity for ECM is dependent on the direct interaction of Zasp with Talin.

3.3 Results

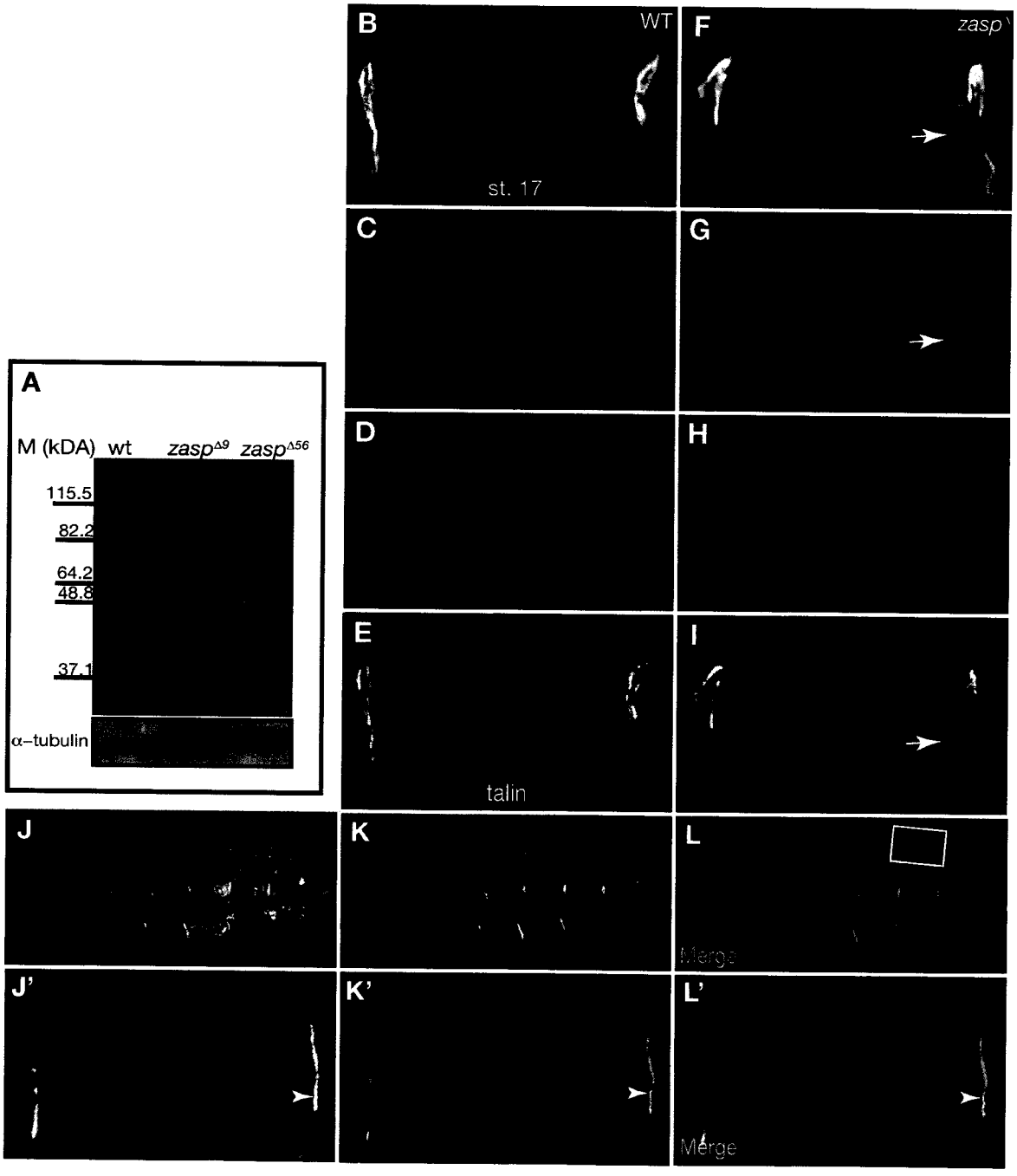
3.3.1 Zasp is required to strengthen the Integrin adhesion to the ECM

We have demonstrated that the muscle protein Zasp is enriched at the sites of Integrin adhesion, such as the myotendinous junctions of developing *Drosophila* embryos, and this localization requires Integrins (Jani and Schöck, 2007). Moreover, Zasp interacts genetically with αPS2 Integrin and late-stage 17 $zasp^{\Delta}$ embryos exhibit a progressive muscle detachment phenotype with lower penetrance, but otherwise similar to βPS Integrin mutant (Jani and Schöck, 2007). Collectively, these data indicates that Zasp is required to maintain Integrin adhesive function during muscle contraction. Thus, we classified Zasp as a new member of the Integrin adhesion complex. Yet, irrespective of this, it remains unanswered how Zasp affects Integrin function. We therefore asked whether Zasp is required to maintain the Integrin-cytoskeletal link or Integrin adhesion to the ECM. We first confirmed the absence of all major Zasp isoforms by Western Blotting of $zasp^{\Delta}$ mutants (Figure 3.3.1 A). In the absence of Zasp, αPS2 Integrin still localizes at the ends of detached muscles, but separates from the ECM ligand Tiggrin (Figure 3.3.1 G), indicating that Zasp plays a role in Integrin adhesion to the ECM.

In *Drosophila* the interaction between Talin head and Integrin cytoplasmic tail is required to strengthen Integrin binding to the ECM (Tanentzap and Brown, 2006). Therefore, we asked if Zasp mediates Integrin

Figure 3.3.1. Absence of Zasp causes defects in Integrin adhesion to the extracellular matrix.

(A) Anti-Zasp Western Blot. Zasp protein isoforms detected in wild type extracts (lane 1) are absent from first instar *zasp*$^{\Delta}$ extracts (lane 2 and 3). α-tubulin serves as a loading control. Comparison of myotendinous junctions in lateral muscles of wild type (WT) (B-E) and zygotic mutant (*zasp*$^{\Delta}$) embryos (F-I). (B-I) Triple staining with rat anti-αPS2 Integrin (green), rabbit anti-Talin (white), mouse anti-Tiggrin (red), and Merge is shown in (B and F). (B-E) In wild-type embryo, the Integrin ECM ligand Tiggrin (D), αPS2 Integrin (C), and Talin (E) colocalize tightly at myotendinous junctions. In contrast, in a Zasp-deficient (*zasp*$^{\Delta}$) embryo (F-I), Talin (I) and αPS2 Integrin (G) become separated from Tiggrin (H). Secondary antibodies against αPS2 Integrin and Talin were labeled with Alexa Flour 488nm and 633nm, respectively, to avoid any possibility of cross-excitation or bleed-through. (J-L) Zasp colocalizes with Talin at myotendinous junctions. (J) Anti-Zasp antibody (red) and anti-Talin antibody (green) (K) and merge of stage 16 embryo (L). Indicated areas are shown enlarged in J'-L'.


A
M (kDA) wt zasp^Δ9 zasp^Δ56
115.5
82.2
64.2
48.8
37.1
α–tubulin
B
WT
st. 17
F
zasp^1
C
G
D
H
E
talin
I
J
K
L
Merge
J'
K'
L'
Merge

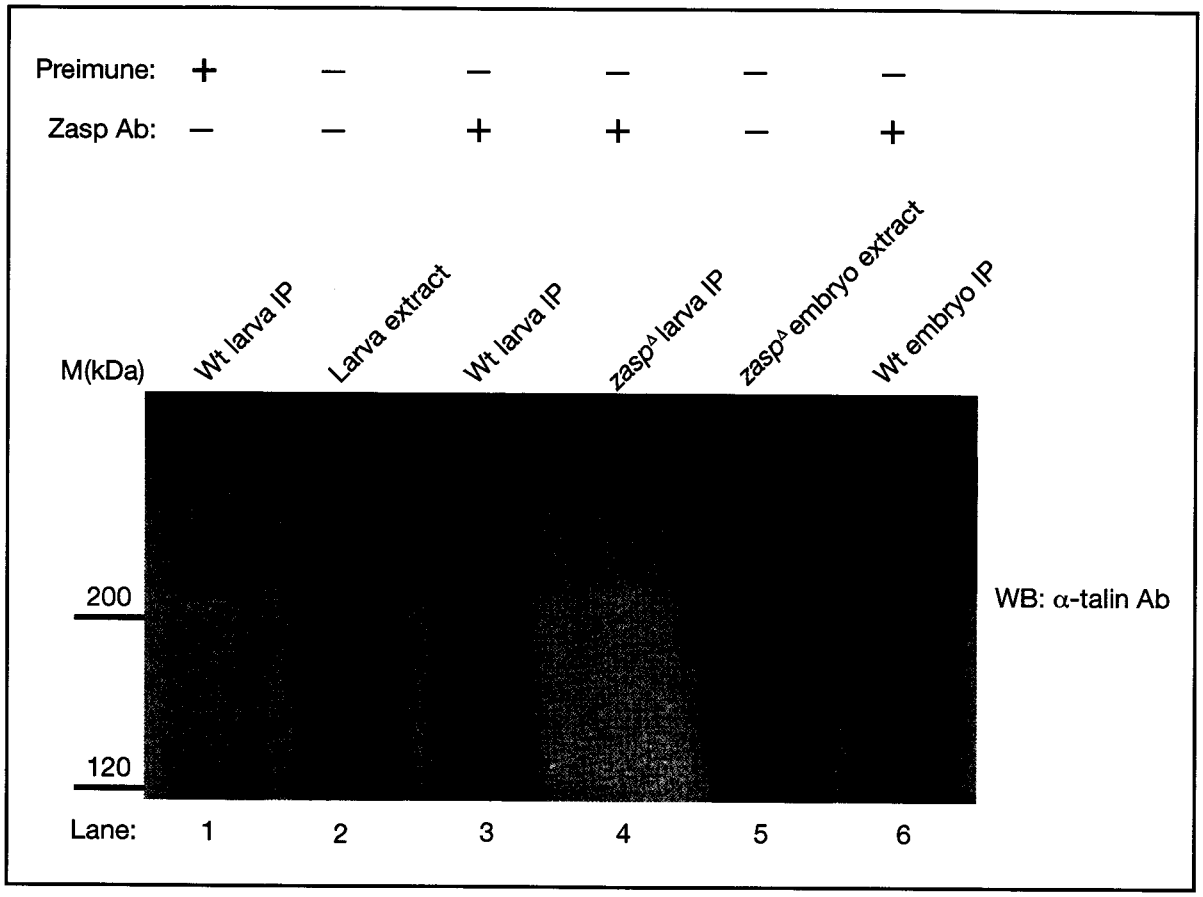
adhesion via Talin. Because Talin is also concentrated at myotendinous junction, we analyzed its localization in relation to Zasp in wild type and mutant embryos. Coimmunostaining with anti-Zasp and anti-Talin antibodies reveals that Zasp tightly colocalizes with Talin at myotendionus junction in the wild type embryo (Figure 3.3.1 L). Interestingly, in *zasp*$^{\Delta}$ embryos Talin is still found at the ends of the detached muscles along with αPS2 Integrin but separated from Tiggrin (Figure 3.3.1 I). This is not surprising, because a mutation in the Talin head domain that prevents Talin head binding to Integrin still results in recruitment of full-length Talin to Integrin adhesions (Tanentzapf and Brown, 2006). The combined data supports the above hypothesis, suggesting the likely possibility of Zasp mediating Integrin adhesion to ECM via Talin.

3.3.2 Zasp complexes *in vivo* with Talin

To explore the hypothesis of Talin bridging Zasp to Integrin we examined the possibility of a Zasp-Talin interaction *in vivo* by means of native coimmunoprecipitation assays. Protein extracts from wild type embryo and first instar larvae were immunoprecipitated with anti-Zasp antibody. Subsequently, the formation of Zasp-Talin complex was examined by immunoblotting using anti-Talin antibody. Figure 3.3.2 shows detection of Talin in immunoprecipitated extracts from wild type larvae and embryo (lane 3 and 6, respectively). In contrast, the absence of Zasp prevents complex formation (lane 4) in the same way as preimmune serum fails to immunoprecipitate tissue extracts (lane 1), demonstrating the specificity of this interaction. Zasp antibody also coimmunoprecipitates α-Actinin (Jani and Schöck, 2007), suggesting that Zasp may be an adaptor linking Talin and α-Actinin. The specificity of the co-precipitation between Zasp and Talin confirms that the two proteins are present in a common molecular complex *in vivo*.

Figure 3.3.2. Zasp forms complex with Talin *in vivo*.

Immunoprecipitation (IP) was performed to investigate if Zasp and Talin interact *in vivo*. Tissue extracts from wild type and $zasp^{\Delta}$ mutant embryo/1-st instar larvae were immunoprecipitated with anti-Zasp antibody or control preimmune and protein complexes were then immunoblotted with anti-Talin or anti-α-Actinin antibodies. Expression of Talin in crude extracts from embryos and larvae were used as controls. Only immunoprecipitated extracts from WT embryos and larvae (lane 6 and 3) form a Zasp-Talin complex in contrast to $zasp^{\Delta}$ (lane 4) or immunoprecipitated extracts with preimmune serum (lane 1).


Preimune: + – – – – –
Zasp Ab: – – + + – +
Wt larva IP
Larva extract
Wt larva IP
zasp^Δ larva IP
zasp^Δ embryo extract
Wt embryo IP
M(kDa)
200
120
WB: α-talin Ab
Lane: 1 2 3 4 5 6

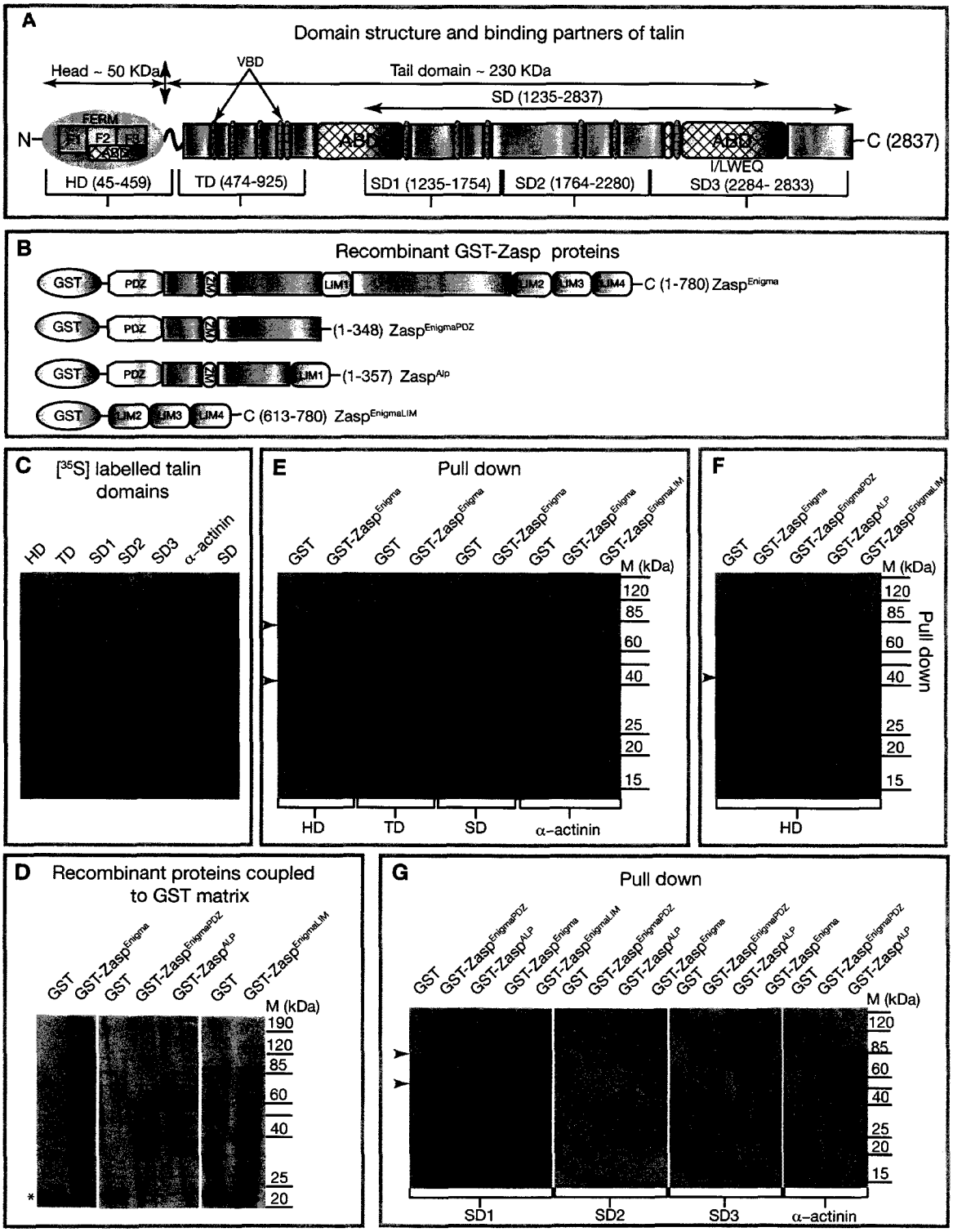
3.3.3 Both the PDZ and LIM domains of Zasp are required for binding Talin

Next, we sought to investigate whether the interaction between Zasp and Talin is direct. We therefore performed *in vitro* binding assays. An *E. coli* cleared lysate from a strain expressing full-length GST-Zasp fusion protein (GST-Zasp^{Enigma}) (Figure 3.3.3 B and D) was immobilized on glutathione-agarose beads and tested for binding to [^{35}S] radiolabeled head (HD), Talin (TD), and tail (SD) regions of Talin and full-length α-Actinin produced by *in vitro* translation (Figure 3.2.3 A and C). Radiolabeled proteins were incubated with equal amounts of GST or GST-Zasp^{Enigma} coupled to beads and bound fractions were recovered and separated by SDS-PAGE analysis (Figure 3.3.3 D). As studies in vertebrates have demonstrated a direct interaction between the N-terminal PDZ domain of Zasp and spectrin repeats of α-Actinin (Xia et al., 1997), we tested the possibility of this interaction with *Drosophila* derived recombinant Zasp protein. As expected, the GST-Zasp^{Enigma} pulls down α-Actinin, whereas the truncated GST-Zasp^{EnigmaLIM} fusion protein, containing the C-terminal LIM domains (Figure 3.3.3 E), fails to so. Intriguingly, in addition to α-Actinin, GST-Zasp^{Enigma} appears to bind the head region of Talin (Figure 3.3.3 E). In contrast, GST alone does not pull down the radiolabeled Talin head region, demonstrating the specificity of this interaction. Thus, Zasp and Talin are able to bind to each other *in vitro* in the absence of other proteins.

