The role of Lasp in the *Drosophila* male stem cell niche and in muscle development

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ABSTRACT

Drosophila Lasp is the only member of the nebulin family in *Drosophila*. *Lasp* has an amino-terminal LIM domain, two actin-binding nebulin repeats and a carboxyl-terminal SH3 domain and exhibits very high homology to human Lasp. To assess Lasp function in vivo, we generated a null mutant in *Drosophila* Lasp, named $Lasp^{1}$. $Lasp^{1}$ mutants are homozygous viable, but male sterile. Lasp localizes to cyst cells, early germ cells, hub cells and actin cones. In $Lasp^{1}$ mutants, the stem cell niche is no longer anchored to the apical tip of the testis, and actin cone migration is perturbed resulting in improper spermatid individualization. *Lasp* colocalizes with βPS integrin and genetically interact with βPS integrin resulting in complete hub cell mislocalization, which indicates that Lasp modulates integrin adhesion in this context.

$Lasp^{1}$ mutant larvae and flies also have impaired crawling, climbing and flying ability. Lasp localizes to Z lines of third instar larval body wall muscles. In $Lasp^{1}$ mutant indirect flight muscle, thin filament and sarcomere length is reduced while sarcomere ultrastructure is not significantly affected. The same applies to larval body wall muscles, where we observe a misregulation of sarcomere length in both absence and overexpression of Lasp. This phenotype is very similar to *nebulin* mutant knock-out mice indicating that Lasp plays a role in regulating thin filament lengths, but with only two nebulin repeats.

ABRÉGÉ

Chez la drosophile, Lasp est la seule protéine représentante de la famille des Nébuline. Lasp contient un domaine LIM, deux répétitions de type Nébuline et un domaine SH3, et présente une forte homologie avec la famille Lasp des mammifères. Afin identifier le rôle de *Lasp*, nous avons généré une mutation nulle, nommée $Lasp^{1}$. Les mutants $Lasp^{1}$ sont homozygotes viables, mais les mâles stériles. Lasp se localise dans cellules kyste, dans les cellules germinales, les cellules hub et au niveau des cônes d'actine. Chez les mutants $Lasp^{1}$, les cellules souches ne sont plus ancré à l'extrémité apicale du testicule, et la migration des cônes d'actine est perturbée, conduisant à une individualisation irrégulière des spermatides. Lasp est colocalisée avec l'intégrine βPS et interagit génétiquement avec l'intégrine βPS, amenant une délocalization des cellules hub, indiquant que Lasp module adhésion intégrine dans ce contexte.

Les larves mutantes pour *Lasp* se déplacent avec difficulté et les adultes ont avec une capacité d'escalade et de vols réduite. Lasp se localise aux lignes Z dans les muscles des larves du troisième stade. Chez les adultes $Lasp^{1}$, les muscles des ailes présentent une longueur réduite des filaments minces ainsi que des sarcomères, alors que l'ultrastructure du sarcomère ne semble pas être significativement affectée. Les muscles larvaires présentent le phenotype. De plus, on observe un dérèglement de la longueur du sarcomère en surexprimant Lasp dans un contexte sauvage. Ce phénotype est très similaire à celui des souris mutantes pour la nébuline, indiquant que Lasp joue un rôle dans la régulation de la longueur du filament mince, mais avec seulement deux répétitions nébuline.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

Chapter 3

LIST OF TABLES

Chapter 2

AUTHOR CONTRIBUTION

My research is presented here in two manuscript-based chapters.

Chapter 2: This chapter has been published as follows:

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I contributed to the male sterility tests (Table 2.3.1) and the quantification of hub cell mislocalization (Table 2.3.2). I performed experiments shown in Figure 2.3.4 F-I and performed experiments shown in Figure 2.3.3, 2.3.5 and 2.3.6. I wrote part of Materials and Methods and prepared figure legends as well.

Dr. Schöck wrote the Abstract, Introduction, Results, and Discussion sections.

Chapter 3: is a manuscript in preparation

I performed experiments shown in Figure 3.3.1, 3.3.3, 3.3.4, and 3.3.6. I wrote part of all sections included in this Chapter.

Dr. Schöck edited all sections included in this Chapter.

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Chapter 1

Introduction

1.1 Integrins in central biological processes

During development, cell adhesion is vital and underlies the architecture of discrete tissues and organs in multicellular organisms. Integrins are cell adhesion receptors that mediate the interaction between cell and extracellular matrix (ECM) (Hynes, 1992) and contribute to many aspects of cellular behavior including proliferation, polarity, adhesion, differentiation, and migration (Bökel and Brown, 2002; Miranti and Brugge, 2002). Integrins can also maintain proper tissue function and integrity during development and pathology, as loss of integrin function leads to cell-spreading defects and muscle detachment (Hughes, 2001; Devenport *et al*., 2007). In addition, integrins can function in outside-in signaling as well as in inside-out signaling by binding of cytoplasmic proteins followed by changing the extracellular conformation (Coppolino and Dedhar, 1999; Miranti and Brugge, 2002).

Integrins are highly conserved in all metazoans (Gotwals *et al*., 1994; Brower *et al*., 1997). *Drosophila* integrins are heterodimeric transmembrane receptors, consisting of five α subunits and two β subunits, with large extracellular domains and short cytoplasmic tails (Luo and Springer, 2006). The extracellular domains contain binding sites for ECM ligands such as laminins, fibronectin, and collagens (Giancotti and Tarone, 2003). Integrins exist in a range of conformations that are in dynamic equilibrium. In the closed conformation, α and β cytoplasmic tails are in close proximity and the extracellular domains are folded back on themselves. In the open conformation, the two cytoplasmic tails are far apart and the extracellular domains are fully extended. Integrins have great affinity for ECM ligands when in the open conformation (Luo *et al*., 2007).

Some of the integrin heterodimers have been well studied: αPS1βPS localizes ectodermally at muscle attachment sites, whereas αPS2βPS integrin localizes to muscle-ends at muscle attachement sites, and both αPS1βPS and αPS3βPS are required for cell migration (Stark and Hynes, 1994; Roote and Zusman, 1995). In Chapter 2, we use a hypomorphic allele of *myospheroid* (*mys*^{nj42}) to do genetic interaction tests with our mutant *Lasp*^{1}. *mys* encodes βPS integrin (Leptin *et al*., 1989).

In *Drosophila* testes, the stem cell niche adheres to the apical tip of the testis by interacting with ECM components. With the lack of integrin function, the attachment of the stem cell niche to the apical tip of the testis is disrupted (Tanentzapf *et al*., 2007). Talin, a well-known cell adhesion protein, directly links integrin to the actin cytoskeleton (Nayal *et al*., 2004), and its mutant also exhibits hub cell mislocalization in the adult testis (Tanentzapf *et al*., 2007). Therefore, these data demonstrate that integrins are involved in anchoring the male stem cell niche to the apical tip of the testis.

1.2 Drosophila spermatogenesis

1.2.1 Studies of the testicular stem cell niche

Sperm are highly specialized cells with unique morphological and cellular characteristics. Spermatogenesis, which describes the origin and development of sperm cells, requires the coordinated action of many different cell biological events to carry out a cell type-specific differentiation process of great complexity (White-Cooper, 2009).

Drosophila testis is a coiled tube 2 mm in length and 0.1 mm in width (Hardy *et al,* 1979). It is closed at the apical end and connected to the rest of the genital tract at the basal end (Fuller, 1993). *Drosophila* spermatogenesis starts at the apical tip of the testis where the stem cell niche resides. The stem cell niche consists of three cell types, hub cells, germline stem cells, and somatic stem cells. The hub cells, a cluster of approximately 10-15 somatic cells, are firmly attached to the basement membrane of the apical testis wall (Hardy *et al*, 1979). Germline and somatic stem cells surround and are in turn attached to the hub cells. Germline stem cells undergo asymmetric division, giving rise to one stem cell that remains in contact with the hub cells and one daughter cell called a gonialblast. The daughter cell that is displaced away from the hub undergoes four mitotic divisions followed by meiosis resulting in a syncitial cluster of 64 spermatids (Hardy *et al.*, 1979; White-Cooper and Bausek, 2010). Somatic stem cells also divide asymmetrically, but the daughter cell divides only once into two cyst cells, which envelop the gonialblast and all later stages of differentiated germ cells (Hardy *et al.*, 1979, Gonczy and DiNardo, 1996; White-Cooper, 2010). After the meiotic divisions, the 64 spermatids undergo dramatic morphological changes to transform themselves into fully elongated cells.