Due to the large molecular weight of Talin tail region (SD~190kDa), which structurally might hinder Zasp binding sites in Talin, we divided the tail region into three subregions. In reciprocal pull-down experiments, the radiolabeled (SD1) region (amino acid 1235-1754) associates with GST Zasp^{Enigma} but not with GST alone (Figure 3.3.3 G). These observations indicate that the Zasp binding site in tail region resides within amino acids 1235-1755. This region of Talin includes an Actin-binding domain predicted by http://blocks.fhcrc.org/, as well as by *in vitro* observations in vertebrates, which

Figure 3.3.3. Zasp interacts physically with Talin *in vitro*

Schematic presentation of Talin domains (A) and of GST tagged deletion constructs of Zasp (B). (C) ^{35}S-labeled Talin regions and full-length α-Actinin. (D) Expression, purification and coupling of GST-tagged Zasp fusion proteins in E. coli cells verified by Coomassie blue staining. Bands corresponding to the size of the recombinant proteins are depicted by an asterisk. (E) Pull-down of GST-Zasp^{Enigma} with each of the ^{35}S-labeled Talin regions and α-Actinin. Zasp interacts with Talin head region and full-length α-Actinin. Note, the GST-Zasp^{EnigmaLIM} fails to bind α-Actinin. (F) Pull-down of Zasp deletion variants with Talin head domain and Talin tail regions. (G) The LIM domain of Zasp interacts with the head region, while the PDZ domain associates with the Actin-binding tail region SD1.


A
Domain structure and binding partners of talin
Head ~ 50 KDa
VBD
Tail domain ~ 230 KDa
SD (1235-2837)
FERM
F1
F2
F3
ABS
N
ABD
ABD
I/LWEQ
C (2837)
HD (45-459)
TD (474-925)
SD1 (1235-1754)
SD2 (1764-2280)
SD3 (2284- 2833)
B
Recombinant GST-Zasp proteins
GST
PDZ
ZM
LIM1
LIM2
LIM3
LIM4
C (1-780) Zasp Enigma
(1-348) Zasp EnigmaPDZ
(1-357) Zasp Alp
C (613-780) Zasp EnigmaLIM
C
[35S] labelled talin domains
HD
TD
SD1
SD2
SD3
α-actinin
SD
D
Recombinant proteins coupled to GST matrix
GST
GST-Zasp Enigma
GST-Zasp EnigmaPDZ
GST-Zasp Alp
GST-Zasp EnigmaLIM
M (kDa)
190
120
85
60
40
25
20
E
Pull down
M (kDa)
120
85
60
40
25
20
15
HD
TD
SD
α-actinin
F
Pull down
HD
G
Pull down
SD1
SD2
SD3
α-actinin

have concluded that the spanning residues 951-1,327 co-sediments with F-Actin (Hemmings et al., 1996). Intriguingly, this region also contains three Vinculin binding sites (Figure 3.3.3 A).

Next, we sought to determine the specificity of Zasp domains required for binding Talin. The C-terminal of Zasp contains three LIM domains that share 50% amino acid identity with its human orthologue (Jani and Schöck). LIM domains are cysteine-rich regions that bind zinc ions, resulting in formation of zinc finger-like structures (Sanchez and Rabbitts, 1994; Kadrmas and Beckerle, 2004). They are found in a diverse group of proteins, including transcription factors and cytoskeletal proteins and they mediate protein-protein interactions (Sanchez and Rabbitts, 1994; Kadrmas and Beckerle, 2004). In mice, *in vitro* assays have established an interaction between Talin and the LIM domain of muscle specific protein N-RAP (Luo et al., 1999). In addition to the conserved LIM region, Zasp contains the consensus sequence for an N-terminal PDZ domain. PDZ domains are usually found in cytoplasmic proteins, which function to anchor transmembrane proteins to the cytoskeleton and to hold together signaling complexes (Ponting et al., 1997).

We assessed domain specificity by using a series of GST recombinant Zasp fragments spanning the entire length of the protein (Figure 3.3.3 A) and subjecting them to *in vitro* binding assays. In each experiment, samples were assayed by Coomassie staining to ensure equal loading of GST and GST fusion proteins (Figure 3.3.3 D). The recombinant proteins GST-Zasp^{EnigmaPDZ} and GST-Zasp^{ALP} containing the PDZ domain of Zasp bind the tail region of Talin (Figure 3.3.3 G), whereas the head domain of Talin interacts with the C-terminal LIM domains of Zasp (Figure 3.3.3 F). Noteworthy, the PDZ domain of Zasp also pulls down α-Actinin (Figure 3.3.3 G), indicating the proficiency of this assay.

3.4 Conclusion

In summary, our results suggest that weakening of Integrin's ability to bind ECM can be attributed to loss of Zasp function. As the possibility of Zasp interacting with Integrin directly remains to be explored, our results envisage the interaction of Zasp with Talin as a potential mechanism for regulating Integrin adhesion to the ECM. Perhaps the direct association of Zasp with the head region of Talin may regulate adhesion of Integrin to its extracellular matrix components, whereas the binding of PDZ domain of Zasp to the Talin's rod may control Talin-mediated Integrin attachment to the cytoskeleton.

Future work directed towards understanding the mechanism of regulation of Integrin adhesion will likely elucidate whether Zasp is a direct Integrin effector or an activator that enhances Talin-induced Integrin activation.

3.5 Materials and methods

3.5.1 Immunoprecipitation assay

Frozen tissues from embryos and first instar larvae were homogenized with precooled Dounce homogenizer in lysis buffer (30mM Hepes, pH 7.4, 150 mM KCl, 1mM EGTA, 1mM MgCl2, 10% glycerol, 0.1% Nonidet-P40, 0.5 mM DTT, plus one tablet of EDTA-free complete protease inhibitor cocktail (Roche Diagnostics) at a concentration of 0.5 ml tissue/ml lysis buffer. 1 ml of protein extract obtained after centrifugation of the homogenate was transferred into a fresh microcentrifuge tube and precleared with 100 μl of homogenous Protein A/G Plus-Agarose (Santa Cruz Biotechnology) suspension (25 μl bed volume) for al least 3 hours at 4°C with rocking. After pelleting the beads by centrifugation the supernatant was transferred into a fresh microcentrifuge

tube and consecutively incubated with 30 μl of preimmune serum or 30 μl of anti-Zasp serum for 4 hours at 4°C with rocking. 50 μl of homogenous Protein A/G Plus-Agarose suspension was added to the mixture and incubated overnight at 4°C with rocking. The agarose-antibody-antigen complexes were collected by gravity sedimentation and beads were washed 4 times with low stringency buffer (1xPBS, 0.1% NP-40) and finally with 1xPBS. The bound proteins were resuspended in 30 μl of 2 X sample elution buffer and eluted by boiling for 4 min. 1/10 of suspension was resolved by 5% SDS-PAGE and blotted on Hybond-C extra nitrocellulose membrane (GE Healthcare) for detection with anti-Talin monoclonal antibody (1-100). Anti-mouse IgG horseradish peroxidase-linked secondary antibody (1-2500) was used together with the ECL detection kit for visualization (GE Healthcare).

3.5.2 RT-PCR

RT-PCR was used to generate cDNAs that cover the Talin head (amino acid 45-459) and TD region (amino acid 474-925). Total RNA, isolated from *Drosophila* embryos using Trizol reagent (Invitrogen) according to the manufacturer's instructions, was reverse transcribed and domain-specific cDNAs were amplified with primer sets containing a start and a stop codon as follows: the primer set ATGCAACCCAGCGAATATGGACT and TTATTCGTGTTGCAAGAAGGTG for the head domain; ATGGAAGTGCAGACCGTGCAGGTA and TTACGTGTTAGCCGCAGTAGTGGTG for Talin domain. Additional PCR amplifications were also performed to introduce T7 promoter sequence (TAATACGACTCACTATAGGG) flanked 5' with an adapter (TCAAT) and *Eco*RI restriction site (GGATCC) sequences and 3' with a spacer (AG) and Kozak (CCACC) sequences.

For the Talin tail domain (amino acid 1235-2832) cDNA was amplified from EST SD07967 as a template with primers dividing it into three nonover-

lapping regions each amplified with primer sets containing a start and a stop codon: SD1: ATGAGTGAATTCCCACCTTCAAGC and TTAAGCTCCAATTGCTCCATT; SD2: ATGCAAATGTCGCTAATTCAGCA and TTATACGCGACGCGACAAATG; SD3: ATGACCGATCTGGTGGCCAT and TTAACTGTGCTTTTCCGTTTTCG. A second round of amplification was performed using forward primers flanked by T7 promoters as described above.

For α-Actinin, cDNA was obtained from EST LD37956 cloned into T7-containing pOT2 vector.

3.5.3 *In vitro* translation

^{35}S-labeled proteins were generated with the TNT Quick-coupled transcription/translation system (Promega) in the presence of 10 mCi/ml [^{35}S] methionine (PerkinElmer). 2 µg pOT2 plasmid DNA containing T7 promoter as well as of purified PCR-generated templates were mixed with 40 µl TNT Quick Master Transcription Mix and 2 µl [^{35}S] methionine. The reaction mixtures were incubated in a 50 µl reaction volume for 60-90 minutes at 30°C. 2 µl of labeled proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to standard procedures and gels after fixing were treated with fluorographic Amplify reagent (Amersham Biosciences) for signal enhancement according to recommendations of the manufacturer. Gels were dried for 2 hours at 80°C (DryEase Gel Drying System, Invitrogen) and translation products were quantified by using a Phosphor Imaging system (Molecular Dynamics). Translation efficiency and activity was directly measured from the reaction mixture containing unlabeled methionine.

3.5.4 Molecular cloning

The GST-Zasp: GST-Zasp^{EnigmaPDZ} (1-348), GST-Zasp^{ALP} (1-357), GST-Zasp^{Enigma} (1-780), and GST-Zasp^{EnigmaLIM} (613-780) fusion proteins were obtained by digesting PCR-generated fragments from *Drosophila* Zasp clones RH03424 (for Enigma constructs) and LP01550 (for ALP) with EcoRI/XhoI followed by cloning into EcoRI/XhoI-digested pGEX-5X-1 (Pharmacia Biotech). Specifically, the PCR products: GST-Zasp^{EnigmaPDZ}, GST-Zasp^{ALP}, and GST-Zasp^{Enigma} were generated with a forward primer TCAAT**GAATTC**GCCCAACCACAG all containing an *Eco*RI restriction site (bold) and reverse primers ATCCGCTCGAGAAATGCCCACGCCGCAC for GST-Zasp^{EnigmaPDZ}, ATCCGCTCGAGTGCCGGAGCAGGGGTG for GST-Zasp^{ALP}, and ATCCGCTCGAGGCGCGCGTGATT for GST-Zasp^{Enigma} all containing a *Xho*I restriction site (underlined). The PCR product GST-Zasp^{EnigmaLIM} was generated with the forward primer TCAAT**GAATTC**AGAGGACCCTTTATC containing an *Eco*RI restriction site (bold) and the reverse primer ATCCGCTCGAGGCGCGCGTGATT containing a *Xho*I restriction site (underlined). The positive clones were verified by PCR, restriction digest analysis, and automated sequencing. Plasmids were transformed into the *Escherichia coli* strain Bl-21 bacteria for protein expression. *E. coli* strains harboring parental or recombinant pGEX plasmids were grown overnight at 37°C in Luria-Bertani medium plus ampicillin. Cultures were diluted 1/20 into 100 ml of fresh medium and grown for 2 h at 37°C, followed by the addition of isopropyl-β-d-thiogalactopyranoside (IPTG) to 1 mM. After induction for 4 hours at 37°C, the cells were pelleted and resuspended in ice-cold lysis buffer (PGK: 50mM sodium phosphate, pH 7.2, 10% glycerol, 100mM KCl, and 0.5% Triton X-100) at a ratio 1g (wet weight) of cells to 20 ml of buffer. Cells were lysed by incubation for 1 hour on ice, followed by sonication and then centrifugation at 16,000 × *g* and 4°C for 20 min. The cleared supernatants

were adjusted in volume such as to produce equal molar ratios between fusion proteins and GST alone. Such volumes were then incubated with 30 µl of Promega slurry magnetic beads (15 µl bed volume) for 4 hours at 4°C with gentle agitation, followed by serial washes in GST Binding/Wash buffer (4.2 mM Na_2HPO_4, 2mM KH_2PO_4, 140 mM NaCl, and 10 mM KCl). The concentration of each recombinant protein was assessed by SDS-PAGE followed by gel staining with Coomassie blue.

3.5.5 GST pull-down assays

Radiolabeled proteins were precleared prior to binding by incubation with GST-bound glutathione-agarose beads for 30 min at 4°C. For binding assays, *in vitro*-translated reaction products were incubated with 15 µl settled GST-fusion beads in GST Binding/Wash buffer containing 0.1% NP-40 to a final volume of 200 µl for 4 h at 4°C with rotation. After 5 washes with 400 µl of GST Binding buffer/Wash buffer containing 0.2% NP-40, bound radioactive proteins were resuspended in 2XSDS sample buffer and resolved by 10% SDS-PAGE, followed by autoradiography.

3.5.6 Immunohistochemistry

Stage 17 embryos were fixed using heat fixation (Jani and Schöck, 2007). In brief, embryos were dechoroniated in 50% bleach for 90 s, rinsed thoroughly in water, immersed in boiling 1 x embryonic wash buffer (70mM NaCl and 0.1% Triton X-100) for 10 sec with agitation, immediately cooled by adding 3 volumes of ice-cold embryonic wash buffer, and placed on ice for at least 30 min. Embryos were devitellinized in methanol/heptane. Primary antibodies were used as follows: rabbit anti-Talin (1:100; provided by N.H. Brown; Brown et al., 2002), rat anti-αPS2 (1:10; 7A10; provided by N.H. Brown, Gurdon

Institute, University of Cambridge, Cambridge UK; Brower et al, 1984), and mouse anti-Tiggrin (1:300; provided by J. H. Fessler; Fogerty et al., 1994). Fluorescently labeled secondary antibodies of the Alexa series (Molecular Probes) were used at 1:300 dilutions.

Chapter 4

General Discussion

4.1 Summary

The work described in this thesis addresses the role of Zasp in Integrin-mediated adhesion. In contrast to classical EMS and P-element insertion screens used to search for genes controlling Integrin-mediated adhesion, we identified Zasp in an RNAi screen using *Drosophila* tissue culture cells. This cell-derived assay is advantageous since it allows to interrogate the biological function of nearly all genes predicted from genomic sequencing (Kiger et al, 2003; Schöck and Perrimon, unpublished data) based on the ability of cells to rapidly take up long dsRNAs added to the medium and to cause efficient depletion of their targeted mRNAs (Clemens et al., 2000).