1.2.2 Individualization of Drosophila spermatids

One final step of spermatogenesis is a process called individualization. During individualization, 64 actin cones move along the spermatid axoneme simultaneously to extrude all cytoplasmic organelles from the cell body in the front and pull in membrane

in the back to generate fully differentiated individual spermatids (Tokuyasu *et al*., 1972; Fabrizio *et al*., 1998). Actin cones are composed of two types of actin cytoskeleton: a branched actin meshwork in front and parallel actin bundles in the rear. Previous work has shown that actin cone movement depends on actin polymerization (Noguchi *et al*., 2003). Supporting this idea, the Arp2/3 complex is involved in promoting actin polymerization and enriched at the front of the actin meshwork. Throughout the entire differentiation process the two cyst cells enclose the germ cells. Eventually, the tail cyst cell collapses and the bundle of mature sperm coils up before being released into the seminal vesicle (Fuller, 1993).

1.3 Muscle cytoarchitecture and mechanisms of contraction

Muscle contraction is accomplished through spatial organization of the cytoskeleton into repetitive contractile units of sarcomeres (Clark *et al*., 2002). The structure and organization of the striated muscle is composed of actin and myosin that are associated with the generation of force, and associated proteins that fine-tune the force generation (Boateng and Goldspink, 2008).

Myofibrils consist of very regular arrays of sarcomeres, the smallest functional unit of muscles. A sarcomere is defined as the segment between two neighboring Z lines. The thin (actin) filaments are anchored at the Z-lines with their barbed end to α-actinin. F-actin, tropomyosin, the troponin complex, tropomodulin, as well as the capping protein CapZ are all required for thin filament assembly and function. In the middle of the

sarcomere are the M line proteins, where the thick (myosin) filaments are anchored. Actomyosin interactions generate contractile force and the sliding of the two filament systems; elastic support is provided by titin, which connects the Z line to the thick filaments (Clark *et al*., 2002; Squire *et al*., 2005).

The Z-line builds up the lateral boundaries of a single sarcomere, which also represents a key interface between the cytoskeleton and the contractile apparatus. In addition, the Z-line largely consists of α-actinin homodimers, which constitutes the backbone for the insertion of actin and titin filaments (Frank *et al*., 2006).

α-actinin is a modular protein that belongs to the spectrin superfamily comprised of a N-terminal acting-binding domain, a central rod domain including four spectrin domains and a C-terminal calmodulin-like domain (Hampton *et al*., 2007). Two α-actinin monomers dimerize via the central rod domain, forming an antiparallel molecule with an actin-binding domain at either ends.

In *Drosophila*, α-actinin may contribute to the stability of the sarcomere, rather than development of its architecture (Vigoreaux, 1994). *Drosophila* α-actinin null mutants are lethal, and those mutants survive into larval development (Fyrberg *et al*., 1998). α-actinin mutants have muscle attachment defects and sarcomeric abnormalities (Dubreuil and Wang, 2000). In non-muscle cells, α-actinin is found in various F-actin containing structures, where it is involved in organizing actin filaments into bundles or networks. α-actinin is also associated with focal adhesions and cell-cell contacts, where it links the actin cytoskeleton to the membrane. In addition, α-actinin interacts with several signaling molecules, suggesting another role as a scaffold bringing interacting proteins together (Otey and Carpen, 2004).

Actin is a ubiquitously expressed protein which plays a fundamental role in many processes of cell motility. Actin fibres represent the main component of the thin filament system. Thin filaments lie on either side of the thick filament and are attached to Z-lines via the barbed end of actin and "capped" by a protein termed capZ (Sellers, 2006).

The heterodimeric actin capping protein CapZ is a major capper of barbed ends of actin filaments, which prevents the G-actin and F-actin association at the capped end and therefore modifies the kinetics of actin polymerization. In fact, capping protein at the barbed end stabilizes actin nuclei, enhancing nucleation rate (Kwiateck *et al*., 2000). In cardiac myocytes, the down-regulation of CapZ is associated with increased myofilament calcium sensitivity and altered Protein Kinase C (PKC) signaling to the myofilaments. Changes in PKC activity are a putative cause of heart failure. Consequently, depressed myocardial contractility of the failing heart is associated with increased activation of PKC (Pyle *et al*., 2006). CapZ has a dual role as both a structural protein and an element of PKC signaling. In *Drosophila* bristles, when capZ function is reduced, F-actin levels increase and the actin cytoskeleton becomes disorganized, causing abnormal bristle morphology (Hopmann and Miller, 2003).

1.4 The Lasp protein family

Lasp-1 was initially identified from screening a cDNA library of breast cancer-derived metastatic axillary lymph nodes (Tomasetto *et al*., 1995a). Lasp-1 is overexpressed in 8% - 12% of human breast carcinomas and ubiquitously expressed in

normal cells with a basal level of expression (Tomasetto *et al*., 1995b; Schreiber et al., 1998). Lasp family proteins are conserved in all metazoans (Nichols *et al*., 2006). In vertebrates, there are two members called Lasp (Lasp-1) and Lasp-2 (LIM-nebulette) (Li *et al*., 2004; Terasaki *et al*., 2004). However, in *Drosophila*, Lasp is the only member of the Lasp family. *Drosophila* Lasp has an amino-terminal LIM domain, two nebulin repeats and a carboxyl-terminal SH3 domain. The LIM domain contains a zinc-finger motif and is involved in many biological functions through its multiple binding partners. (Kadrmas *et al*., 2004). At cell membrane extensions Lasp is directly bound to actin via its nebulin repeats, and the SH3 domain is involved in several protein-protein interactions through binding to proline-rich sequences (Traenka *et al*., 2009). Lasp-1 is involved in the regulation of cytoskeletal architecture, and localized within multiple sites of actin assembly including focal adhesions (Salvi *et al*., 2009). Depletion of Lasp-1 causes the loss of zyxin, thus inhibits proliferation and cell migration (Li *et al*., 2004; Grunewald *et al*., 2006). In addition, Lasp-2/LIM-nebulette is expressed in heart and skeletal muscle and localizes to the sarcomeric Z-lines, intercalated discs, and to focal adhesions. Lasp-2 also binds to α-actinin, which mediates its Z-line localization (Zieseniss *et al*., 2008). Based on these studies, the Schöck laboratory decided to investigate the function of Lasp in Drosophila development.

1.5 Previous work done on Lasp

A previous member of the lab, Soojin Lee, generated a *Drosophila* Lasp null mutant called $Lasp^{1}$. $Lasp^{1}$ mutants are homozygous viable, but male sterile. $Lasp^{1}$ adult flies display several other phenotypes: a detached posterior crossvein in wings, less lateral branches in aristae, and flightlessness. Lasp is expressed in the cell cortex, and in *Drosophila* testis. $Lasp^{1}$ displays a testicular hub cell mislocalization defect in the stem cell niche and disruption of coiling cysts at the basal end. $Lasp^{1}$ also displays actin cone defects during spermatid individualization (Lee, S. (2008) *Lasp is required for anchoring of the male stem cell niche and spermatid individualization in Drosophila* M.Sc. thesis). In addition, Lasp is also expressed in the ovary, where Lasp anchors Oskar to the actin cytoskeleton (Suyama *et al*., 2009). When I took over her work, we asked if hub cell mislocalization is progressive, and what the effect of hub cell detachment is on male sterility. Additionally, since Soojin found that $Lasp^{1}$ mutants are flightless, I then hypothesized that this defect may be due to defects in muscles, and therefore I started to investigate Lasp's function in *Drosophila* myofibrils.