To identify novel genes involved in Integrin-mediated cell spreading we used the adherent S2R+ cells because of their capacity to assume morphological changes upon gene targeting (Kiger et al., 2003; Kunda et al., 2003) and of the presence of putative Integrin adhesion complexes composed of focal adhesion proteins like Talin, Vinculin, and Phosphotyrosine (Jani and Schöck, 2007). Disrupting the function of Talin leads to spreading defects similar to those observed during Integrin depletion, stressing S2R+ cell independence in Integrin-mediated spreading (Jani and Schöck, 2007).

The exclusive spreading/adhesion defects observed in Zasp-depleted S2R+ cells as well as its localization to Integrin adhesion sites are the initial indications for Zasp as a regulator of Integrin-mediated adhesion (Figure 2.3.2). Our data from developing *Drosophila* embryos supports this finding, as they demonstrate that Zasp colocalizes with Integrin at myotendinous junctions (Figure 2.3.5), which are elaborations of focal adhesion in culture, and that $zasp^{\Delta}$ mutants exhibit muscle attachment defects similar to Integrin mutants (Figure 2.3.9). Moreover, the strong genetic interaction observed between Zasp and Integrin during muscle contraction (Figure 2.3.10) and wing formation reinforces the hypothesis of Zasp functioning as a regulator of

Integrin.

In the course of understanding the mechanism by which Zasp controls Integrin-mediated adhesion, we discovered that in the absence of Zasp the most upstream adhesion component Talin, still localizes at the end of detached muscles along with Integrin but separated from ECM (Figure 3.3.2). This phenotype becomes apparent at embryonic stage 16 and is enhanced during subsequent development, as the force of muscle contraction increases.

The absence of Zasp also disrupts the sarcomere cytoarchitecture. At the ultrastructural level, *zasp*$^{\Delta}$ mutant larvae muscle fibers either lack striation or show a rudimentary striated pattern (Figure 2.3.7), suggesting a flawed cytoskeletal assembly. In addition, the Actin filaments are often disorganized and no longer aligned in bundles (Figure 2.3.7). A plausible function for Zasp, describing this phenotype may be the organization of the Actin cytoskeleton. This hypothesis is in line with our finding that Zasp physically interacts with the Actin cross-linking protein α-Actinin.

Collectively, our results point to a dual role for Zasp as a structural scaffold. First it regulates Integrin adhesion to the extracellular matrix by interacting with the head domain of Talin at the myotendinous junctions. Second, Zasp controls sarcomere assembly by tethering the presarcomeric α-Actinin component to the tail domain of Talin.

In the following sections, I will highlight some unanswered inquiries arising from this work, which will facilitate the direction of future investigations.

4.2 Identification of Zasp targets required in Integrin-mediated adhesion

From our investigation, Zasp appears to function as a structural scaffold, assembling the protein complexes comprised of Integrin-Actin linkers. The combination of the PDZ and LIM domains allows Zasp to interact with different molecular partners, potentially forming protein networks at the membrane as well as at Z-lines.

In Chapters 2 and 3, I demonstrated that Zasp binds to and colocalizes with α-Actinin, which is a component of both Integrin adhesion sites and sarcomeric Z-lines. Zasp protein interacts with α-Actinin through its PDZ domain. Yet, in vertebrates has been demonstrated that Zasp translocation to the Z-lines is seemingly not dependent on this domain (te Velthuis and Bagowski, 2007). Instead, the Zasp motif (ZM) is shown to be the primary structure involved in its translocation (te Velthuis and Bagowski, 2007; Klaavuniemi et al., 2004; Zhou et al., 2001). Although, shorter splice variants of Zasp, encoding the PDZ domain and ZM motif only, can rescue the phenotypes caused by Zasp depletion in both zebrafish and mouse embryos (van der Meer et al., 2006; Huang et al, 2003). So why would *zasp* encode LIM domains if they are apparently not essential for Z-line assembly? We assume that the LIM domains are required to maintain the integrity of the myotendinous junctions upon muscle contraction, and we propose diverse models, by which Zasp mediates this function.

First, Zasp may act as a direct activator of Integrin. It is accepted that transition from low to a high affinity state for ligand is triggered intracellularly by binding one of two NPXY motifs of the Integrin β cytoplasmic tail (Calderwood, 2004; Schwartz et al., 1995). The third LIM domain of the vertebrate Zasp orthologue Enigma associates with the NPXY motif of the insulin receptor cytoplasmic tail (Wu and Gill, 1994). In *Drosophila,* we demonstrate that Zasp colocalizes with Integrins at myotendinous junctions of

developing embryos, and that this localization is dependent on Integrins. Moreover, in the absence of Zasp Integrin detaches from the extracellular matrix. Perhaps, docking of the LIM domain of Zasp at NPXY motif is required to alter the affinity of Integrin for ligands.

Second, the nonphosphorylated N-terminal NPXY-motif in Integrin tail is proposed to be the first target of Talin binding (Garcia-Alvarez et al., 2003). Therefore, we also envisage Zasp as a Talin co-activator that enhances Talin-induced Integrin activation. This prediction is consistent with our *in vitro* finding that the LIM domain of Zasp binds the head of Talin (Figure 3.3.5). Two likely explanations suggest that either the occupation of the β cytoplasmic tail of Integrin by Zasp could position it in a more favorable conformation for interaction with Talin or Zasp binding to Talin may increase its affinity for the Integrin cytoplasmic tail. Such functional synergism between Zasp and Talin may eventually strengthen the Integrin link to the extracellular matrix.

Finally, Zasp enriched at the muscle ends may also serve as a scaffold for other Integrin binding partners or activators. It is extensively believed that the regulation of Integrin-mediated adhesion depends largely on phosphorylation and dephosphorylation of specific linkage proteins to enable modifications of their interactions. *In vitro* and *in vivo* studies in vertebrate systems have demonstrated that PKC phosphorylates proteins localized to focal adhesions, including Talin (Watters et al., 1996; Pietromonaco et al., 1998; Critchley, 2004; Ratnikov et al., 2005), Vinculin (Werth et al., 1983; Ziegler et al., 2002; Perez-Moreno et al., 1998), and Integrin (Gimond et al., 1995). PKC-mediated phosphorylation of Talin appears to influence Talin binding to Integrin (Burn et al., 1988) and phosphorylation of Integrin affects Integrin ligand-binding affinity. Based on our results, we assume that weakening of Integrin's ability to bind ECM in the absence of Zasp can be attributed in part to inability of PKC recruitment to adhesion complexes, presumably preventing subsequent phosphorylation of PKC targets. Support

for this hypothesis comes from studies in mice demonstrating that Enigma, a Zasp orthologue, targets PKC directly to the sarcomere via its C-terminal LIM domains (Kuroda et al., 1996; Zhou et al., 1999). Temporal sequestration of PKC by Zasp at the sites of integrin adhesions may, in turn, mediate specific phosphorylation of the Integrin tail as well as the Talin head along with its tail region. As such, it is reasonable to posit that subsequent phosphorylation of Talin by PKC may influence Talin binding to Integrins and the Actin cytoskeleton.

Mechanistically, we speculate the PKC-Zasp interaction to be transient to ensure that PKC phosphorylates only appropriate targets *in vivo*. Mutation in the third LIM domain of Cypher (the mouse homologue of Zasp) increases its affinity for PKC compared with the wild-type Zasp protein (Arimura et al, 2004). Furthermore, Enigma phosphorylation by PKC leads to its translocation from the membrane to the cytosol *in vivo* (Kuroda et al., 1996). Therefore, Zasp phosphorylation may decrease its affinity for Integrin and Talin, consequentially leading to its detachment and translocation to other locations.

In *Drosophila*, investigation of PKC-mediated regulation, unlike studies in vertebrates, is far from being revealing. There are 6 known PKC *Drosophila* isoforms: PKCδ, PKC98E, PKC53E, inaC, aPKC, and Pkn (protein with a PKC-like kinase domain) and very little is known about their expression pattern during embryonic development (Shieh et al., 2002). Future work will address the specificity of PKC isoforms that act on the same molecular pathway as Zasp, by attempting to perform a co-RNAi enhancer screen in S2R+ cells, which will allow us to identify modifiers of the Zasp dsRNA-induced cell adhesion phenotype. By using a lower concentration of Zasp dsRNA, we can observe a less severe adhesion phenotype. Our aim is to find those isoforms that will enhance Zasp RNA-induced phenotype from mild to severe. To tackle the redundancy we will perform a screen by co-incubating dsRNA(s) of PKC isoforms in all possible combinations. In case this approach fails to be concl-

usive, we will perform some additional genetic approach with PKC98E and PKCδ, which are homologues of vertebrate PKCε and PKCδ respectively. These homologues are shown to localize in muscle Z-lines and play roles in Integrin-mediated adhesion and sarcomerogenesis (Borg et al., 2000; Strait et al., 2001).

We cannot exclude the possibility of Zasp binding to other proteins. In flies, Zasp was demonstrated to be a component of myotendinous junction and Z-lines, which are structures that contain multiple protein complexes. Thereby, identifying these components will be an important point to address in the future. A feasible approach could be the purification of complexes immunoprecipitated with Zasp antibody. Proteomic techniques, such as mass spectrometry, can in principle be used to identify proteins that are enriched following immunoprecipitation. Subsequently, follow-up genetic experiments could test whether the regulation of these candidates is affected in *zasp*$^{\Delta}$ mutants.

4.3 Zasp regulates the sarcomere assembly

Our data supports the hypothesis that Zasp may play a direct role during sarcomerogenesis. Live imaging of the endogenous GFP-Zasp fusion protein (Jani and Schöck, 2007) shows a rather dynamic distribution of Zasp in the embryo (unpublished data). First, a punctate distribution of GFP-Zasp, exemplifying the sarcomere precursor, is observed in muscle ends at the sites of myotendinous junctions. Then, the growing sarcomeres aggregated in "pearl on the string" structures appear along the muscle fiber. As differentiation proceeds, these structures are oriented into transverse banded patterns representing the striations of mature sarcomere.

This dynamic distribution is consistent with the finding that loss of Zasp disrupts sarcomere assembly (Jani and Schöck, 2007). As such, we specula-

te that Zasp acts as a structural scaffold that regulates the assembly of α-Actinin and Actin into sarcomere precursors (refer to Introduction for further information on sarcomerogenesis). Supportively, we show that the PDZ domain of Zasp interacts directly with α-Actinin (Figure 3.3.3). Yet, the absence of Zasp does not fully inhibit α-Actinin binding to Actin filaments (unpublished data; Klaavuniemi et al., 2006). Thus, Zasp is possibly required to potentiate the α-Actinin-mediated Actin cross-linking. However, this Zasp function may not explain the absence of Z-lines observed in *zasp*$^{\Delta}$ mutant muscle fibers, since α-Actinin mutant larvae exhibit Z-line organization, indicating that the cytoskeletal assembly is initiated but is not maintained.

Given that tethering of the earliest sarcomere ancestors to the sarcolemma is the first crucial step of sarcomerogenesis (Dabiri et al., 1997; Rhee et al, 1994; Carroll et al., 2004), we suggest that the Z-line defects are likely caused by perturbations in interactions of sarcomere precursors with the myotendious junctions complexes. This hypothesis is based on our findings that Zasp is enriched at the myotendinous junctions (Jani and Schöck, 2007) and binds directly to Talin *in vitro* (Figure 3.3.4). Perhaps, Zasp binding to Talin is required for recruitment of α-Actinin to the sarcolemma, followed by α-Actinin and Actin organization into sarcomere precursors. Zasp-mediated positioning of presarcomeric components to the membrane may eventually prompt the recruitment of other sarcomeric structural components, leading to sarcomere maturation. In essence, Zasp via its interaction with Talin, seems to provide a positional cue for catalyzing precursor assembly.

4.4 Investigation of Zasp function in cytoskeletal remodelling

The major function of Actin cytoskeleton in muscle cells is to act as contractile machinery, whereas in non-muscle cells it is involved in multiple processes including cell polarity, cell shape change, and motility. Experiments on cultur-

ed mammalian cells have suggested that the PDZ-LIM domain protein family is not only required for focal adhesion assembly but also for cytoskeletal remodelling (Tamura et al., 2007).

In *Drosophila*, a non-muscle model of cytoskeletal remodelling is the single layered follicular epithelium that surrounds the oocyte (Wahlström et al., 2006). The follicle cells are polarized with the apical face facing the germ line and the basal side facing the epithelial sheath surrounding the egg chamber (Horne-Badovinac and Bilder, 2005). During egg elongation a rapid increase of the oocyte volume and a concomitant expansion of the follicular epithelium occurs, causing most of the follicle cells to take on a columnar morphology instead of cuboidal (Horne-Badovinac and Bilder, 2005). These morphological changes are likely dependent on the reorganization of the cytoskeleton elements, in particular Actin (Gutzeit, 1990).

During stage 10, the Actin filaments at the basal side of follicle cells (bordering the basement membrane) are organized in parallel bundles oriented perpendicular to the long axis of the follicle (Gutzeit, 1990). By late stage 14, the basal Actin filaments disappear coinciding with follicular flattening and degeneration. Therefore, Actin bundles maintain the columnar shape by restricting growth in the short axis (Bateman et al., 2001).

However, the alignment of basal Actin bundles in columnar follicle cells requires their attachment to the basement membrane that surrounds the follicle cells (Gutzeit, 1990; Gutzeit and Eberhardt, 1991). In addition to Integrins (Bateman et al., 2001), many focal adhesion components have been implicated in this process such as Talin (Bécam et al, 2005), Tensin (Lee et al., 2003), and α-Actinin (Wahlström et al., 2006), which directly link Integrin to Actin cytoskeleton.

Preliminary findings have shown that Zasp displays a rather dynamic distribution in the adhesion structures of follicle cells that form at the onset of egg elongation (unpublished data). As demonstrated in muscles, Zasp coloc-

alizes with Integrin and α-Actinin at these adhesion sites. Future work will be focused on studying whether Zasp promotes the attachment of the α-Actinin cross-linked Actin filaments to the basement membrane. Further insight on this topic can be gained by examining Zasp localization in clones of follicle cells lacking β-Integrin. Based on our previous work in muscles (Jani and Schöck, 2007), we would expect a rather disturbed pattern of Zasp distribution. This finding may suggest that the dynamic accumulation of Zasp in late-stage follicle cells depends on Integrin adhesion. Furthermore, investigation of Integrin and Actin filament assembly in clones of follicle cells lacking Zasp might further provide insights on cytoskeletal remodeling.

4.5 How is Zasp regulated?

Zasp contributes to the formation of adhesion complexes and the progression of sarcomerogenesis. These processes require strict spatiotemporal control, assuming that Zasp activity must be regulated.

4.5.1 Phosphorylation

We hypothesize that Zasp activity is regulated through phosphorylation. Enigma, the vetebrate Zasp orthologue, associates with PKC through its LIM domain and is phophorylated by PKC *in vitro* (Kuroda et al., 1996). We may address the topic of posttranslational modification of Zasp by using 2D SDS-PAGE in combination with phosphatase treatment. This could be investigated in embryo and larval extracts. For example, a slower migrating form of Zasp, which might correspond to the posttranslationally modified form of protein, in the presence of phosphatases would be expected to lead to dephosphorylation of Zasp and reversion to a faster migrating form of the protein. Subsequently, if it is established that Zasp is modified through phos-

phorylation, the next step would be to try to map the residues that are phosphorylated. Further insight on this topic can be gained by mapping the amino acid residues that are phosphorylated using mass spectrometry and then conducting mutagenesis through substitution or deletion of the phosphorylated residues to create phospho-mimicking and phospho-inhibiting mutations. These specific mutations could then be examined transgenetically in the background of Zasp alleles, which would allow for an *in vivo* understanding of the role of Zasp phosphorylation.

We hypothesize that phosphorylation of Zasp by PKC modulates Zasp function. Indeed, translocation of Zasp from membrane to cytosol is observed upon its phosphorylation (Kuroda et al., 1996). Thus, we are interested to further explore whether there is a localization/function relationship upon Zasp phosphorylation.

4.5.2 Zasp regulation by its splice variants

The fact that *zasp* gene encodes different domains allows for alternative processing that can generate different combinations of these domains and motifs. At least three different alternatively spliced forms of Zasp have been identified (Figure 3.3.1 A). In addition to RNA and protein analysis, expression of GFP-tagged isoforms could be useful for analysis of their function *in vivo*. Apart from information on their localization, the expression of the fluorescently-labelled splice variants in Zasp mutant background may provide insights on domain specificity required for muscle functioning.