Chapter 2

Lasp anchors the Drosophila male stem cell niche and mediates spermatid individualization

2.1 Abstract

Lasp family proteins contain an amino-terminal LIM domain, two actin-binding nebulin repeats and a carboxyl-terminal SH3 domain. Vertebrate Lasp-1 localizes to focal adhesions and the leading edge of migrating cells, and is required for cell migration. To assess the in vivo function of Lasp, we generated a null mutant in *Drosophila* Lasp. $Lasp^{1}$ is homozygous viable, but male sterile. In *Lasp* mutants the stem cell niche is no longer anchored to the apical tip of the testis, and actin cone migration is perturbed resulting in improper spermatid individualization. Hub cell mislocalization can by phenocopied by expressing Lasp or βPS integrin RNAi transgenes in somatic cells, and Lasp genetically interacts with βPS integrin, demonstrating that Lasp functions together with integrins in hub cells to anchor the stem cell niche. Finally, we show that the stem cell niche is maintained even if it is not properly localized.

2.2 Introduction

Drosophila spermatogenesis starts at the apical tip of the testis where the stem cell niche resides. The stem cell niche consists of three cell types, hub cells that are firmly attached to the basement membrane of the apical testis wall, and germ-line and somatic stem cells that are in turn attached to the hub cells (Hardy *et al*., 1979). Division of a somatic stem cell gives rise to a daughter cell that is displaced away from the hub and will differentiate into a cyst cell. Division of a germ-line stem cell gives rise to a daughter cell displaced away from the hub that undergoes four mitotic divisions followed by meiosis resulting in a syncitial cluster of 64 spermatids. Spermatids then undergo elongation, which is followed by individualization. During individualization, 64 actin cones move along the spermatid axoneme simultaneously pulling in plasma membranes around each spermatid and extruding the cytoplasm to generate fully differentiated individual spermatids (Tokuyasu *et al.*, 1972). Throughout the entire differentiation process two cyst cells enclose the germ cells forming a cyst. Eventually, the tail cyst cell collapses and the bundle of mature sperm coils up before being released into the seminal vesicle (Fuller, 1993).

Here we investigate the in vivo function of Lasp (LIM and SH3 domain protein). Lasp was initially identified from a cDNA library of breast cancer metastases (Tomasetto et al., 1995). Lasp proteins contain an amino-terminal LIM domain, followed by two nebulin repeats and a carboxyl-terminal SH3 domain and are conserved from sponges to humans (Nichols *et al*., 2006). The Lasp nebulin repeats mediate binding to filamentous actin (Chew *et al*., 2002; Schreiber *et al*., 1998). The Lasp SH3 domain interacts with

zyxin, which causes localization of Lasp to focal adhesions (Keicher *et al.*, 2004; Li *et al*., 2004). RNAi-mediated depletion of Lasp inhibits cell migration and proliferation (Grunewald *et al*., 2006; Lin *et al*., 2004).

2.3 Results and discussion

In *Drosophila*, CG3849 encodes the single member of the nebulin and the Lasp family, whereas in vertebrates the nebulin and Lasp families consist of five members, nebulin, nebulette, NRAP, Lasp-1, and LIM-nebulette/Lasp-2, (the last two members comprise the Lasp family (Grunewald and Butt, 2008; Moncman and Wang, 1995; Wang and Williamson, 1980). Drosophila Lasp exhibits very high homology to human Lasp-1 (Fig. 2.3.1). To analyze Lasp function in vivo, we generated a *Lasp* mutant by FRT-mediated recombination of piggyBac elements (Fig. 2.3.1). This deletes the second exon encompassing 31 amino acids of the LIM domain and results in a frameshift. We verified the deletion by PCR and refer to this mutant as $Lasp^{1}$. $Lasp^{1}$ mutants are homozygous viable, but sterile with only rare escapers. Females have reduced fertility, whereas males are almost completely sterile (Table 2.3.1). To check Lasp expression, we stained testes with anti-Lasp antibody. Lasp protein is strongly expressed in testes, but is undetectable in $Lasp^{1}$ mutants (Fig. 2.3.1). Our deletion also deletes CG9692, a gene without homologies sitting in the first intron of Lasp. The *Drosophila* gene disruption project (Bellen et al., 2004) recently generated a *minos* element (*MB03281*) that disrupts the predicted open reading frame of *CG9692*. This transposable element does not show male

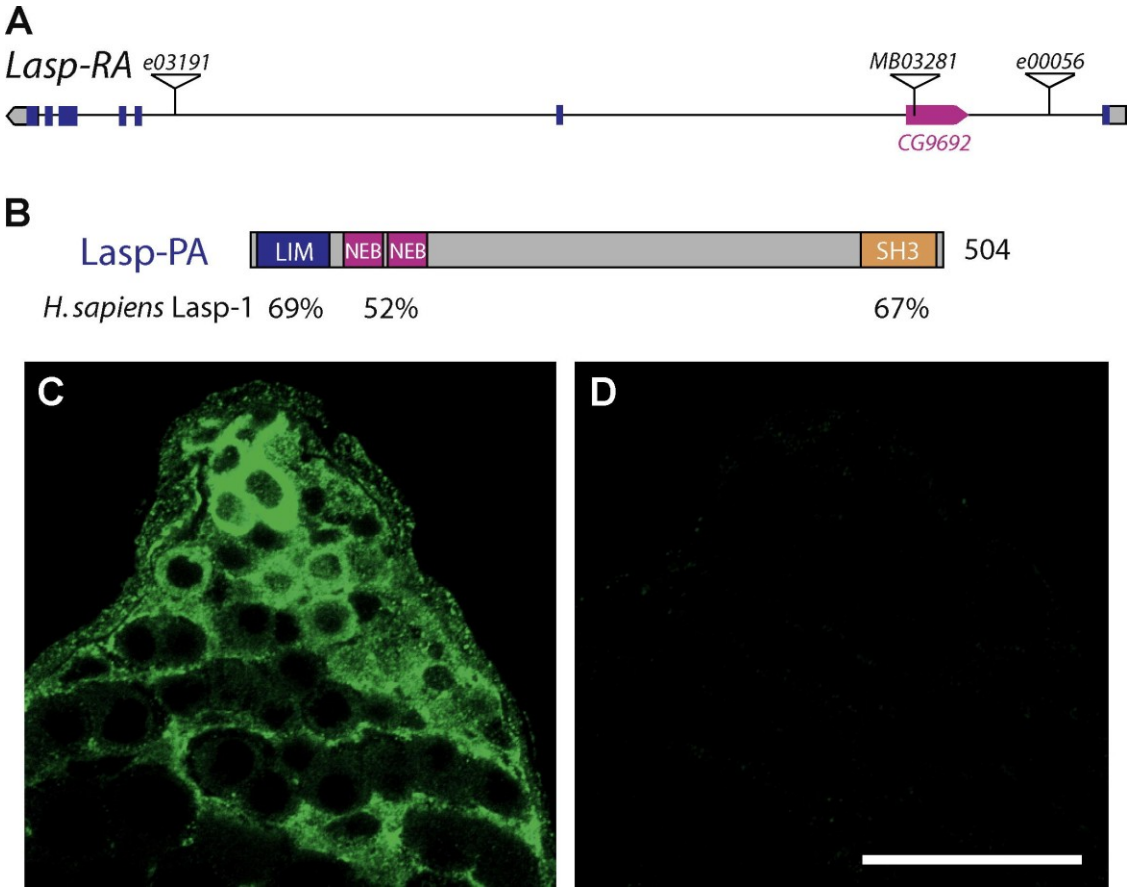

Fig. 2.3.1. The *Lasp* gene.
(A)Schematic presentation of the *Lasp* gene. Only the short isoform *Lasp-RA* is depicted. Translated exons are shown in blue and untranslated exons in gray. piggyBac insertions used to generate $Lasp^{1}$ and the minos element *MB03281* disrupting *CG9692* (in purple) are indicated. (B) The *D. melanogaster Lasp* gene encodes two isoforms containing the same domains. The short isoform Lasp-PA is depicted with the length in amino acids on the right. Below three conserved domains, we show the percent identity between Lasp and its human orthologue. (C and D) Immunostaining of the apical testis tip with rabbit anti-Lasp antibody in wild-type (C), and $Lasp^{1}$ mutant (D). Bar: 30 μm.