Alternatively, spliced variants of Zasp could also serve as modulators of full-length Zasp. In vertebrates, the shorter splice variants of Enigma are suggested to play a dominant negative role toward the longer variant, possibly by competing with the PDZ domain docking sites (i.e., α-Actinin), ultimately resulting in inhibition of scaffold formation between the PDZ and LIM binding

proteins (e.g. PKC) (te Velthuis and Bagowski, 2007; Nakagawa et al., 2000). We could thus explore the possibility for dimer formation through their expression in *Drosophila* S2R+ cells. Individual- and co-expression of HA and V5 tagged-splice variants in Zasp depleted cells (RNAi targeting the 3'UTR of endogenous Zasp) and subsequent protein analysis with anti-HA and anti-V5 may give rise to a higher molecular mass complex compared to the single transfected cells. Likewise, examination of spreading behavior in single transfected and co-transfected cells with given splice variants may reveal the domain specificity required for Zasp localization and function at Integrin adhesion sites.

4.5.3 Zasp regulation by growth factors

In general, the cross talk between Integrins and growth factor receptors regulates cellular responses that converge at focal adhesion sites (Eliceiri, 2001). Growth factors can regulate Integrin-mediated events such as cell adhesion, cell spreading, and cell migration through alterations in Integrin localization and activation (Klemke, et al., 1994; Mainiero et al., 1996; Trusolino et al., 1998). Agonist stimuli cause alterations in Integrin affinity for ligand (Kim et al., 2003; Han et al., 2006; Caldewood, 2004; Qin et al., 2004).

Apart from a direct association between Integrin and growth factors (Schutt et al., 2004; Narita et al., 1996) an intersection between the growth factors and Integrin resides at their downstream signaling molecules. The agonist receptors (e.g., G protein-coupled receptors, tyrosine-kinase-coupled receptors) induce activation of PKC that in turn, phosphorylates target proteins that complex with Talin, thus unmasking the Integrin binding site in Talin leading to Integrin activation (Han et al., 2006).

In *Drosophila*, the synergistic effect between Integrin and growth factors regulates muscles attachment to the epidermal tendon cells (Sastry et al.,

1996; Volk, 1999; Becker et al., 1997). The epidermal cells signal the myotubes and induce myotube attraction and adhesion to their target cells (Becker et al., 1997). During extension, the myotube projects the elongated filopodia towards the epidermal cells (Bate, 1990). After attachment, the muscles send signals to the epidermal cells for their further differentiation into tendon-like cells (Becker et al., 1997). Tendon precursors are characterized by the expression of *stripe* gene that encodes an early growth response-like transcription factor (De la Pompa et al., 1989; Frommer et al., 1996). Embryos that carry a *stripe* mutation exhibit defects in somatic muscle patterning in which the myotubes often send filopodia in incorrect directions. In late stages of embryonic development, most of the myotubes do not attach to the epidermis (Frommer et al., 1996).

The hypothesis that growth factor signaling might regulate Zasp derives from our observation that Zasp mutants exhibit muscle-pattering defects with muscles missing in some segments (unpublished data). Thus, Zasp under the control of tendon cells likely may contribute to proper targeting and adhesion of myotubes to the tendon cell.

4.5.4 Zasp regulation by phorbol esters

There is a diverse literature on the effects of phorbol esters on cell adhesion and morphology. Phorbols, the analogue of DAG (Braun et al., 2005), in culture cells cause an increase in Integrin adhesiveness (Danilov and Juliano, 1989; Boudignon-Proudhon et al., 1996) by modifying Integrin affinity for ligand (Kucik et al., 1996) and this seems to correlate with Talin binding to Integrin (Burn et al., 1988). In other cases, phorbol ester treatment causes disruption of the cytosketon and cell rounding during cell spreading (Schliwa et al., 1988). Finally, their signal perturbs the assembly of striated myofibrilis in cultured myotubes (Lin et al, 1987; Lin et al., 1989).

Phorbol esters are potent activators of protein kinase C (Nishizuka, 1984, Braun et al., 2005). The hallmark of PKC activation by phorbols is its translocation to the subcellular compartments or membrane (Braun et al., 2005). The activated PKC may then phosphorylate substrates or interact with target proteins in a location-dependent fashion. Phorbol ester effects on Integrin-mediated adhesion are possibly due to the phosphorylation of cytoskeletal proteins involved in Integrin-mediated adhesion (Danilov and Juliano, 1989). Indeed, Talin is phosphorylated *in vivo* upon exposure to a tumor-promoting phorbol ester (Beckerle, 1990, Burn et al, 1988).

Zasp seems to be a direct target of PKC phosphorylation mediated by phorbol esters. Upon treatment of cells with phorbols, ENH, a human Enigma family protein closely related to Zasp, interacts with PKC and activation of PKC results in phosphorylation and subsequent translocation of ENH from membrane to cytosol (Kuroda et al., 1996). Thus, Zasp emerges at the crossroad for integrating PKC signaling initiated by growth factors with other cellular activities.

4.6 Synopsis

Zasp function is highly conserved throughout eukaryotes. Analysis of Zasp in *Drosophila* has demonstrated several functional aspects that may provide insights on the mechanisms of human diseases related to the PDZ/LIM domain family of proteins.

4.6.1 Skeletal muscle development and disease

Skeletal muscle organization and integrity are regulated through protein networks assembled in complexes at different cell compartments. Analyses of muscle diseases caused by mutations in genes encoding for such complex

components have revealed their function in muscle development. This thesis has demonstrated that the PDZ/LIM domain protein Zasp localizes at the multiprotein complex-containing myotendinous junctions where it complexes *in vivo* with the cytoskeletal proteins Talin and α-Actinin possibly through its LIM and PDZ domain, respectively. In addition, from studies in vertebrate orthologues of Zasp, it appears as a scaffold for the signaling protein PKC (Kuroda et al., 1996; Zhou et al., 1999; Dyson et al., 2001). These Zasp-mediated interactions are essential for keeping the protein complexes at the sites of their functions or for regulating the activity of Zasp binding partners. Indeed, in the absence of Zasp dissociation of these complexes is observed, leading to skeletal muscle dysfunction. Collectively, these findings demonstrate a role for Zasp as a core component of these complexes.

4.6.2 Cell migration and cancer

Cell adhesion and migration are not only important during development, but also are critical steps in tumor invasion and metastasis (Ramsay et al., 2007; te Velthuis and Bagowski, 2007). Organization of adhesion complexes and Actin cytoskeleton are the most common mechanisms that control cell motility (te Velthuis and Bagowski, 2007). It is likely that Zasp, which is linked to focal adhesions and Actin cytoskeleton, plays an important role in the development of cancer. Elucidating the mechanisms by which Zasp influences cell adhesion would be beneficial for future research in cancer.

LIST OF REFERENCES

Alatortsev, V. E., I. A. Kramerova, et al. (1997). "Vinculin gene is non-essential in *Drosophila* melanogaster." Febs Letters **413**(2): 197-201.

Arber, S., G. Halder, et al. (1994). "Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation." Cell **79**(2): 221-231.

Arber, S., J. J Hunter, et al. (1997). "MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure." Cell **88** (3): 393 -403

Arimura, T., T. Hayashi, et al. (2004). "A Cypher/ZASP mutation associated with dilated cardiomyopathy alters the binding affinity to protein kinase C." Journal of Biological Chemistry **279**(8): 6746-6752.

Arnold, H. H. and T. Braun (2000). "Genetics of muscle determination and development." Current Topics in Developmental Biology, Vol 48 **48**: 129-164.

Artero, R., A. Prokop, et al. (1998). "The *muscleblind* gene participates in the organization of Z-bands and epidermal attachments of *Drosophila* muscles and is regulated by DMef2." Developmental Biology **195**(2): 131-143.

Au, Y. H., R. A. Atkinson, et al. (2004). "Solution structure of ZASP PDZ domain: Implications for sarcomere ultrastructure and Enigma family redundancy." Structure **12**(4): 611-622.

Ayme-Southgate, A., R. Southgate, et al. (1995). "Both synchronous and asynchronous muscle isoforms of projectin (the *Drosophila bent* locus product) contain functional kinase domains." Journal of Cell Biology **128**(3): 393-403.

Ayme-Southgate, A., J. Vigoreaux, et al. (1991). "*Drosophila* has a *twitchin* titin-related gene that appears to encode projectin." Proceedings of the National Academy of Sciences of the United States of America **88**(18): 7973-7977.

Ayoob, J. C., K. K. Turnacioglu, et al. (2000). "Targeting of cardiac muscle titin fragments to the Z-bands and dense bodies of living muscle and non-muscle cells." Cell Motility and the Cytoskeleton **45**(1): 67-82.

Backer, J. M., S. E. Shoelson, et al. (1992). "The insulin-receptor juxtamembrane region contains 2 independent tyrosine beta-turn internalization signals." Journal of Cell Biology **118**(4): 831-839.

Balaban, N. Q., U. S. Schwarz, et al. (2001). "Force and focal adhesion assembly: A close relationship studied using elastic micropatterned substrates." Nature Cell Biology **3**(5): 466-472

Bass, M. D., B. J. Smith, et al. (1999). "Talin contains three similar vinculin-binding sites predicted to form an amphipathic helix." Biochemical Journal **341**: 257-263.

Bate, M. (1990). "The embryonic-development of larval muscles in *Drosophila*." Development **110**(3): 791-804.

Bateman, J., R. S. Reddy, et al. (2001). "The receptor tyrosine phosphatase dlar and integrins organize actin filaments in the *Drosophila* follicular epithelium." Current Biology **11**(17): 1317-1327.

Bauer, K., M. Kratzer, et al. (2000). "Human Clp36, a PDZ-domain and LIM-domain protein, binds to alpha-actinin-1 and associates with actin filaments and stress fibers in activated platelets and endothelial cells." Blood **96**(13): 4236-4245.

Becam, I. E., G. Tanentzapf, et al. (2005). "Integrin-independent repression of cadherin transcription by talin during axis formation in *Drosophila*." Nature Cell Biology **7**(5): 510-516.

Becker, S., G. Pasca, et al. (1997). "Reciprocal signaling between *Drosophila* epidermal muscle attachment cells and their corresponding muscles." Development **124**(13): 2615-2622.

Beckerle, M. C. (1990). "The adhesion plaque protein, talin, is phosphorylated *in vivo* in chicken-embryo fibroblasts exposed to a tumor-promoting phorbol ester." Cell Regulation **1**(2): 227-236.

Beckerle, M. C., D. E. Miller, et al. (1989). "Activation-dependent redistribution of the adhesion plaque protein, talin, in intact human-platelets." Journal of Cell Biology **109**(6): 3333-3346.

Belkin, A. M., N. I. Zhidkova, et al. (1986). "Localization of talin in skeletal and cardiac muscles." Febs Letters **200**(1): 32-36.

Bendig, G., M. Grimmler, et al. (2006). "Integrin-linked kinase, a novel component of the cardiac mechanical stretch sensor, controls contractility in the zebrafish heart." Genes & Development **20**(17): 2361-2372.

Bernstein, S. I., E. A. Fyrberg, et al. (1978). "Isolation and partial characterization of *Drosophila* myoblasts from primary cultures of embryonic cells." Journal of Cell Biology **78**(3): 856-865.

Bertagnolli, M. E., S. J. Locke, et al. (1993). "Talin distribution and phosphorylation in thrombin-activated platelets." Journal of Cell Science **106**: 1189-1199.

Bloor, J. W. and N. H. Brown (1998). "Genetic analysis of the *Drosophila* alpha-PS2 integrin subunit reveals discrete adhesive, morphogenetic and sarcomeric functions." Genetics **148**(3): 1127-1142.

Bloor, J. W. and D. P. Kiehart (2001). "*Zipper* nonmuscle myosin-II functions downstream of PS2 integrin in *Drosophila* myogenesis and is necessary for myofibril formation." Developmental Biology **239**(2): 215-228.

Boateng, S. Y. and P. H. Goldspink (2008). "Assembly and maintenance of the sarcomere night and day." Cardiovascular Research **77**(4): 667-675.

Bogaert, T., N. Brown, et al. (1987). "The *Drosophila* PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments." Cell **51**(6): 929-940.

Bois, P. R. J., R. A. Borgon, et al. (2005). "Structural dynamics of alpha-actinin-vinculin interactions." Molecular and Cellular Biology **25**(14): 6112-6122.

Bois, P. R. J., R. A. Borgon, et al. (2007). "Structural dynamics of alpha-actinin-vinculin interactions." Molecular and Cellular Biology **27**(15): 5606-5606.

Bois, P. R. J., B. P. O'Hara, et al. (2006). "The vinculin binding sites of talin and alpha-actinin are sufficient to activate vinculin." Journal of Biological Chemistry **281**(11): 7228-7236.

Bokel, C. and N. H. Brown (2002). "Integrins in development: Moving on, responding to, and sticking to the extracellular matrix." Developmental Cell **3**(3): 311-321.

Borg, T. K., E. C. Goldsmith, et al. (2000). "Specialization at the Z line of cardiac myocytes." Cardiovascular Research **46**(2): 277-285.

Boudignon-Proudhon, C., P. M. Patel, et al. (1996). "Phorbol ester enhances integrin alpha II beta 3-dependent adhesion of human erythroleukemic cells to activation-dependent monoclonal antibodies." Blood **87**(3): 968-976.

Brabant, M. C. and D. L. Brower (1993). "PS2 integrin requirements in *Drosophila* embryo and wing morphogenesis." Developmental Biology **157**(1): 49-59.

Braun, D. C., Y. Y. Cao, et al. (2005). "Role of phorbol ester localization in determining protein kinase C or RasGRP3 translocation: Real-time analysis using fluorescent ligands and proteins." Molecular Cancer Therapeutics **4**(1): 141-150.

Brower, D. L., M. Wilcox, et al. (1984). "Related cell-surface antigens expressed with positional specificity in *Drosophila* imaginal disks." Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences **81**(23): 7485-7489.

Brown, M. C., J. A. Perrotta, et al. (1998). "Serine and threonine phosphorylation of the paxillin LIM domains regulates paxillin focal adhesion localization and cell adhesion to fibronectin." Molecular Biology of the Cell **9**(7): 1803-1816.

Brown, N. H. (1993). "Integrins hold *Drosophila* together." Bioessays **15**(6): 383-390.

Brown, N. H. (1994). "Null mutations in the alphaPS2 and betaPS integrin subunit genes have distinct phenotypes." Development **120**(5): 1221-1231.

Brown, N. H., S. L. Gregory, et al. (2000). "Integrins as mediators of morphogenesis in *Drosophila*." Developmental Biology **223**(1): 1-16.

Brown, N. H., S. L. Gregory, et al. (2002). "Talin is essential for integrin function in *Drosophila*." Developmental Cell **3**(4): 569-579.

Bunch, T. A., M. W. Graner, et al. (1998). "The PS2 integrin ligand tiggrin is required for proper muscle function in *Drosophila*." Development **125**(9): 1679-1689.

Bunch, T. A., T. L. Helsten, et al. (2006). "Amino acid changes in *Drosophila* alphaPS2 betaPS integrins that affect ligand affinity." Journal of Biological Chemistry **281**(8): 5050-5057.

Burn, P., A. Kupfer, et al. (1988). "Dynamic membrane cytoskeletal interactions - specific association of integrin and talin arises *in vivo* after phorbol ester treatment of peripheral-blood lymphocytes." Proceedings of the National Academy of Sciences of the United States of America **85**(2): 497-501.

Burridge, K. and M. Chrzanowska-Wodnicka (1996). "Focal adhesions, contractility, and signaling." Annual Review of Cell and Developmental Biology **12**: 463-518.

Burridge, K., K. Fath, et al. (1988). "Focal adhesions - transmembrane junctions between the extracellular-matrix and the cytoskeleton." Annual Review of Cell Biology **4**: 487-525.

Calderwood, D. A. (2004). "Integrin activation." Journal of Cell Science **117**(5): 657-666.

Calderwood, D. A. (2004). "Talin controls integrin activation." Biochemical Society Transactions **32**: 434-437.

Calderwood, D. A. (2005). "Integrin activation - a final common pathway." Journal of Neurochemistry **94**: 120-120.

Calderwood, D. A., V. Tai, et al. (2004). "Competition for talin results in trans-dominant inhibition of integrin activation." Journal of Biological Chemistry **279**(28): 28889-28895.

Calderwood, D. A., R. Zent, et al. (1999). "The talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation." Journal of Biological Chemistry **274**(40): 28071-28074.

Campbell, I. D. and M. H. Ginsberg (2004). "The talin-tail interaction places integrin activation on FERM ground." Trends in Biochemical Sciences **29**(8): 429-435.

Cariati, M., A. Naderi, et al. (2008). "Alpha-6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line." International Journal of Cancer **122**: 298-304.

Carroll, S., S. J. Lu, et al. (2004). "N-RAP scaffolds I-Z-I assembly during myofibrillogenesis in cultured chick cardiomyocytes." Journal of Cell Science **117**(1): 105-114.

Chen, C. H., M. O. Gray, et al. (1999). "Cardioprotection from ischemia by a brief exposure to physiological levels of ethanol: Role of epsilon protein kinase C." Proceedings of the National Academy of Sciences of the United States of America **96**(22): 12784-12789.

Chen, H., X. Y. N. Huang, et al. (2005). "Role of the integrin-linked kinase/PINCH1/alpha-parvin complex in cardiac myocyte hypertrophy." Laboratory Investigation **85**(11): 1342-1356.

Chen, H. C., P. A. Appeddu, et al. (1995). "Interaction of focal adhesion kinase with cytoskeletal protein talin." Journal of Biological Chemistry **270**(28): 16995-16999.

Chen, W. J., J. L. Goldstein, et al. (1990). "NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low-density-lipoprotein receptor." Journal of Biological Chemistry **265**(6): 3116-3123.

Choquet, D., D. P. Felsenfeld, et al. (1997). "Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages." Cell **88**(1): 39-48.

Chrzanowska-Wodnicka, M. and K. Burridge (1996). "Rho-stimulated contractility drives the formation of stress fibers and focal adhesions." Journal of Cell Biology **133**(6): 1403-1415.

Chuang, J. Z., D. C. Lin, et al. (1995). "Molecular-cloning, expression, and mapping of the high-affinity actin-capping domain of chicken cardiac tensin." Journal of Cell Biology **128**(6): 1095-1109.

Clark, K. A., J. M. Bland, et al. (2007). "The *Drosophila* muscle LIM protein, Mlp84b, cooperates with D-titin to maintain muscle structural integrity." Journal of Cell Science **120**(12): 2066-2077.

Clark, K. A., A. S. McElhinny, et al. (2002). "Striated muscle cytoarchitecture: An intricate web of form and function." Annual Review of Cell and Developmental Biology **18**: 637-706.

Clark, K. A., M. McGrail, et al. (2003). "Analysis of PINCH function in *Drosophila* demonstrates its requirement in integrin-dependent cellular processes." Development **130**(12): 2611-2621.

Clemens, J. C., C. A. Worby, et al. (2000). "Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways." Proceedings of the National Academy of Sciences of the United States of America **97**(12): 6499-6503.

Conti, F. J., A. Felder, et al. (2008). "Progressive myopathy and defects in the maintenance of myotendinous junctions in mice that lack talin 1 in skeletal muscle." Development **135**(11): 2043-2053.

Cooper, L. A., T. L. Shen, et al. (2003). "Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction." Molecular and Cellular Biology **23**(22): 8030-8041.

Cram, E. J. and J. E. Schwarzbauer (2004). "The talin wags the dog: New insights into integrin activation." Trends in Cell Biology **14**(2): 55-57.

Critchley, D. R. (2004). "Cytoskeletal proteins talin and vinculin in integrin-mediated adhesion." Biochemical Society Transactions **32**: 831-836.

Critchley, D. R. and A. R. Gingras (2008). "Talin at a glance." Journal of Cell Science **121**(9): 1345-1347.

Dabiri, G. A., J. C. Ayoob, et al. (1999). "Use of green fluorescent proteins linked to cytoskeletal proteins to analyze myofibrillogenesis in living cells." Green Fluorescent Protein **302**: 171-186.

Dabiri, G. A., K. K. Turnacioglu, et al. (1997). "Myofibrillogenesis visualized in living embryonic cardiomyocytes." Proceedings of the National Academy of Sciences of the United States of America **94**(17): 9493-9498.

Danilov, Y. N. and R. L. Juliano (1989). "Phorbol ester modulation of integrin-mediated cell-adhesion - a postreceptor event." Journal of Cell Biology **108**(5): 1925-1933.

Danowski, B. A., K. Imanakayoshida, et al. (1992). "Costameres are sites of force transmission to the substratum in adult-rat cardiomyocytes." Journal of Cell Biology **118**(6): 1411-1420.

de la Pompa, J. L., J. R. Garcia, et al. (1989). "Genetic analysis of muscle development in *Drosophila melanogaster*." Developmental Biology **131**(2): 439-454

Delon, I. and N. Brown (2008). "Cell-matrix adhesion: The Wech connection." Current Biology **18**(9): R389-R391.

Delon, I. and N. H. Brown (2007). "Integrins and the actin cytoskeleton." Current Opinion in Cell Biology **19**(1): 43-50.

Devenport, D. and N. H. Brown (2004). "Morphogenesis in the absence of integrins: Mutation of both *Drosophila* beta subunits prevents midgut migration." Development **131**(21): 5405-5415.

Devenport, D., T. A. Bunch, et al. (2007). "Mutations in the *Drosophila* alphaPS2 integrin subunit uncover new features of adhesion site assembly." Developmental Biology **308**(2): 294-308.

Diamond, M.S, and T. A. Springer (1994). "The dynamic regulation of integrin adhesiveness." Current Biology **4**(6): 506-517

Dhume, A., S. J. Lu, et al. (2006). "Targeted disruption of N-RAP gene function by RNA interference: A role for N-RAP in myofibril organization." Cell Motility and the Cytoskeleton **63**(8): 493-511.

Djinovic-Carugo, K., P. Young, et al. (1999). "Structure of the alpha-actinin rod: Molecular basis for cross-linking of actin filaments." Cell **98**(4): 537-546.

Dorn, G. W., M. C. Souroujon, et al. (1999). "Sustained *in vivo* cardiac protection by a rationally designed peptide that causes epsilon protein kinase C translocation." Proceedings of the National Academy of Sciences of the United States of America **96**(22): 12798-12803.

Du, A. P., J. M. Sanger, et al. (2003). "Myofibrillogenesis in the first cardiomyocytes formed from isolated quail precardiac mesoderm." Developmental Biology **257**(2): 382-394.

Dubreuil, R. R. and P. Wang (2000). "Genetic analysis of the requirements for alpha-actinin function." Journal of Muscle Research and Cell Motility **21**(7): 705-713.

Durieux, A. C., D. Desplanches, et al. (2007). "Mechanotransduction in striated muscle via focal adhesion kinase." Biochemical Society Transactions **35**: 1312-1313.

Dyson, J. M., C. J. O'Malley, et al. (2001). "The SH2-containing inositol polyphosphate 5-phosphatase, SHIP-2, binds filamin and regulates submembraneous actin." Journal of Cell Biology **155**(6): 1065-1079.

Ehler, E., B. M. Rothen, et al. (1999). "Myofibrillogenesis in the developing chicken heart: Assembly of Z-disk, M-line and the thick filaments." Journal of Cell Science **112**(10): 1529-1539.

Eliceiri, B. P. (2001). "Integrin and growth factor receptor crosstalk." Circulation Research **89**(12): 1104-1110.

Erb, E. M., K. Tangemann, et al. (1997). "Integrin alpha IIb beta 3 reconstituted into lipid bilayers is nonclustered in its activated state but clusters after fibrinogen binding." Biochemistry **36**(24): 7395-7402.

Ervasti, J. M. (2003). "Costameres: The Achilles' Heel of Herculean Muscle." Journal of Biological Chemistry **278**(16): 13591-13594.

Fässler, R. and M. Meyer (1995). "Consequences of lack of beta-1 integrin gene-expression in mice." Genes & Development **9**(15): 1896-1908.

Faulkner, G., A. Pallavicini, et al. (1999). "Zasp: A new Z-band alternatively spliced PDZ-motif protein." Journal of Cell Biology **146**(2): 465-475.

Fernandes, J. J., S.E. Celniker, et al. (1996). "Development of the indirect flight muscle attachment sites in *Drosophila*: role of the PS integrins and the stripe gene. Dev. Biol. **176**(2): 166-84

Fogerty, F. J., L. I. Fessler, et al. (1994). "Tiggrin, a novel *Drosophila* extracellular-matrix protein that functions as a ligand for *Drosophila* alphaPS2betaPS integrins." Development **120**(7): 1747-1758.

Fonseca, P. M., R. Y. Inoue, et al. (2005). "Targeting to C-terminal myosin heavy chain may explain mechanotransduction involving focal adhesion kinase in cardiac myocytes." Circulation Research **96**(1): 73-81.

Francis, G. R. and R. H. Waterston (1985). "Muscle organization in *Caenorhabditis elegans* - localization of proteins implicated in thin filament attachment and I-band organization." Journal of Cell Biology **101**(4): 1532-1549.

Franco, S. J., M. A. Rodgers, et al. (2004). "Calpain-mediated proteolysis of talin regulates adhesion dynamics." Nature Cell Biology **6**(10): 977-+.

Frank, D., C. Kuhn, et al. (2006). "The sarcomeric Z-disc: A nodal point in signalling and disease." Journal of Molecular Medicine-Jmm **84**(6): 446-468.

Frommer, G., G. Vorbruggen, et al. (1996). "Epidermal EGR-like zinc finger protein of *Drosophila* participates in myotube guidance." Embo Journal **15**(7): 1642-1649.

Fürst, D.O., M. Osborn, et al. (1988). "The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: A map of ten nonrepetitive epitopes starting at the Z-line extends close to the M line." Journal of Cell Biology **106**(5):1563-1572

Fyrberg, C., A. Ketchum, et al. (1998). "Characterization of lethal *Drosophila* melanogaster alpha-actinin mutants." Biochemical Genetics **36**(9-10): 299-310.

Fyrberg, E., M. Kelly, et al. (1990). "Molecular-genetics of *Drosophila* alpha-actinin - mutant alleles disrupt Z-disk integrity and muscle insertions." Journal of Cell Biology **110**(6): 1999-2011.

Galbraith, C. G., K. M. Yamada, et al. (2002). "The relationship between force and focal complex development." Journal of Cell Biology **159**(4): 695-705.

Garamvol.N (1965). "Inter Z-bridges in flight muscle of bee." Journal of Ultrastructure Research **13**(5-6): 435-&.

Geiger, B., A. Bershadsky, et al. (2001). "Transmembrane extracellular matrix-cytoskeleton crosstalk." Nature Reviews Molecular Cell Biology **2**(11): 793-805.

Georges-Labouesse, E., N. Messaddeq, et al. (1996). "Absence of integrin alpha 6 leads to epidermolysis bullosa and neonatal death in mice." Nature Genetics **13**(3): 370-373.

Gheyara, A. L., A. Vallejo-Illarramendi, et al. (2007). "Deletion of integrin-linked kinase from skeletal muscles of mice resembles muscular dystrophy due to alpha 7 beta 1-integrin deficiency." American Journal of Pathology **171**: 1966-1977.

Giancotti, F. G. and G. Tarone (2003). "Positional control of cell fate through joint integrin/receptor protein kinase signaling." Annual Review of Cell and Developmental Biology **19**: 173-206.

Gilmore, A. P., C. Wood, et al. (1993). "The cytoskeletal protein talin contains at least 2 distinct vinculin binding domains." Journal of Cell Biology **122**(2): 337-347.

Gimond, C., A. Demelker, et al. (1995). "The cytoplasmic domain of alpha 6a integrin subunit is an *in vitro* substrate for protein kinase C." Experimental Cell Research **216**(1): 232-235.

Ginsberg, M. H., A. Partridge, et al. (2005). "Integrin regulation." Current Opinion in Cell Biology **17**(5): 509-516.

Gonsior, S. M., M. Gautel, et al. (1998). "A six-module human nebulin fragment bundles actin filaments and induces actin polymerization." Journal of Muscle Research and Cell Motility **19**(3): 225-235.

Gotwals, P. J., L. I. Fessler, et al. (1994). "*Drosophila* PS1 integrin is a laminin receptor and differs in ligand specificity from PS2." Proceedings of the National Academy of Sciences of the United States of America **91**(24): 11447-11451.

Grabbe, C., C. G. Zervas, et al. (2004). "Focal adhesion kinase is not required for integrin function or viability in *Drosophila*." Development **131**(23): 5795-5805.

Graner, M. W., T. A. Bunch, et al. (1998). "Splice variants of the *Drosophila* PS2 integrins differentially interact with RGD-containing fragments of the extracellular proteins tiggrin, ten-m, and D-laminin alpha 2." Journal of Biological Chemistry **273**(29): 18235-18241.

Gregorio, C. C., K. Trombitas, et al. (1998). "The NH2 terminus of titin spans the Z-disc: Its interaction with a novel 19-kd ligand (t-cap) is required for sarcomeric integrity." Journal of Cell Biology **143**(4): 1013-1027.

Grinnell, F. and M. K. Feld (1979). "Initial adhesion of human-fibroblasts in serum-free medium - possible role of secreted fibronectin." Cell **17**(1): 117-129.

Gu, X. and S. P. Bishop (1994). "Increased protein kinase C and isozyme redistribution in pressure-overload cardiac-hypertrophy in the rat." Circulation Research **75**(5): 926-931.

Guo, C., M. Willem, et al. (2006). "Absence of alpha 7 integrin in dystrophin-deficient mice causes a myopathy similar to Duchenne muscular dystrophy." Human Molecular Genetics **15**(6): 989-998.

Gutzeit, H. O. (1990). "The microfilament pattern in the somatic follicle cells of mid-vitellogenic ovarian follicles of *Drosophila*." European Journal of Cell Biology **53**(2): 349-356.

Gutzeit, H.O., W. Eberhardt, et al. (1991). "Laminin and basement membrane-associated microfilaments in wild-type and mutant *Drosophila* ovarian follicles." Journal of Cell Science **100** (4): 781–78

Guy, P. M., D. A. Kenny, et al. (1999). "The PDZ domain of the LIM protein Enigma binds to beta-tropomyosin." Molecular Biology of the Cell **10**(6): 1973-1984.

Han, J. W., C. J. Lim, et al. (2006). "Reconstructing and deconstructing agonist-induced activation of integrin alpha IIb beta 3." Current Biology **16**(18): 1796-1806.

Hannigan, G. E., C. Leung Hagesteijn, et al. (1996). "Regulation of cell adhesion and anchorage-dependent growth by a new beta(1)-integrin-linked protein kinase." Nature **379**(6560): 91-96.

Harris, A. K., P. Wild, et al. (1980). "Silicone-rubber substrata - new wrinkle in the study of cell locomotion." Science **208**(4440): 177-179.

Harris, B. Z. and W. A. Lim (2001). "Mechanism and role of PDZ domains in signaling complex assembly." Journal of Cell Science **114**(18): 3219-3231.

Hayashi, I., K. Vuori, et al. (2002). "The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin." Nature Structural Biology **9**(2): 101-106.

Heidkamp, M. C., A. L. Bayer, et al. (2003). "Activation of focal adhesion kinase by protein kinase C epsilon in neonatal rat ventricular myocytes." American Journal of Physiology-Heart and Circulatory Physiology **285**(4): H1684-H1696.

Hemmings, L., D. J. G. Rees, et al. (1996). "Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site." Journal of Cell Science **109**: 2715-2726.

Henderson, J. R., P. Pomies, et al. (2003). "ALP and MLP distribution during myofibrillogenesis in cultured cardiomyocytes." Cell Motility and the Cytoskeleton **54**(3): 254-265.

Hirsch, E., L. Lohikangas, et al. (1998). "Mouse myoblasts can fuse and form a normal sarcomere in the absence of beta 1 integrin expression." Journal of Cell Science **111**: 2397-2409.

Holtzer, H., T. Hijikata, et al. (1997). "Independent assembly of 1.6 microns long bipolar MHC filaments and I-Z-I bodies." Cell Structure and Function **22**(1): 83-93.

Horne-Badovinac, S. and D. Bilder (2005). "Mass transit: Epithelial morphogenesis in the *Drosophila* egg chamber." Developmental Dynamics **232**(3): 559-574.

Horwitz, A., K. Duggan, et al. (1986). "Interaction of plasma-membrane fibronectin receptor with talin - a transmembrane linkage." Nature **320**(6062): 531-533.

Huang, C. Q., Q. Zhou, et al. (2003). "Characterization and *in vivo* functional analysis of splice variants of Cypher." Journal of Biological Chemistry **278**(9): 7360-7365.

Huang, S. and D. E. Ingber (1999). "The structural and mechanical complexity of cell-growth control." Nature Cell Biology **1**(5): E131-E138.

Huelsmann, S., C. Hepper, et al. (2006). "The PDZ-GEF dizzy regulates cell shape of migrating macrophages via Rap1 and integrins in the *Drosophila* embryo." Development **133**(15): 2915-2924.

Hughes, A. L. (2001). "Evolution of the integrin alpha and beta protein families." Journal of Molecular Evolution **52**(1): 63-72.

Hutson, M. S., Y. Tokutake, et al. (2003). "Forces for morphogenesis investigated with laser microsurgery and quantitative modeling." Science **300**(5616): 145-149.

Hyatt, S. L., L. Liao, et al. (1994). "Identification and characterization of alpha-protein kinase C binding-proteins in normal and transformed REF52 cells." Biochemistry **33**(5): 1223-1228.

Imanakayoshida, K., B. A. Danowski, et al. (1992). "Vinculin-containing costameres - parts of contraction forces transmitting site of cardiomyocyte." Journal of Molecular and Cellular Cardiology **24**: S52-S52.