Genotype	Days of egglay	Average number of offspring	Number of single males tested
Oregon R	1	64	18
$Lasp^{1}/Lasp^{1}$	1	0	20
$Lasp^{1}/+$	1	84	20
MB03281/MB03281	1	61	26
$Lasp^{1}$/MB03281	1	113	28
$Lasp^{1}$/Df(3L)st7	1	0	20
$Lasp^{1}$ UASLasp-GFP/$Lasp^{1}$ UASLasp-GFP	2	0	21
ptcGal4; $Lasp^{1}$ UASLasp-GFP/$Lasp^{1}$ UASLasp-GFP	2	63	20
$Lasp^{1}$ nosGal4/$Lasp^{1}$ nosGal4	2	0	22
$Lasp^{1}$ nosGal4/$Lasp^{1}$ UASLasp-GFP	2	2	21
mys^{nj42}	1	57	19
mys^{nj42}; $Lasp^{1}/+$	1	81	14

Table 2.3.1: Male Sterility

sterility both homozygously or as a transheterozygous over *Lasp*^{1} (Table 2.3.1). Furthermore, *Lasp*^{1} over the small deficiency *Df(3L)st7* is also homozygous viable and male-sterile and displays the same phenotypes. To unambiguously verify that a mutation in *Lasp*^{1} is responsible for the observed sterility, we created a UAStLasp-GFP transgene with the short Lasp isoform from a testis cDNA library. Expressing UAStLasp-GFP with the germ-line driver nanos-Gal4 rescues slightly, while expression with the somatic driver ptc-Gal4 rescues fertility to a level about half that observed with wild-type (Table 2.3.1). Our combined data demonstrate that we most likely created a null mutant in *Lasp*.

The antibody staining indicates the presence of Lasp protein in both germ cells and somatic cells. To better define the expression of Lasp, we conducted co-immunostainings with well-characterized markers for different testis cell types. Vasa, a germ cell marker (Lasko and Ashburner, 1988), colocalizes with Lasp in early germ cells, but barely in later germ cells (Fig. 2.3.2 A–C). We observe a gradient of Lasp, with its strongest expression observed in early germ cells. Lasp is also expressed in hub cells, as seen by its colocalization with the hub cell marker Fasciclin III, and is likely expressed in stem cells, because cells directly adjacent to hub cells express Lasp (Fig. 2.3.3). In mammalian cells, Lasp localizes to the leading edge of migrating cells and to focal adhesions (Grunewald *et al*., 2006; Lin *et al.*, 2004). Focal adhesions are integrin adhesion sites in tissue culture, where heterodimeric integrin receptors connect cells to the extracellular matrix. We therefore tested colocalization of Lasp with βPS integrin, the major βintegrin subunit in *Drosophila* (Devenport and Brown, 2004). Lasp fully colocalizes with βPS integrin in germ cells and cyst cells (Fig. 2.3.2 D–F). Fax-GFP, another marker for somatic cyst cells (Decotto and Spradling, 2005), also colocalizes

with Lasp demonstrating that Lasp is expressed in cyst cells (not depicted). Finally, Lasp very strongly localizes to actin cones of individualizing spermatids, where it partially colocalizes with actin in all stages of actin cone migration (Fig. 2.3.2 G and H).

We next analyzed the phenotypes observed in $Lasp^{1}$ mutants. In unsquashed wild-type testes hub cells always localize to the apical most tip of the testis. However, hub cells fail to localize to the apical tip of the testis in $Lasp^{1}$ mutants (Fig. 2.3.4 A and B, and Table 2.3.2). This phenotype is similar to a talin mutant that also shows a mislocalization of hub cells (Tanentzapf *et al*., 2007). However, the phenotype is considerably milder, and germ cells are still present in 14-day-old $Lasp^{1}$ males (Fig. 2.3.4 F and Table 2). We can phenocopy hub cell mislocalization by expressing *Lasp* RNAi transgenes targeting different regions of *Lasp* in somatic cells with *ptc-Gal4* and *c587-Gal4*, but not by expressing it with a germ-line driver (Table 2.3.2). This independently confirms that Lasp is responsible for the observed hub cell mislocalization in $Lasp^{1}$ mutants, and it also demonstrates that Lasp acts in somatic cells, most likely the hub cells, to mediate their attachment to the testis wall at the apical tip. We did not analyze hub cell localization in the embryonic gonad, because the Lasp phenotype is already mild in the adult and *Lasp* mRNA expression is not detectable during embryogenesis (BDGP Gene Expression Report).

As Lasp colocalizes with βPS integrin in somatic and germ-line cells, and mutants in both Lasp and talin cause hub cell mislocalization, we asked if Lasp acts together with βPS integrin. We therefore first tested if βPS integrin, which is required for hub cell localization in embryos (Tanentzapf *et al*., 2007), is also required in adult testes. We analyzed a *mys* (encoding β PS integrin) RNAi UAS transgene, which we expressed in