Imanaka-Yoshida, K., M. Enomoto-Iwamoto, et al. (1999). "Vinculin, talin, integrin alpha 6 beta 1, and laminin can serve as components of attachment complex mediating contraction force transmission from cardiomyocytes to extracellular matrix." Cell Motility and the Cytoskeleton **42**(1): 1-11.

Ingber, D. E. (1994). "Mechanochemical transduction across integrins and through the cytoskeleton." Biophysical Journal **66**(2): A224-A224.

Isenberg, W. M., R. P. McEver, et al. (1987). "The platelet fibrinogen receptor-an immunogold-surface replica study of agonist-induced ligand-binding and receptor clustering." Journal of Cell Biology **104**(6): 1655-1663.

Ivaska, J., S. Kermorgant, et al. (2003). "Integrin-protein kinase C relationships." Biochemical Society Transactions **31**: 90-93.

Ivaska, J., K. Vuoriluoto, et al. (2005). "PKC epsilon-mediated phosphorylation of vimentin controls integrin recycling and motility." Embo Journal **24**(22): 3834-3845.

Ivaska, J., R. D. H. Whelan, et al. (2002). "PKC epsilon controls the traffic of beta 1 integrins in motile cells." Embo Journal **21**(14): 3608-3619.

Izard, T., G. Evans, et al. (2004). "Vinculin activation by talin through helical bundle conversion." Nature **427**(6970): 171-175.

Izard, T. and C. Vonrhein (2004). "Structural basis for amplifying vinculin activation by talin." Journal of Biological Chemistry **279**(26): 27667-27678.

Jaken, S., K. Leach, et al. (1989). "Association of type-3 protein kinase C with focal contacts in rat embryo fibroblasts." Journal of Cell Biology **109**(2): 697-704.

Jani, K. and F. Schöck (2007). "Zasp is required for the assembly of functional integrin adhesion sites." Journal of Cell Biology **179**: 1583-1597.

Jelen, F., A. Oleksy, et al. (2003). "PDZ domains - common players in the cell signaling." Acta Biochimica Polonica **50**(4): 985-1017.

Johnson, R. P. and S. W. Craig (1994). "An intramolecular association between the head and tail domains of vinculin modulates talin binding." Journal of Biological Chemistry **269**(17): 12611-12619.

Johnson, R. P. and S. W. Craig (1995). "F-actin binding-site masked by the intramolecular association of vinculin head and tail domains." Nature **373**(6511): 261-264.

Kadrmas, J. L. and M. C. Beckerle (2004). "The LIM domain: From the cytoskeleton to the nucleus." Nature Reviews Molecular Cell Biology **5**(11): 920-931.

Katsumi, A., T. Naoe, et al. (2005). "Integrin activation and matrix binding mediate cellular responses to mechanical stretch." Journal of Biological Chemistry **280**(17): 16546-16549.

Katsumi, A., A. W. Orr, et al. (2004). "Integrins in mechanotransduction." Journal of Biological Chemistry **279**(13): 12001-12004.

Keenan, C. and D. Kelleher (1998). "Protein kinase C and the cytoskeleton." Cellular Signalling **10**(4): 225-232.

Kiehart, D. P. and R. Feghali (1986). "Cytoplasmic myosin from *Drosophila melanogaster*." Journal of Cell Biology **103**(4): 1517-1525.

Kiger, A., B. Baum, S. Jones, M. Jones, A. Coulson, C. Echeverri, and N. Perrimon. 2003. A functional genomic analysis of cell morphology using RNA interference. Journal of Biology **2**(4):27

Kim, M., C. V. Carman, et al. (2003). "Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins." Science **301**(5640): 1720-1725

Kinashi, T. (2005). "Intracellular signalling controlling integrin activation in lymphocytes." Nature **5**(7): 546-559

Klaavuniemi, T., A. Kelloniemi, et al. (2004). "The ZASP-like motif in actinin-associated LIM protein is required for interaction with the alpha-actinin rod and for targeting to the muscle Z-line." Journal of Biological Chemistry **279**(25): 26402-26410.

Klemke, R. L., M. Yebra, et al. (1994). "Receptor tyrosine kinase signaling required for integrin alpha-v-beta-5-directed cell motility but not adhesion on vitronectin." Journal of Cell Biology **127**(3): 859-866.

Knoll, R., R. Postel, et al. (2007). "Integrin-linked kinase and laminin alpha 4 mutations cause human cardiomyopathy." Circulation **116**: 221-221.

Kobayashi, T., W. J. Dong, et al. (2004). "Effects of protein kinase C dependent phosphorylation and a familial hypertrophic cardiomyopathy-related mutation of cardiac troponin I on structural transition of troponin C and myofilament activation." Biochemistry **43**(20): 5996-6004.

Kornberg, L. J., H. S. Earp, et al. (1991). "Signal transduction by integrins - increased protein tyrosine phosphorylation caused by clustering of beta-1 integrins." Proceedings of the National Academy of Sciences of the United States of America **88**(19): 8392-8396.

Kovacic-Milivojevic, B., F. Roediger, et al. (2001). "Focal adhesion kinase and p130Cas mediate both sarcomeric organization and activation of genes associated with cardiac myocyte hypertrophy." Molecular Biology of the Cell **12**(8): 2290-2307.

Kucik, D. F., M. L. Dustin, et al. (1996). "Adhesion-activating phorbol ester increases the mobility of leukocyte integrin lfa-1 in cultured lymphocytes." Journal of Clinical Investigation **97**(9): 2139-2144.

Kulke, M., S. Fujita-Becker, et al. (2001). "Interaction between PEVK-titin and actin filaments - origin of a viscous force component in cardiac myofibrils." Circulation Research **89**(10): 874-881.

Kunda, P., G. Craig, et al. (2003). "Abi, Sra1, and Kette control the stability and localization of SCAR/WAVE to regulate the formation of actin-based protrusions." Current Biology **13**(21): 1867-1875.

Kuroda, S., C. Tokunaga, et al. (1996). "Protein-protein interaction of zinc finger LIM domains with protein kinase C." Journal of Biological Chemistry **271**(49): 31029-31032.

Labeit, S. and B. Kolmerer (1995). "Titins - giant proteins in charge of muscle ultrastructure and elasticity." Science **270**(5234): 293-296.

Lange, S., E. Ehler, et al. (2006). "From A to Z and back? Multicompartment proteins in the sarcomere." Trends in Cell Biology **16**(1): 11-18.

Lecroisey, C., L. Segalat, et al. (2007). "The *C.elegans* dense body: Anchoring and signaling structure of the muscle." Journal of Muscle Research and Cell Motility **28**(1): 79-87.

Lee, S. B., K. S. Cho, et al. (2003). "*Blistery* encodes *Drosophila* tensin protein and interacts with integrin and the JNK signaling pathway during wing development." Development **130**(17): 4001-4010.

Lele, T. P., J. E. Sero, et al. (2007). "Tools to study cell mechanics and mechanotransduction." Cell Mechanics **83**: 443-+.

Leptin, M., T. Bogaert, et al. (1989). "The function of PS integrins during *Drosophila* embryogenesis." Cell **56**(3): 401-408.

Lewis, J. M. and M. A. Schwartz (1995). "Mapping *in vivo* associations of cytoplasmic proteins with integrin beta-1 cytoplasmic domain mutants." Molecular Biology of the Cell **6**(2): 151-160.

Li, F. G., Y. J. Zhang, et al. (1999). "Integrin-linked kinase is localized to cell-matrix focal adhesions but not cell-cell adhesion sites and the focal adhesion localization of integrin-linked kinase is regulated by the PINCH-binding AMK repeats." Journal of Cell Science **112**(24): 4589-4599.

Li, S., M. Kim, et al. (1997). "Fluid shear stress activation of focal adhesion kinase - linking to mitogen-activated protein kinases." Journal of Biological Chemistry **272**(48): 30455-30462.

Liddington, R. C. and M. H. Ginsberg (2002). "Integrin activation takes shape." Journal of Cell Biology **158**(5): 833-839.

Lin, Y. H., Z. Y. Park, et al. (2004). "Regulation of cell migration and survival by focal adhesion targeting of Lasp-1." Journal of Cell Biology **165**(3): 421-432.

Lin, Z. X., J. R. Eshelman, et al. (1987). "Sequential disassembly of myofibrils induced by myristate acetate in cultured myotubes." Journal of Cell Biology **105**(3): 1365-1376.

Lin, Z. X., J. Eshleman, et al. (1989). "Differential response of myofibrillar and cytoskeletal proteins in cells treated with phorbol-myristate acetate." Journal of Cell Biology **108**(3): 1079-1091.

Ling, K., R. L. Doughman, et al. (2002). "Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions." Nature **420**(6911): 89-93.

Linke, W. A., M. Kulke, et al. (2002). "PEVK domain of titin: An entropic spring with actin-binding properties." Journal of Structural Biology **137**(1-2): 194-205.

Liu, S., S. M. Thomas, et al. (1999). "Binding of paxillin to alpha 4 integrins modifies integrin-dependent biological responses." Nature **402**(6762): 676-681.

Liu, S. C., D. A. Calderwood, et al. (2000). "Integrin cytoplasmic domain-binding proteins." Journal of Cell Science **113**(20): 3563-3571.

Lo, S. H., E. Weisberg, et al. (1994). "Tensin - a potential link between the cytoskeleton and signal-transduction." Bioessays **16**(11): 817-823.

Löer, B., R. Bauer, et al. (2008). "The NHL-domain protein Wech is crucial for the integrin-cytoskeleton link." Nature Cell Biology **10**(4): 422-U103.

Lu, M. H., C. Dilullo, et al. (1992). "The vinculin sarcomeric-alpha-actinin alpha-actin nexus in cultured cardiac myocytes." Journal of Cell Biology **117**(5): 1007-1022.

Lu, S. J., S. L. Carroll, et al. (2003). "New N-RAP-binding partners alpha-actinin, filamin and Krp1 detected by yeast two-hybrid screening: Implications for myofibril assembly." Journal of Cell Science **116**(11): 2169-2178.

Luo, B. H., C. V. Carman, et al. (2007). "Structural basis of integrin regulation and signaling." Annual Review of Immunology **25**: 619-647.

Luo, G., A. H. Herrera, et al. (1999). "Molecular interactions of N-RAP, a nebulin-related protein of striated muscle myotendon junctions and intercalated disks." Biochemistry **38**(19): 6135-6143.

Ma, Y. Q., J. Qin, et al. (2008). "Kindlin-2 (Mig-2): a co-activator of beta 3 integrins." Journal of Cell Biology **181**(3): 439-446

Machado, C., C. E. Sunkel, et al. (1998). "Human autoantibodies reveal titin as a chromosomal protein." Journal of Cell Biology **141**(2): 321-333.

Mainiero, F., A. Pepe, et al. (1996). "The intracellular functions of alpha 6 beta 4 integrin are regulated by EGF." Journal of Cell Biology **134**(1): 241-253.

Mansour, H., P. P. de Tombe, et al. (2004). "Restoration of resting sarcomere length after uniaxial static strain is regulated by protein kinase C epsilon and focal adhesion kinase." Circulation Research **94**(5): 642-649.

Martel, V., C. Racaud-Sultan, et al. (2001). "Conformation, localization, and integrin binding of talin depend on its interaction with phosphoinositides." Journal of Biological Chemistry **276**(24): 21217-21227.

Martin-Bermudo, M. D. (2000). "Integrins modulate the EGFR signaling pathway to regulate tendon cell differentiation in the *Drosophila* embryo." Development **127**(12): 2607-2615.

Martin-Bermudo, M. D. and N. H. Brown (2000). "The localized assembly of extracellular matrix integrin ligands requires cell-cell contact." Journal of Cell Science **113**(21): 3715-3723.

McGregor, A., A. D. Blanchard, et al. (1994). "Identification of the vinculin-binding site in the cytoskeletal protein alpha-actinin." Biochemical Journal **301**: 225-233.

McKeown, C. R., H. F. Han, et al. (2006). "Molecular characterization of the *Caenorhabditis elegans* ALP/Enigma gene *alp-1*." Developmental Dynamics **235**(2): 530-538.

Mery, A., O. Taghli-Lamallem, et al. (2008). "The *Drosophila* muscle LIM protein, Mlp84b, is essential for cardiac function." Journal of Experimental Biology **211**: 15-23.

Miyamoto, S., S. K. Akiyama, et al. (1995). "Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function." Science **267**(5199): 883-885.

Miyamoto, S., H. Teramoto, et al. (1995). "Integrin function - molecular hierarchies of cytoskeletal and signaling molecules." Journal of Cell Biology **131**(3): 791-805.

Mochly-Rosen, D. and A. S. Gordon (1998). "Anchoring proteins for protein kinase C: A means for isozyme selectivity." Faseb Journal **12**(1): 35-42.

Moes, M., S. Rodius, et al. (2007). "The integrin binding site 2 (IBS2) in the talin rod domain is essential for linking integrin beta subunits to the cytoskeleton." Journal of Biological Chemistry **282**(23): 17280-17288.

Moore, J. R., J. O. Vigoreaux, et al. (1999). "The *Drosophila* projectin mutant, *bentD*, has reduced stretch activation and altered indirect flight muscle kinetics." Journal of Muscle Research and Cell Motility **20**(8): 797-806.

Morin, X., R. Daneman, et al. (2001). "A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*." Proceedings of the National Academy of Sciences of the United States of America **98**(26): 15050-15055.

Muguruma, M., S. Nishimuta, et al. (1995). "Organization of the functional domains in membrane cytoskeletal protein talin." Journal of Biochemistry **117**(5): 1036-1042.

Musa, H., S. Meek, et al. (2006). "Targeted homozygous deletion of M-band titin in cardiomyocytes prevents sarcomere formation." Journal of Cell Science **119**(20): 4322-4331.

Nakagawa, N., M. Hoshijima, et al. (2000). "ENH, containing PDZ and LIM domains, heart/skeletal muscle-specific protein, associates with cytoskeletal proteins through the PDZ domain." Biochemical and Biophysical Research Communications **272**(2): 505-512.

Narasimha, M. and N. H. Brown (2004). "Novel functions for integrins in epithelial morphogenesis." Current Biology **14**(5): 381-385.

Narita, T., N. KawakamiKimura, et al. (1996). "Alteration of integrins by heparin-binding EGF-like growth factor in human breast cancer cells." Oncology **53**(5): 374-381.

Newton, A. C. (1995). "Protein kinase C- structure, function, and regulation." Journal of Biological Chemistry **270**(48): 28495-28498.

Ng, T., D. Shima, et al. (1999). "PKC alpha regulates beta 1 integrin-dependent cell motility through association and control of integrin traffic." Embo Journal **18**(14): 3909-3923.

Nikolopoulos, S. N. and C. E. Turner (2001). "Integrin-linked kinase (ILK) binding to paxillin LD1 motif regulates ILK localization to focal adhesions." Journal of Biological Chemistry **276**(26): 23499-23505.

Nishizuka, Y. (1984). "The role of protein kinase C in cell-surface signal transduction and tumor promotion." Nature **308**(5961): 693-698.

Nobes, C. D. and A. Hall (1995). "Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia." Cell **81**(1): 53-62.

Ohtsuka, H., H. Yajima, et al. (1997). "The N-terminal Z repeat 5 of connectin/titin binds to the C-terminal region of alpha-actinin." Biochemical and Biophysical Research Communications **235**(1): 1-3.

Ojima, K., Z. X. Lin, et al. (1999). "Initiation and maturation of I-Z-I bodies in the growth tips of transfected myotubes." Journal of Cell Science **112**(22): 4101-4112.

Otey, C. A. and O. Carpen (2004). "Alpha-actinin revisited: A fresh look at an old player." Cell Motility and the Cytoskeleton **58**(2): 104-111.

Otey, C. A., F. M. Pavalko, et al. (1990). "An interaction between alpha-actinin and the beta-1 integrin subunit invitro." Journal of Cell Biology **111**(2): 721-729.

Otey, C. A., G. B. Vasquez, et al. (1993). "Mapping of the alpha-actinin binding-site within the beta-1 integrin cytoplasmic domain." Journal of Biological Chemistry **268**(28): 21193-21197.

Palecek, S. P., C. E. Schmidt, et al. (1996). "Integrin dynamics on the tail region of migrating fibroblasts." Journal of Cell Science **109**: 941-952.

Papagrigoriou, E., A. R. Gingras, et al. (2004). "Activation of a vinculin-binding site in the talin rod involves rearrangement of a five-helix bundle." Embo Journal **23**(15): 2942-2951.

Pardo, J. V., J. D. Siliciano, et al. (1983). "A vinculin-containing cortical lattice in skeletal-muscle - transverse lattice elements (costameres) mark sites of attachment between myofibrils and sarcolemma." Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences **80**(4): 1008-1012.

Parker, K. K. and D. E. Ingber (2007). "Extracellular matrix, mechanotransduction and structural hierarchies in heart tissue engineering." Philosophical Transactions of the Royal Society B-Biological Sciences **362**(1484): 1267-1279.

Parks, A. L., K. R. Cook, et al. (2004). "Systematic generation of high-resolution deletion coverage of the *Drosophila* melanogaster genome." Nature Genetics **36**(3): 288-292.

Parsons, J. T. (2003). "Focal adhesion kinase: The first ten years." Journal of Cell Science **116**(8): 1409-1416.

Parsons, M., M. D. Keppler, et al. (2002). "Site-directed perturbation of protein kinase C-integrin interaction blocks carcinoma cell chemotaxis." Molecular and Cellular Biology **22**(16): 5897-5911.

Pashmforoush, M., P. Pomies, et al. (2001). "Adult mice deficient in actinin-associated LIM-domain protein reveal a developmental pathway for right ventricular cardiomyopathy." Nature Medicine **7**(5): 591-597.

Pavalko, F. M. and S. Laroche (1993). "Activation of human neutrophils induces an interaction between the integrin beta-2 subunit (CD18) and actin-filaments via alpha-actinin." Journal of Cellular Biochemistry: 340-340.

Pawson, T. and P. Nash (2000). "Protein-protein interactions define specificity in signal transduction." Genes & Development **14**(9): 1027-1047.

Perez-Moreno, M., A. Avila, et al. (1998). "Vinculin but not alpha-actinin is a target of PKC phosphorylation during junctional assembly induced by calcium." Journal of Cell Science **111**: 3563-3571.

Perrimon, N., L. Engstrom, et al. (1985). "Developmental genetics of the 2C-D region of the *Drosophila* x-chromosome." Genetics **111**(1): 23-41.

Petrich, B. G., P. Fogelstrand, et al. (2007). "The antithrombotic potential of selective blockade of talin-dependent integrin alpha IIb beta 3 (platelet GPIIb-IIIa) activation." Journal of Clinical Investigation **117**(8): 2250-2259.

Pietromonaco, S. F., P. C. Simons, et al. (1998). "Protein kinase C-theta phosphorylation of moesin in the actin-binding sequence." Journal of Biological Chemistry **273**(13): 7594-7603.

Pomies, P., T. Macalma, et al. (1998). "Identification and purification of an alpha-actinin binding protein that is upregulated during muscle differentiation." Molecular Biology of the Cell **9**: 25A-25A.

Pomies, P., T. Macalma, et al. (1999). "Purification and characterization of an alpha-actinin-binding PDZ-LIM protein that is up-regulated during muscle differentiation." Journal of Biological Chemistry **274**(41): 29242-29250.

Ponting, C. P., C. Phillips, et al. (1997). "PDZ domains: Targeting signalling molecules to sub-membranous sites." Bioessays **19**(6): 469-479.

Poole, A. A. W., G. Pwa, et al. (2004). "PKC-interacting proteins: From function to pharmacology." Trends in Pharmacological Sciences **25**(10): 528-535.

Postel, R., P. Vakeel, et al. (2008). "Zebrafish integrin-linked kinase is required in skeletal muscles for strengthening the integrin-ECM adhesion complex." Developmental Biology **318**(1): 92-101.

Prekeris, R., R. M. Hernandez, et al. (1998). "Molecular analysis of the interactions between protein kinase C-epsilon and filamentous actin." Journal of Biological Chemistry **273**(41): 26790-26798.

Prekeris, R., M. W. Mayhew, et al. (1996). "Identification and localization of an actin-binding motif that is unique to the epsilon isoform of protein kinase C and participates in the regulation of synaptic function." Journal of Cell Biology **132**(1-2): 77-90.

Pyle, W. G. and R. J. Solaro (2004). "At the crossroads of myocardial signaling - the role of Z-discs in intracellular signaling and cardiac function." Circulation Research **94**(3): 296-305.

Qin, J., O. Vinogradova, et al. (2004). "Integrin Bidirectional Signaling: A Molecular View." PLoS Biology **2**(6): 726-729

Quach, N., M. Disatnik, et al. (2004). "Focal adhesion kinase (FAK) localization at focal adhesions is essential for myoblast fusion and sarcomere assembly." Molecular Biology of the Cell **15**: 33A-33A.

Quach, N. L. and T. A. Rando (2006). "Focal adhesion kinase is essential for costamerogenesis in cultured skeletal muscle cells." Developmental Biology **293**(1): 38-52.

Ratnikov, B., C. Ptak, et al. (2005). "Talin phosphorylation sites mapped by mass spectrometry." Journal of Cell Science **118**(21): 4921-4923.

Reedy, M. C. and C. Beall (1993). "Ultrastructure of developing flight-muscle in *Drosophila* .II. Formation of the myotendon junction." Developmental Biology **160**(2): 466-479.

Rees, D. J. G., S. E. Ades, et al. (1990). "Sequence and domain-structure of talin." Nature **347**(6294): 685-689.

Reszka, A. A., Y. Hayashi, et al. (1992). "Identification of amino-acid-sequences in the integrin-beta-1 cytoplasmic domain implicated in cytoskeletal association." Journal of Cell Biology **117**(6): 1321-1330.

Rhee, D., J. M. Sanger, et al. (1994). "The premyofibril - evidence for its role in myofibrillogenesis." Cell Motility and the Cytoskeleton **28**(1): 1-24.

Riveline, D., E. Zamir, et al. (2001). "Focal contacts as mechanosensors: Externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism." Journal of Cell Biology **153**(6): 1175-1185.

Robia, S. L., J. Ghanta, et al. (2001). "Localization and kinetics of protein kinase C-epsilon anchoring in cardiac myocytes." Biophysical Journal **80**(5): 2140-2151.

Rogers, S. L., U. Wiedemann, et al. (2003). "Molecular requirements for actin-based lamella formation in *Drosophila* S2 cells." Journal of Cell Biology **162**(6): 1079-1088.

Roote, C. E. and S. Zusman (1995). "Functions for PS integrins in tissue adhesion, migration, and shape changes during early embryonic-development in *Drosophila*." Developmental Biology **169**(1): 322-336.

Ross, R. S. and T. K. Borg (2001). "Integrins and the myocardium." Circulation Research **88**(11): 1112-1119.

Roulier, E. M., C. Fyrberg, et al. (1992). "Perturbations of *Drosophila* alpha-actinin cause muscle paralysis, weakness, and atrophy but do not confer obvious nonmuscle phenotypes." Journal of Cell Biology **116**(4): 911-922.

Sadoshima, J. and S. Izumo (1993). "Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes - potential involvement of an autocrine paracrine mechanism." Embo Journal **12**(4): 1681-1692.

Saide, J. D., S. Chinbow, et al. (1989). "Characterization of components of Z-bands in the fibrillar flight-muscle of *Drosophila melanogaster*." Journal of Cell Biology **109**(5): 2157-2167.

Samarel, A. M. (2005). "Costameres, focal adhesions, and cardiomyocyte mechanotransduction." American Journal of Physiology-Heart and Circulatory Physiology **289**(6): H2291-H2301.

Sanchez-Garcia, I. and T. H. Rabbitts (1994). "The LIM domain - a new structural motif found in zinc-finger-like proteins." Trends in Genetics **10**(9): 315-320.

Sanger, J. W., P. Chowrashi, et al. (2002). "Myofibrillogenesis in skeletal muscle cells." Clinical Orthopaedics and Related Research(403): S153-S162.

Sanger, J. W., G. A. Dabiri, et al. (1998). "Using green fluorescent probes to study myofibrillogenesis in living muscle cells." Faseb Journal **12**(4): A313-A313.

Sastry, S. K., M. Lakonishok, et al. (1996). "Integrin alpha subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation." Journal of Cell Biology **133**(1): 169-184.

Sawada, Y. and M. P. Sheetz (2002). "Force transduction by triton cytoskeletons." Journal of Cell Biology **156**(4): 609-615.

Schaller, M. D., J. D. Hildebrand, et al. (1994). "Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2 dependent binding of pp60(src)." Molecular and Cellular Biology **14**(3): 1680-1688.

Schaller, M. D., C. A. Otey, et al. (1995). "Focal adhesion kinase and paxillin bind to peptides mimicking beta-integrin cytoplasmic domains." Journal of Cell Biology **130**(5): 1181-1187.

Scheswohl, D. M., J. R. Harrell, et al. (2008). "Multiple paxillin binding sites regulate FAK function." J. Mol. Signal. **3**: 1-11

Schliwa, M., T. Nakamura, et al. (1984). "A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured-cells." Journal of Cell Biology **99**(3): 1045-1059.

Schneide.I (1972). "Cell lines derived from late embryonic stages of *Drosophila melanogaster*." Journal of Embryology and Experimental Morphology **27**(APR): 353-&.

Schöck, F. and N. Perrimon (2003). "Retraction of the *Drosophila* germ band requires cell-matrix interaction." Genes & Development **17**(5): 597-602.

Schoenwaelder, S. M. and K. Burridge (1999). "Bidirectional signaling between the cytoskeleton and integrins." Current Opinion in Cell Biology **11**(2): 274-286.

Schultheiss, T., J. Choi, et al. (1992). "A sarcomeric alpha-actinin truncated at the carboxyl end induces the breakdown of stress fibers in PtK2 cells and the formation of nemaline-like bodies and breakdown of myofibrils in myotubes." Proceedings of the National Academy of Sciences of the United States of America **89**(19): 9282-9286.

Schultheiss, T., Z. X. Lin, et al. (1990). "Differential distribution of subsets of myofibrillar proteins in cardiac nonstriated and striated myofibrils." Journal of Cell Biology **110**(4): 1159-1172.

Schutt, B. S., M. Langkamp, et al. (2004). "Integrin-mediated action of insulin-like growth factor binding protein-2 in tumor cells." Journal of Molecular Endocrinology **32**(3): 859-868.

Schwander, M., M. Leu, et al. (2003). "Beta 1 integrins regulate myoblast fusion and sarcomere assembly." Developmental Cell **4**(5): 673-685.

Schwartz, M. A. and R. K. Assoian (2001). "Integrins and cell proliferation: Regulation of cyclin-dependent kinases via cytoplasmic signaling pathways." Journal of Cell Science **114**(14): 2553-2560.

Schwartz, M. A. and D. E. Ingber (1994). "Integrating with integrins." Molecular Biology of the Cell **5**(4): 389-393.

Senetar, M. A., C. L. Moncman, et al. (2007). "Talin2 is induced during striated muscle differentiation and is targeted to stable adhesion complexes in mature muscle." Cell Motility and the Cytoskeleton **64**(3): 157-173.

Shieh, B. H., L. Parker, et al. (2002). "Protein kinase C (PKC) isoforms in *Drosophila*." Journal of Biochemistry **132**(4): 523-527.

Sieg, D. J., C. R. Hauck, et al. (2000). "FAK integrates growth-factor and integrin signals to promote cell migration." Nature Cell Biology **2**(5): 249-256.

Sink, H. (2006). "Muscle development in *Drosophila*." Molecular Biology Intelligence Unit

Soriani, A., B. Moran, et al. (2006). "A role for PKC-theta in outside-in alpha IIb beta 3 signaling." Journal of Thrombosis and Haemostasis **4**(3): 648-655.

Sorimachi, H., A. Freiburg, et al. (1997). "Tissue-specific expression and alpha-actinin binding properties of the Z-disc titin: Implications for the nature of vertebrate Z-discs." Journal of Molecular Biology **270**(5): 688-695.

Strait, J. B., J. L. Martin, et al. (2001). "Role of protein kinase C-epsilon in hypertrophy of cultured neonatal rat ventricular myocytes." American Journal of Physiology-Heart and Circulatory Physiology **280**(2): H756-H766.

Stronach, B. E., S. E. Siegrist, et al. (1996). "Two muscle-specific LIM proteins in *Drosophila*." Journal of Cell Biology **134**(5): 1179-1195.

Subramanian, A., B. Wayburn, et al. (2007). "Thrombospondin-mediated adhesion is essential for the formation of the myotendinous junction in *Drosophila*." Development **134**(7): 1269-1278.

Tadokoro, S., S. J. Shattil, et al. (2003). "Talin binding to integrin beta tails: A final common step in integrin activation." Science **302**(5642): 103-106.

Tamura, N., K. Ohno, et al. (2007). "The PDZ-LIM protein Clp36 is required for actin stress fiber formation and focal adhesion assembly in BeWo cells." Biochemical and Biophysical Research Communications **364**(3): 589-594.

Tanentzapf, G. and N. H. Brown (2006). "An interaction between integrin and the talin FERM domain mediates integrin activation but not linkage to the cytoskeleton." Nature Cell Biology **8**(6): 601-606.

te Velthuis, A. and C. P. Bagowski (2007). "PDZ and LIM domain-encoding genes: Molecular interactions and their role in development." Thescientificworldjournal **7**: 1470-1492.

Tepass, U. (1996). "Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of *Drosophila*." Developmental Biology **177**(1): 217-225.

Terai, M., M. Komiyama, et al. (1989). "Myofibril assembly is linked with vinculin, alpha-actinin, and cell-substrate contacts in embryonic cardiac myocytes *in vitro*." Cell Motility and the Cytoskeleton **12**(4): 185-194.

Tidball, J. G., T. Ohalloran, et al. (1986). "Talin at myotendinous junctions." Journal of Cell Biology **103**(4): 1465-1472.

Tidball, J. G. and M. J. Spencer (1993). "PDGF stimulation induces phosphorylation of talin and cytoskeletal reorganization in skeletal-muscle." Journal of Cell Biology **123**(3): 627-635.

Torgler, C. N., M. Narasimha, et al. (2004). "Tensin stabilizes integrin adhesive contacts in *Drosophila*." Developmental Cell **6**(3): 357-369.

Torsoni, A. S., S. S. Constancio, et al. (2003). "Focal adhesion kinase is activated and mediates the early hypertrophic response to stretch in cardiac myocytes." Circulation Research **93**(2): 140-147.

Trusolino, L., G. Serini, et al. (1998). "Growth factor-dependent activation of alpha v beta 3 integrin in normal epithelial cells: Implications for tumor invasion." Journal of Cell Biology **142**(4): 1145-1156.

Tskhovrebova, L. and J. Trinick (2003). "Titin: Properties and family relationships." Nature Reviews Molecular Cell Biology **4**(9): 679-689.

Tu, Y. Z., F. G. Li, et al. (1999). "The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells." Molecular and Cellular Biology **19**(3): 2425-2434.

Tullio, A. N., D. Accili, et al. (1997). "Nonmuscle myosin II-b is required for normal development of the mouse heart." Proceedings of the National Academy of Sciences of the United States of America **94**(23): 12407-12412.

Turner, C. E. (2000). "Paxillin interactions." Journal of Cell Science **113**(23): 4139-4140.

Turner, C. E., J. R. Glenney, et al. (1990). "Paxillin - a new vinculin-binding protein present in focal adhesions." Journal of Cell Biology **111**(3): 1059-1068.

Upla, P., V. Marjomaki, et al. (2004). "Clustering induces a lateral redistribution of alpha 2 beta 1 integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization." Molecular Biology of the Cell **15**(2): 625-636.

Vallenius, T., B. Scharm, et al. (2004). "The PDZ-LIM protein RIL modulates actin stress fiber turnover and enhances the association of alpha-actinin with F-actin." Experimental Cell Research **293**(1): 117-128.

van der Meer, D. L. M., I. J. Marques, et al. (2006). "Zebrafish Cypher is important for somite formation and heart development." Developmental Biology **299**(2): 356-372.

Vatta, M., B. Mohapatra, et al. (2003). "Mutations in Cypher/ZASPin patients with dilated cardiomyopathy and left ventricular non-compaction." Journal of the American College of Cardiology **42**(11): 2014-2027.

Vigoreaux, J. O. (1994). "The muscle Z-band - lessons in stress management." Journal of Muscle Research and Cell Motility **15**(3): 237-255.

Vinogradova, O., A. Velyvis, et al. (2002). "A structural mechanism of integrin alpha IIb beta 3 "inside-out" activation as regulated by its cytoplasmic face." Cell **110**(5): 587-97

Volk, T. (1999). "Singling out *Drosophila* tendon cells - a dialogue between two distinct cell types." Trends in Genetics **15**(11): 448-453.

Volk, T., L. I. Fessler, et al. (1990). "A role for integrin in the formation of sarcomeric cytoarchitecture." Cell **63**(3): 525-536.

Wahlstrom, G., H. L. Norokorpi, et al. (2006). "*Drosophila* alpha-actinin in ovarian follicle cells is regulated by EGFR and Dpp signalling and required for cytoskeletal remodelling." Mechanisms of Development **123**(11): 801-818.