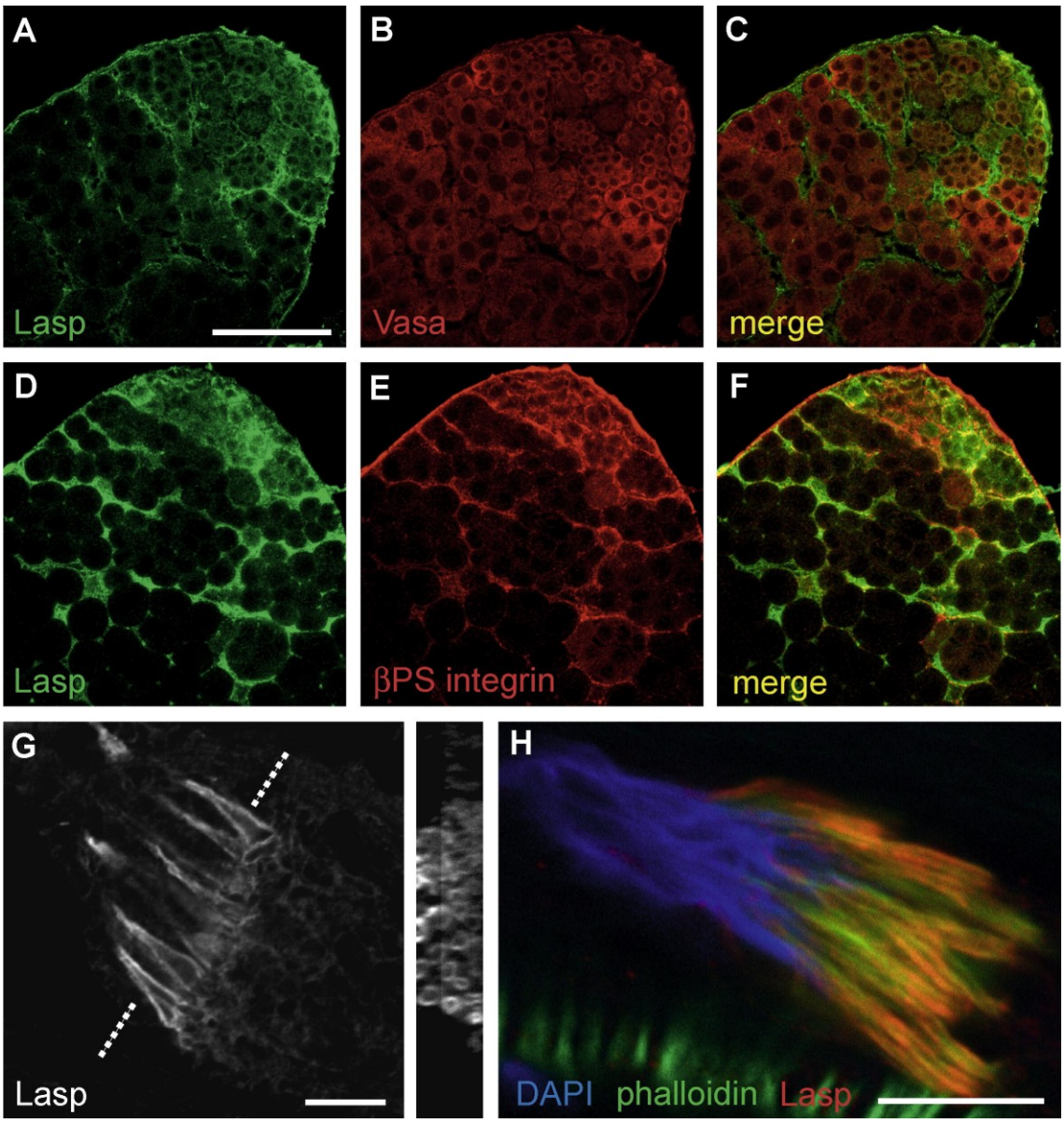

Fig. 2.3.2. Lasp localizes to early germ cells, cyst cells and actin cones.
(A) Anti-Lasp antibody staining, (B) anti-vasa antibody staining, (C) merge of the testis apical tip. (D) Anti-Lasp antibody staining, (E) anti-βPS integrin antibody staining, (F) merge of the testis apical tip. (G) Anti-Lasp antibody staining of actin cones. A z-section of 41 confocal planes at the position indicated by the dashed lines is shown on the right. (H) A triple staining for DAPI to visualize spermatid DNA, phalloidin and anti-Lasp antibody indicates colocalization of actin filaments and Lasp. All images are from squashed testis preparations. Bars: (A–F) 50 μm; (G and H) 10 μm.

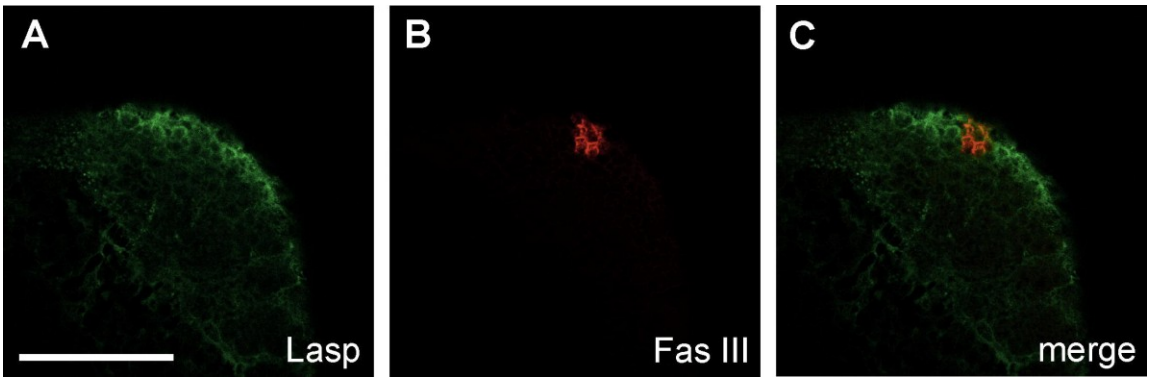

Fig. 2.3.3. Lasp is expressed in hub cells.
(A) Anti-Lasp antibody staining, (B) anti-fasciclin III antibody staining outlining hub cells. (C) Merge. Also note that cells directly adjacent to hub cells, which are likely stem cells, strongly express Lasp. Bar: 50 μm.

Genotype	Mislocalized hub cells 3-day-old (n)	Mislocalized hub cells 14-day-old (n)
Oregon R	0% (20)	ND
$Lasp^{1}/Lasp^{1}$ §	85% (20)	60% (20)
UASiLasp1; c587Gal4§	25% (20)	25% (20)
UASiLasp2; c587Gal4§	30% (20)	30% (20)
UASiLasp1; ptcGal4§	25% (20)	20% (20)
UASiLasp1; nosGal4::VP16§	0% (20)	ND
UASimys; c587Gal4§	30% (20)	30% (20)
mys^{nj42} §	95% (19)	85% (20)
$Lasp^{1}/+$#	0% (16)	0% (20)
mys^{nj42} #	0% (19)	20% (20)
mys^{nj42}; $Lasp^{1}/+$#	50% (18)	39% (18)

Table 2.3.2: Hub cell mislocalization*

* Hub cell localization was analyzed in unsquashed testes.
§ The percentages for 14-day-old males are most likely an underestimate because of the generally perturbed morphology of testes at that age.
# Only hubs completely mislocalized at least 100 µm from the apical tip or absent were counted.

hub cells with the somatic driver *c587-Gal4*. Similar to Lasp and in line with the talin phenotype, we observe a mislocalization of the stem cell niche (Table 2.3.2). Furthermore, mys^{nj42}, a hypomorphic viable βPS integrin mutant, displays a mislocalization of hub cells (Fig. 2.3.4 C and Table 2.3.2). We checked βPS integrin expression in mys^{nj42} by antibody staining, but could not detect a difference compared to wild-type (data not shown), suggesting that complete absence of βPS integrin will result in a stronger phenotype. The mys^{nj42} phenotype is mildly progressive, as we observe a stronger mislocalization in 14-day-old males (Fig. 2.3.4 G and Table 2.3.2). When we additionally remove one copy of $Lasp^{1}$, we observe a complete mislocalization of the stem cell niche in 50% of 3-day-old males (Fig. 2.3.4 E and Table 2.3.2). Importantly, this does not affect fertility, because 13/14 (93%) individual mys^{nj42}; $Lasp^{1}$/+ males exhibit wild-type fertility (Table 2.3.1). The phenotype is also slightly progressive, as we observed one testis out of 18 without hub and germ cells and two testes with hub and germ cells halfway between the apical and basal end in 14-day-old males. To better analyze if the stem cell niche still functions when it is mislocalized, we stained 14-day-old males with anti-vasa and anti-Fas III antibody. Both germ cells and hub cells are still present in slightly (Fig. 2.3.4 F) or completely mislocalized hubs (Fig. 2.3.4 G and H), even when they are halfway between the apical and the basal end of the testis (Fig. 2.3.4 I). Furthermore, we do not observe a disintegration of the hub cell cluster in mislocalized hub cells (Fig. 2.3.4 B, C, E–I). Our data argue that localization of the stem cell niche is not important for stem cell proliferation and maintenance and that the occasional loss of germ cells occurs because the entire stem cell niche is swept into the seminal vesicle and lost. The stronger loss of germ cells previously observed with a talin RNAi transgene in