Wang, K., J. G. Forbes, et al. (2001). "Single molecule measurements of titin elasticity." Progress in Biophysics & Molecular Biology **77**(1): 1-44.

Wang, K., J. McClure, et al. (1979). "Titin - major myofibrillar components of striated-muscle." Proceedings of the National Academy of Sciences of the United States of America **76**(8): 3698-3702.

Wang, N., J. P. Butler, et al. (1993). "Mechanotransduction across the cell-surface and through the cytoskeleton." Science **260**(5111): 1124-1127.

Watters, D., B. Garrone, et al. (1996). "Bistratene A causes phosphorylation of talin and redistribution of actin microfilaments in fibroblasts: Possible role for PKC-delta." Experimental Cell Research **229**(2): 327-335.

Weinert, S., N. Bergmann, et al. (2006). "M line-deficient titin causes cardiac lethality through impaired maturation of the sarcomere." Journal of Cell Biology **173**(4): 559-570.

Werth, D. K., J. E. Niedel, et al. (1983). "Vinculin, a cytoskeletal substrate of protein kinase C." Journal of Biological Chemistry **258**(19): 1423-1426.

West, K. A., H. Y. Zhang, et al. (2001). "The LD4 motif of paxillin regulates cell spreading and motility through an interaction with paxillin kinase linker (PKL)." Journal of Cell Biology **154**(1): 161-176.

Wilcox, M., A. Diantonio, et al. (1989). "The function of PS integrins in *Drosophila* wing morphogenesis." Development **107**(4): 891-897.

Woods, A. and J. R. Couchman (1992). "Protein kinase C involvement in focal adhesion formation." Journal of Cell Science **101**: 277-290.

Woods, A. J., D. P. White, et al. (2004). "PKD1/PKCmu promotes alpha v beta 3 integrin recycling and delivery to nascent focal adhesions." Embo Journal **23**(13): 2531-2543.

Wright, T. R. F. (1960). "The phenogenetics of the embryonic mutant, lethal myospheroid, in *Drosophila* melanogaster." Journal of Experimental Zoology **143**(1): 77-99.

Wu, R. Y., K. Durick, et al. (1996). "Specificity of LIM domain interactions with receptor tyrosine kinases." Journal of Biological Chemistry **271**(27): 15934-15941.

Wu, R. Y. and G. N. Gill (1994). "LIM domain recognition of a tyrosine-containing tight turn." Journal of Biological Chemistry **269**(40): 25085-25090.

Xia, H. H., S. T. Winokur, et al. (1997). "Actinin-associated LIM protein: Identification of a domain interaction between PDZ and spectrin-like repeat motifs." Journal of Cell Biology **139**(2): 507-515.

Xing. B., A. Jedsadayanmata, et al. (2001). "Localization of an integrin binding site to the C-terminus of talin." Journal of Biological Chemistry **276**(48): 44373-44378

Yagi, R., S. Ishimaru, et al. (2001). "A novel muscle LIM-only protein is generated from the paxillin gene locus in *Drosophila*." Embo Reports **2**(9): 814-820.

Yamaji, S., A. Suzuki, et al. (2004). "Affixin interacts with alpha-actinin and mediates integrin signaling for reorganization of F-actin induced by initial cell-substrate interaction." Journal of Cell Biology **165**(4): 539-551.

Yan, B., D. A. Calderwood, et al. (2001). "Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain." Journal of Biological Chemistry **276**(30): 28164-28170.

Yanagawa, S., J. S. Lee, et al. (1998). "Identification and characterization of a novel line of *Drosophila* Schneider S2 cells that respond to wingless signaling." Journal of Biological Chemistry **273**(48): 32353-32359.

Yoshigi, M., L. M. Hoffman, et al. (2005). "Mechanical force mobilizes zyxin from focal adhesions to actin filaments and regulates cytoskeletal reinforcement." Journal of Cell Biology **171**(2): 209-215.

Young, P., C. Ferguson, et al. (1998). "Molecular structure of the sarcomeric Z-disk: Two types of titin interactions lead to an asymmetrical sorting of alpha-actinin." Embo Journal **17**(6): 1614-1624.

Yu, J. G., and B. Russell. (2005)." Cardiomyocyte remodeling and sarcomere addition after uniaxial static strain *in vitro*." Journal of Histochemistry and Cytochemistry **53**(7): 839-844

Zaidel-Bar, R., S. Itzkovitz, et al. (2007). "Functional atlas of the integrin adhesome." Nature Cell Biology **9**(8): 858-868.

Zervas, C. G., S. L. Gregory, et al. (2001). "*Drosophila* integrin-linked kinase is required at sites of integrin adhesion to link the cytoskeleton to the plasma membrane." Journal of Cell Biology **152**(5): 1007-1018.

Zhang, M., J. Liu, et al. (2007). "Identification of CAP as a costameric protein that interacts with filamin C." Molecular Biology of the Cell **18**: 4731-4740.

Zhang, S. J. Y., W. Kraus, et al. (2006). "Integrin beta 1D regulates skeletal myogenesis and mechanotransduction." Faseb Journal **20**(4): A391-A391.

Zhang, Y., D. Featherstone, et al. (2000). "*Drosophila* D-titin is required for myoblast fusion and skeletal muscle striation." Journal of Cell Science **113**(17): 3103-3115.

Zhang, Y. J., K. Chen, et al. (2002). "Characterization of PINCH-2, a new focal adhesion protein that regulates the PINCH-1-ILK interaction, cell spreading, and migration." Journal of Biological Chemistry **277**(41): 38328-38338.

Zhang, Y. J., K. Chen, et al. (2002). "Assembly of the PINCH-ILK-CH-ILKBP complex precedes and is essential for localization of each component to cell-matrix adhesion sites." Journal of Cell Science **115**(24): 4777-4786.

Zhou, Q., P. H. Chu, et al. (2001). "Ablation of Cypher, a PDZ-LIM domain Z-line protein, causes a severe form of congenital myopathy." Journal of Cell Biology **155**(4): 605-612.

Zhou, Q., P. Ruiz-Lozano, et al. (1999). "Cypher, a striated muscle-restricted PDZ and LIM domain-containing protein, binds to alpha-actinin-2 and protein kinase C." Journal of Biological Chemistry **274**(28): 19807-19813.

Ziegler, W. H., A. R. Gingras, et al. (2008). "Integrin connections to the cytoskeleton through talin and vinculin." Biochemical Society Transactions **36**: 235-239.

Ziegler, W. H., R. C. Liddington, et al. (2006). "The structure and regulation of vinculin." Trends in Cell Biology **16**(9): 453-460.

Ziegler, W. H., U. Tigges, et al. (2002). "A lipid-regulated docking site on vinculin for protein kinase c." Journal of Biological Chemistry **277**(9): 7396-7404.

Zieseniss, A., A. G. Terasaki, et al. (2008). "Lasp-2 expression, localization, and ligand interactions: A new Z-disc scaffolding protein." Cell Motility and the Cytoskeleton **65**: 59-72.

Appendix

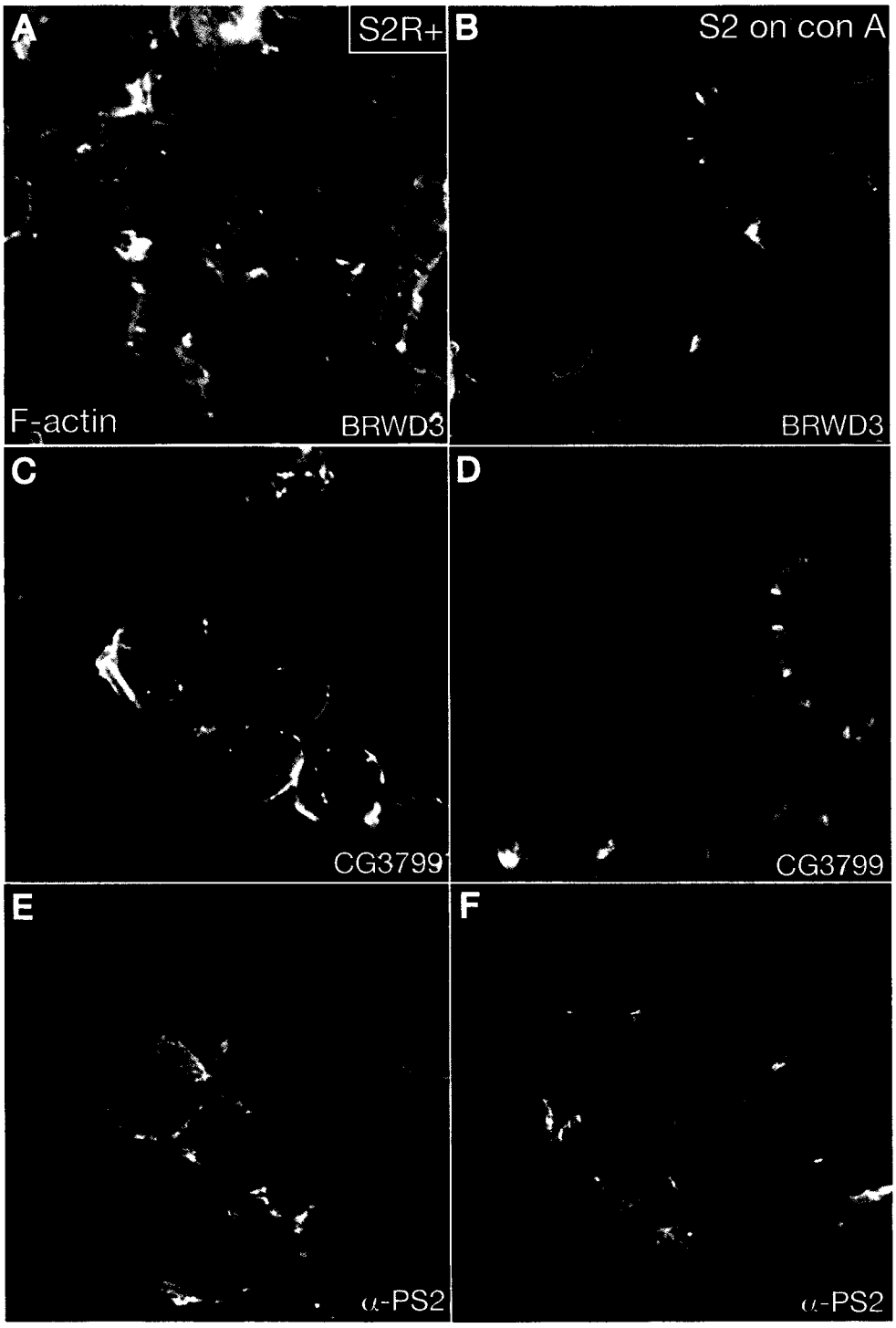


Figure S1. Exclusive spreading defects in S2R+ cells upon depletion of three candidate genes.
Left, S2R+ cells; right, S2 cells spread on concanavalin A. (A and B) BRWD3 RNAi. (C and D) CG3799 RNAi. (E and F) if (αPS2 integrin) RNAi. All cells are stained with Alexa 594–labeled phalloidin to visualize filamentous actin.

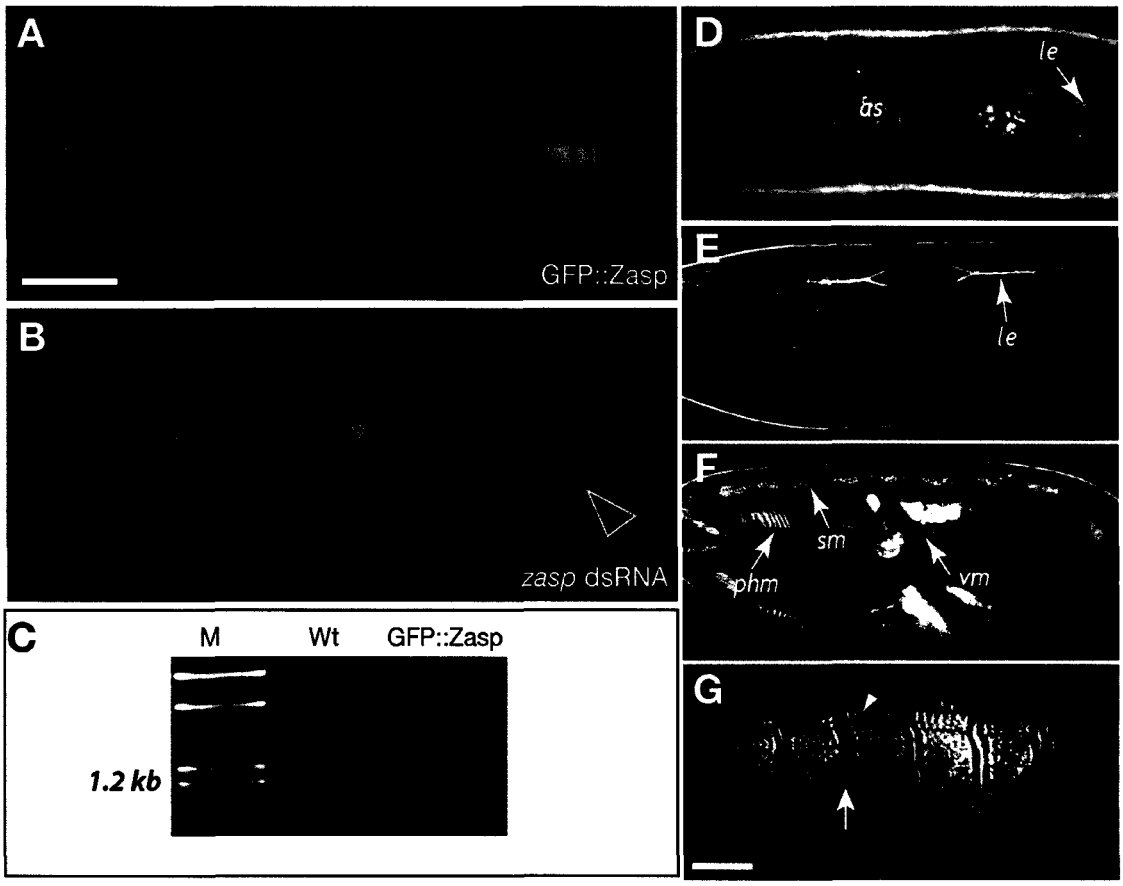


Figure S2. G00189 expresses a functional endogenous GFP-Zasp fusion protein.

(A and B) Live G00189 embryos. GFP-Zasp expression in muscle Z lines disappears in a gradient from the injection site (arrowhead) upon injection of zasp dsRNA against exon 7. Compare uninjected embryo (A) with embryo injected into the posterior (B). Note that the anterior two thirds of the embryo in B is placed on an autofluorescing agar block. (C) RT-PCR with a forward primer binding to GFP-coding sequences (CTACCTGTTCCATGGCCAAC) and a reverse primer binding to zasp-coding sequences (CTCCTGGCTGCTCTCAGACT) shows specific amplification only in the GFP-Zasp fusion line. (D–G) Live imaging of homozygously viable G00189 embryos. GFP-Zasp fusion protein expression follows the expression of endogenous Zasp as detected by antibody staining. (D) Lateral view of an embryo at stage 11 shows GFP -Zasp protein localization in the amnioserosa (as) and the leading edge (le). (E) Dorsolateral view of a stage-14 embryo. The arrow indicates GFP-Zasp expression in the leading edge during dorsal closure. (F) An optical horizontal section of a stage-17 embryo showing GFP-Zasp expression in pharyngeal muscles (phm), somatic muscles (sm), and the visceral mesoderm (vm). (G) An optical section of a stage-17 embryo close to the surface indicating GFP-Zasp expression in Z lines (arrowhead) and myotendinous junctions (arrow). Bars, 50 μm.

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Attended Seminar on Safe Use of Biological Safety Cabinets? If yes, indicate date of attendance.
KLODIANA JANI	BIOLOGY	GRADUATE	YES MAY 2004
JUDIT PANDUR	BIOLOGY	TECHNICIAN	
AMILA SARAC	CONCORDIA	UNDERGRAD	

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

ESCHERICHIA COLI CLASS I
DROSOPHILA TISSUE CULTURE CLASS II
MAMMALIAN TISSUE CULTURE CLASS II
NO USE OF VIRUSES OR ANY OTHER INFECTIOUS AGENTS

ii) the procedures involving biohazards

~~NONE~~ TRANSFECTION OF DNA TO EXPRESS DROSOPHILA PROTEINS

iii) the protocol for decontaminating spills

BLEACH
95% ETHANOL

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards of the infectious agent(s)?

NO

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

YES

9. What precautions will be taken to reduce production of infectious droplets and aerosols?

CLASS II BIOLOGICAL SAFETY CABINET FOR TISSUE CULTURE

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
STEWART	W517	THERMOFORMA	1284	101056-3270	15/12/2003