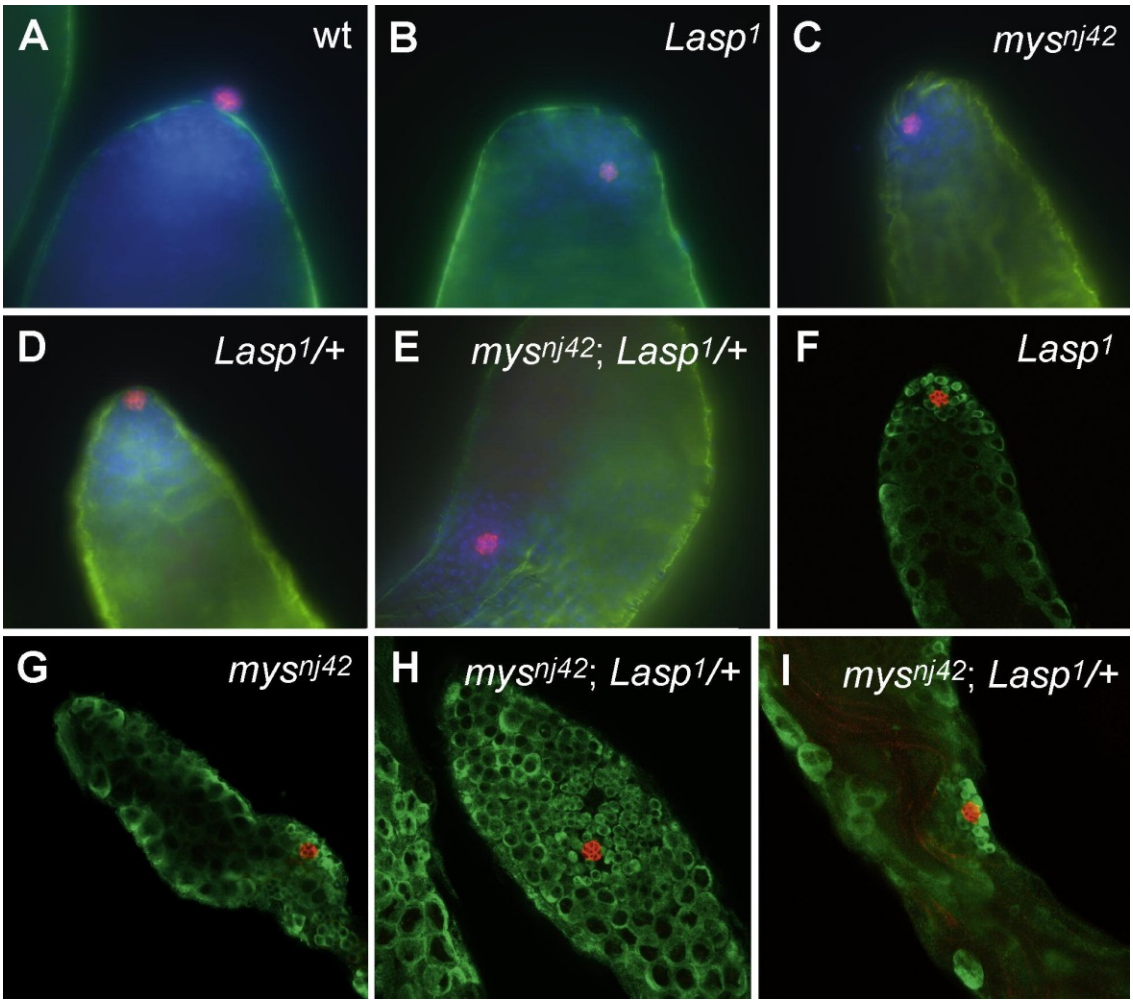

Fig. 2.3.4. In *Lasp*^{1} testes the stem cell niche is not anchored to the apical tip.

(A–E) 3-day-old unsquashed testes stained with anti-Fas III antibody (red) to visualize hub cells, phalloidin-Alexa 488 to visualize filamentous actin, and DAPI to visualize DNA. (A) While the hub cell in wild-type is anchored to the tip, (B) hub cells are no longer attached to the apical tip in *Lasp*^{1} mutants. (C) Hub cells are also mislocalized in *mys*^{nj42}. (D) Hub cells localize normally in a *Lasp*^{1} heterozygous mutant. (E) Hub cells completely mislocalize in *mys*^{nj42}; *Lasp*^{1}/+. (F–I) 14-day-old unsquashed testes stained with anti-vasa to visualize germ cells and anti-Fas III to visualize hub cells. (F) *Lasp*^{1}. (G) *mys*^{nj42}. (H and I) *mys*^{nj42}; *Lasp*^{1}/+. In (I) the stem cell niche is located halfway between the apical and the basal end.

14-day-old cells (Tanentzapf *et al.*, 2007) may be explained in two ways: the talin RNAi transgene likely acts as a stronger allele compared to the mild hypomorph mys^{nj42}, and additionally or alternatively, talin may not only function in integrin-dependent cell-matrix adhesion, but also in cell–cell adhesion (Becam *et al*., 2005). Intriguingly, Lasp is also specifically expressed in cap cells, the stem cell niche in the ovary, suggesting that Lasp may play a more general role in the attachment of stem cell niches (Fig. 2.3.5).

We also observe premature coiling of cysts and a premature disintegration of cysts into individual sperm at the coiling stage in $Lasp^{1}$ mutants (Fig. 2.3.6 A and B). This phenotype is most likely due to a function of Lasp in cyst cells, because the somatic driver ptc-Gal4 completely rescues premature coiling and disintegration, whereas the germ-line driver nos-Gal4 does not rescue this phenotype at all (Fig. 2.3.6 C and D). Lasp may function in cyst cell maintenance or contribute to proper adhesion of cyst cells to the spermatid bundle, which could indirectly contribute to a breakdown of cyst cells after loss of adhesion.

Finally, we analyzed if Lasp plays a role in spermatid individualization. In wild-type actin cones, Lasp localizes to the actin cone colocalizing with filamentous actin. In $Lasp^{1}$ mutants, the actin cytoskeleton in the cone is affected: filamentous actin is only weakly visible at the assembly stage of actin cones (Fig. 2.3.7 A and B). We calculated the average mean gray value of pixels in actin cones stained and imaged under identical conditions and observe that filamentous actin staining is approximately twice as strong in wild-type (76 on a scale of 0–255; n = 53) than in $Lasp^{1}$ mutants (31; n = 30). At the migrating stage filamentous actin is barely or not visible at all (not depicted). This phenotype is similar to the myosin VI mutant (Noguchi et al., 2006). Weaker F-actin

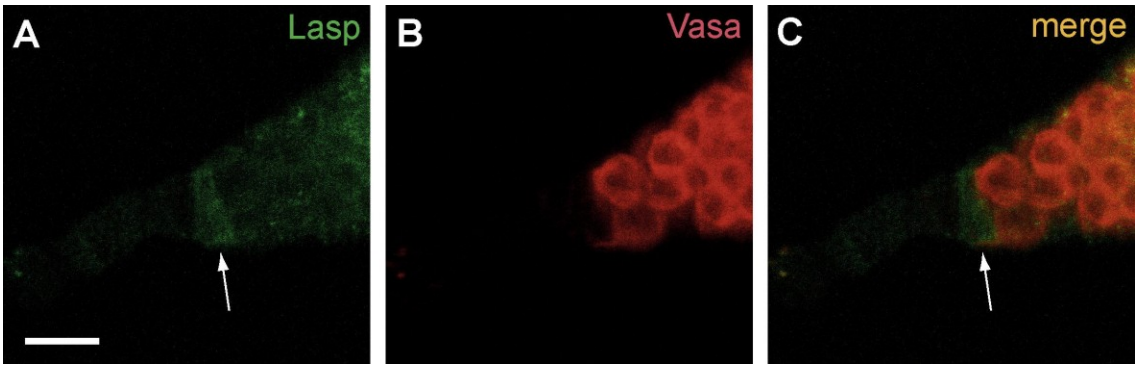

Fig.2.3.5. Lasp is expressed in cap cells in the ovary.
(A) Anti-Lasp antibody staining, (B) anti-vasa antibody staining, and (C), merge of the germarium. Lasp is expressed anterior and adjacent to germ cells (arrows). Bar: 10 μm.

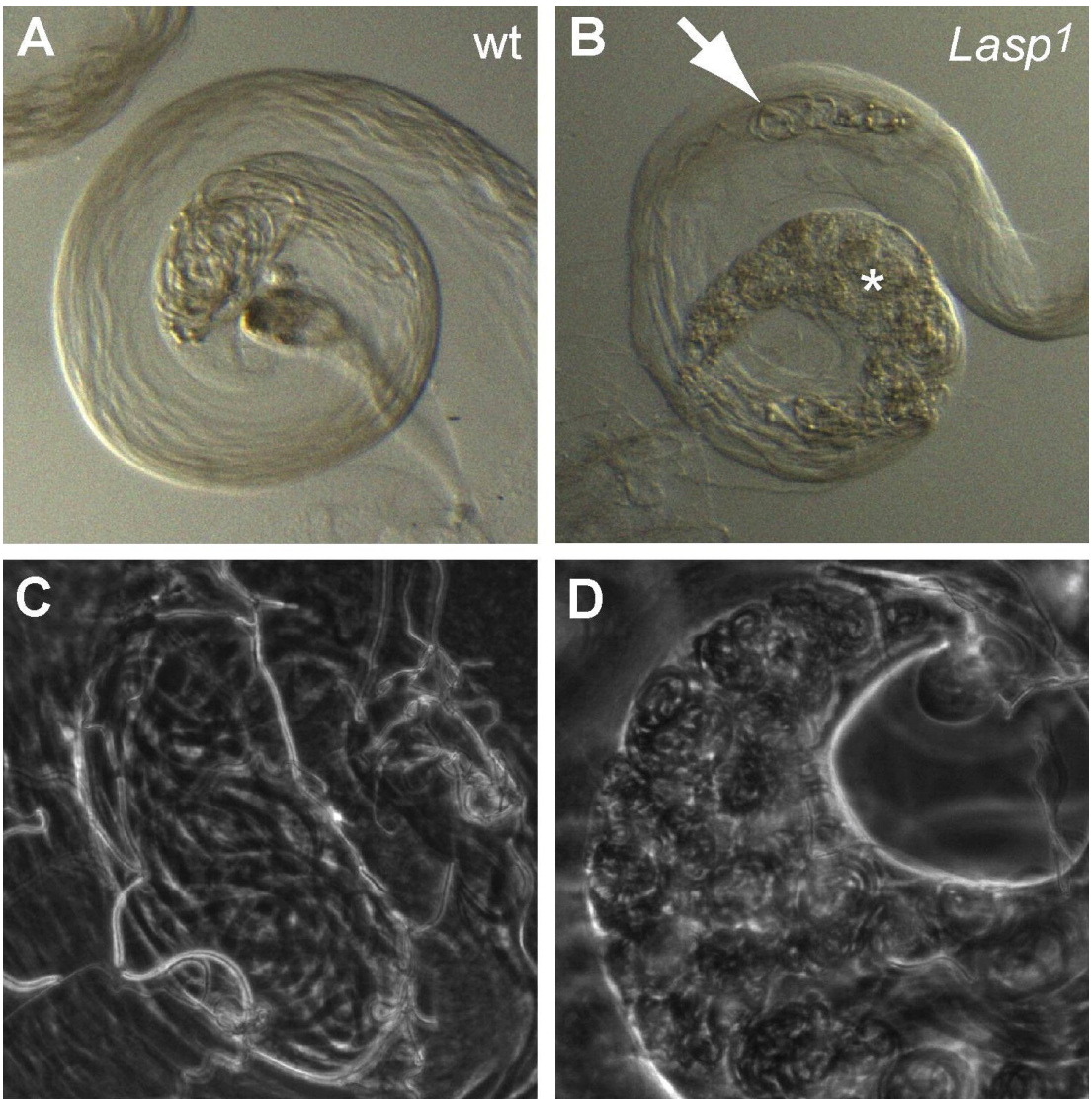

Fig. 2.3.6. Cyst cells disintegrate prematurely in *Lasp*^{1} mutants.
(A) Bright-field image of a wild-type testis showing the coiling stage of cysts in the basal end. (B) Cysts coil prematurely (arrow) and disintegrate into individual sperm at the coiling stage (asterisk) in *Lasp*^{1} mutant. (C) Differential interference contrast image of the basal end of a *Lasp*^{1} mutant rescued by ptcGal4-driven expression of UASLasp-GFP. Coiling defects are completely rescued. (D) Differential interference contrast image of the basal end of a *Lasp*^{1} mutant. Defects are not rescued by nosGal4-driven expression of UASLasp-GFP.

staining is not enhanced in a *mys*^{nj42} background, consistent with the absence of integrin staining in actin cones (data not shown). Given Lasp's dual function in binding to filamentous actin and localizing to the leading edge of migrating cells in vertebrates (Chew *et al*., 2002; Lin *et al*., 2004), we suggest that Lasp may tether the actin cytoskeleton to the plasma membrane of the actin cone. To support this notion, we investigated cross sections of cysts by transmission electron microscopy. Apart from incomplete and slower spermatid individualization (Fig. 2.3.7C), we always observe incomplete extrusion of the cytoplasm indicated by a loose membrane surrounding individual spermatids and additional membrane fragments in between spermatids, indicating a failure to properly pull in the membrane around individual spermatids (Fig. 2.3.7 D and E).

Finally, we wanted to determine the phenotype responsible for the observed sterility. To this end, we compared the rescue of actin cone defects and cyst disintegration in ptc-Gal4 rescued flies and nos-Gal4 rescued flies. We could not evaluate the role of hub cell localization with these driver lines, but we already know that mislocalization of hub cells does not cause sterility. The actin cone defects are rescued with nos-Gal4 (data not shown), but nos-Gal4 barely rescues sterility (Table 2.3.1), indicating that the actin cone defect contributes only slightly to the observed sterility of *Lasp*^{1}. Even though actin cones are rescued as judged by phalloidin staining, it is possible that using UASp or the long isoform of Lasp may result in a better rescue. In contrast, actin cone defects are not rescued with the ptc-Gal4 driver, while premature cyst cell coiling and disintegration is completely rescued and sterility is substantially rescued suggesting that this phenotype is largely responsible for the observed sterility (Fig. 2.3.6, and Table 2.3.1).

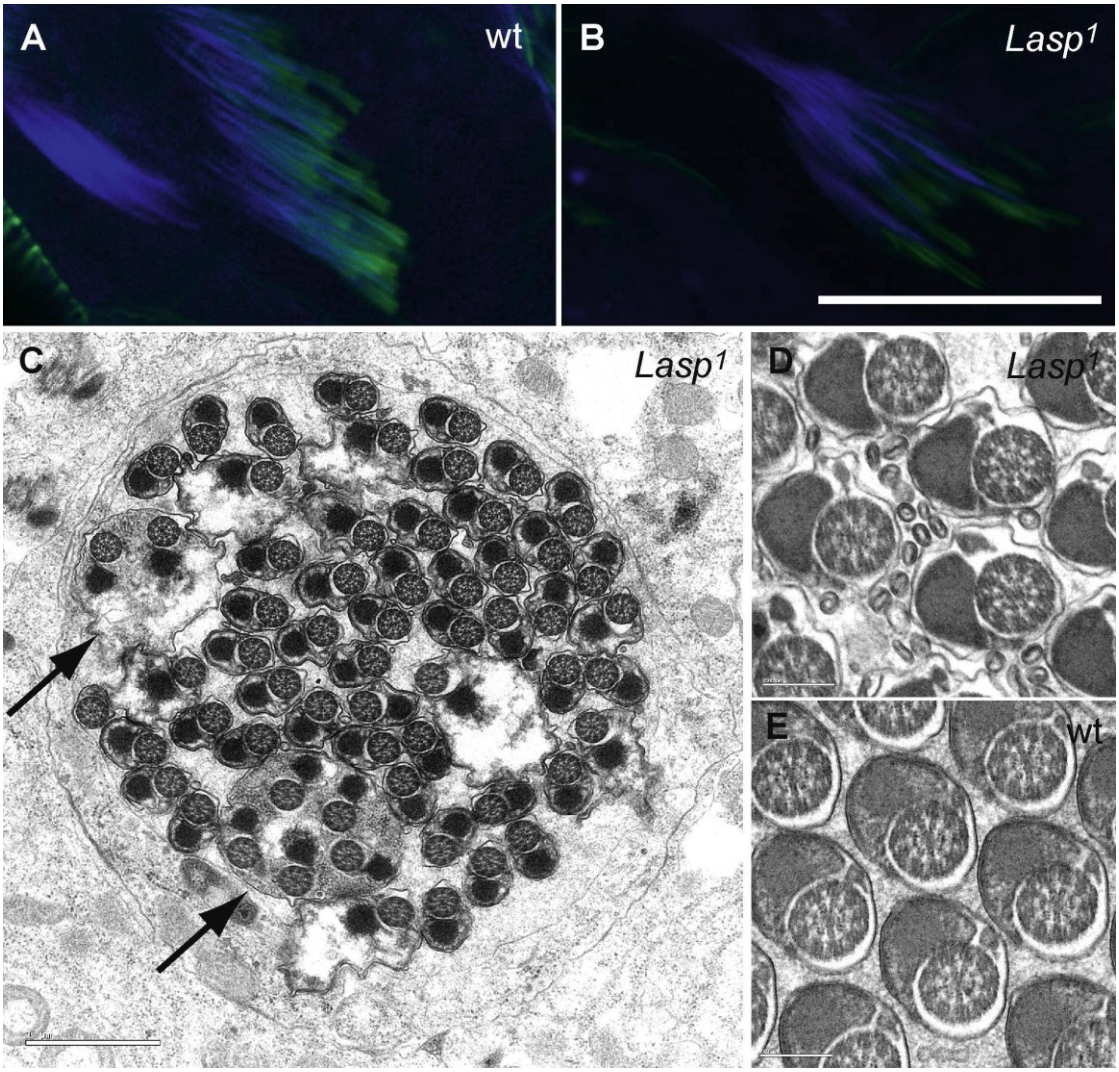

Fig. 7. Actin cones are perturbed causing individualization defects in *Lasp*^{1} mutant testis.

(A and B) Phalloidin and DAPI double staining. Compared to actin cones in wild-type (A), *Lasp*^{1} mutant actin cones are weakly visible (B). (C–E) Transmission electron micrographs of testes cross-sections. (C) Spermatids are not completely individualized in *Lasp*^{1} (arrows indicate membranes surrounding two and five spermatids, respectively). (D) Individualized spermatids are surrounded by a loose membrane and membrane fragments are found in between spermatids in *Lasp*^{1}. (E) In wild-type, membranes are tightly apposed and spermatids are closely spaced. Bars: (A and B) 25 μm; (C) 1 μm; (D and E) 200 nm.

In conclusion, Lasp has an integrin-dependent function in anchoring the stem cell niche, where Lasp likely functions at the periphery of the integrin adhesion site, given its weak phenotype compared to βPS integrin. Lasp also exhibits an integrin-independent function in actin cone migration. Intriguingly, human Lasp-1 similarly localizes to focal adhesions and to the cortical actin cytoskeleton in the leading edge of migrating cells, and it is the leading edge localization that appears to mediate cell migration (Lin *et al*., 2004). Lasp function in whole animals is restricted to highly specialized tissues, suggesting that integrin adhesion sites and actin organization differ depending on tissue and function, which may be one reason for the large number of biochemically defined components of integrin adhesion sites (Zaidel-Bar *et al*., 2007) and actin-binding proteins (Winder and Ayscough, 2005). Furthermore, our data show that the stem cell niche does not require a specific location to function properly, and by extension, that the male stem cell niche in *Drosophila* requires only one type of support cells, the hub cells.

2.4 Materials and methods

2.4.1 Fly stocks and genetics

The following fly stocks were used: *e00056* and *e03191* from the Exelixis Drosophila stock collection, 47127 (*UASiLasp1*), 21500 (*UASiLasp2*), and 29619 (*UASimys*) from the Vienna Drosophila RNAi center (Dietzl *et al*., 2007), mys^{nj42} from Nick Brown, *MB03281* from Hugo Bellen, *Df(3L)st7* from the Bloomington *Drosophila* stock center, *nos-Gal4::VP16* from David Dansereau, *ptc-Gal4* from Leanne Jones, and *c587-Gal4*

from Allan Spradling. The following recombinants and stocks were made by standard genetic crosses: *UASLasp-GFP Lasp*^{1}*, ptc-Gal4; UASLasp-GFP Lasp*^{1} *and nosGal4::VP16 Lasp*^{1}.

To generate the UAStLasp-GFP construct, the short isoform of Lasp encoding a 504 amino acid protein isolated from an adult testes cDNA library (AT23571) was amplified by primers CACCATGAATAAAACCTGTGCCCCGT and TATAACCGCCTGCTCCACGTA and cloned into the Gateway pENTR/D-TOPO vector (Invitrogen), then recombined into pTWG from the *Drosophila* Gateway vector collection (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html).

A *Lasp* mutant was generated by using piggyBac elements *e00056* and *e03191* for heat shock-mediated FLP/FRT recombination (Parks et al., 2004). The *Lasp* mutant fly line was verified by genomic PCRs for the presence of *e03191* (with primers TCCAAGCGGCGACTGAGATG and CTGACGAGCATTTTCATTTATATA), absence of exon 2 (with primers ATGGCACAAAACGTG and TGCTGCACTTTTTGC), absence of *CG9692* (with primers ATTAGGGTTCAGCCACATGC and GATTCCAACGCCTATCTGGA) and presence of exon 1 (with primers CGTTTTAAGCCGCTCTTTTG and AACACCGAAGCAGAGC).

To test male sterility, one male fly and four OreR female flies (1–2 days of age) were mated in a vial for two days at 25 °C. Then the flies were transferred to a fresh vial and allowed to lay eggs for 24 or 48 h at 25 °C. Only vials with all flies alive were analyzed.

2.4.2 Histochemistry and microscopy

Testes dissected from 3-day-old flies were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, 8 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl) for 15 min, and permeabilized in 0.1% Triton X-100 in PBS for 1 min after fixation. After preincubation with blocking solution (3% goat serum, 0.05% Tween 20 in PBS) for 30 min, testes were incubated at 4 °C overnight with the following primary antibodies: rabbit anti-Lasp-C terminal (1:4000 provided by Anne Ephrussi), rat anti-vasa (1:10,000 provided by Paul Lasko) (Styhler *et al*., 1998), mouse anti-βPS integrin (1:10, CF.6G11 obtained from Developmental Studies Hybridoma Bank) (Brower *et al*., 1984), and mouse anti-fasciclin III (1:50, 7G10 obtained from DSHB) (Patel *et al*., 1987). After washing for 1 h in PBT (0.05% Tween 20 in PBS) next day, the testes were incubated in secondary antibodies of the Alexa-Fluor series (1:400, Invitrogen) for 2 h at room temperature. The testes were mounted in Prolong Gold Antifade solution (Invitrogen) after 1 h of washing in PBT. Some testes were squashed prior to fixation. Testes were placed on a poly-l-lysine-coated slide in Testis Buffer (TB; 15 mM potassium phosphate pH 6.7, 80 mM KCl, 16 mM NaCl, 5 mM MgCl2, 1% PEG 8000). A siliconized cover slip was dropped onto the sample and pressed softly. Then the slide was frozen in liquid nitrogen for 3 min, and washed in 95% ethanol for 10 min. Finally, testes were fixed and stained in a wet chamber like the unsquashed samples.

Images were obtained on an upright microscope (DM6000B, Leica) with a digital camera (Orca-ER, Hamamatsu) and OpenLab software or a confocal microscope (LSM510 Meta, Zeiss), and processed with ImageJ and Adobe Photoshop software. To

measure actin cone density, images were taken at the confocal microscope with a 63× C-Apochromat NA 1.2 water objective at three times zoom, and ImageJ was used to measure the mean pixel values in a 150 × 150 pixel square, which covered the basal ends of multiple actin cones.

2.4.3 Electron microscopy

To obtain cross-section images of cysts, testes were dissected from 3-day-old adult flies and fixed in 1.5% glutaraldehyde for 2 h on ice. After fixation, testes were washed for three times in PBT for 10 min. Then they were postfixed in 1% osmium tetroxide for 2 h. After washing, testes were dehydrated in acetone and embedded in epon-815 (Electron Microscopy Sciences). Samples were cut with a Reichert Ultracut AV microtome. EM grids were stained in 4% aqueous uranyl acetate for 8 min, and in Reynold's Lead for 5 min. Images were obtained on a transmission electron microscope (Tecnai-12, FEI) with a wide-angle multiscan CCD camera (792 Bioscan 1k × 1k, Gatan).

2.5 Acknowledgements

We particularly thank Anne Ephrussi for providing the anti-Lasp antibody before publication. We also thank Beili Hu, Judit Pandur, and Jeannie Mui for technical assistance; Hugo Bellen, Nick Brown, David Dansereau, Leanne Jones, Paul Lasko, and Allan Spradling for reagents, Julie Brill for helping us learn the squash technique, and

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Connecting text

In the research presented in Chapter 2, we showed that in *Drosophila*, Lasp is the single member of the nebulin family. *Drosophila* Lasp colocalizes with integrin adhesion sites and structures of the actin cytoskeleton in a tissue-specific manner. In the testis, Lasp colocalizes with actin in actin cones before and throughout migration. Null mutations in *Lasp* are homozygous viable, cannot properly anchor the stem cell niche, and have defects in actin cone migration during spermatid individualization. We could show that attachement of the stem cell niche to the apical tip of the testis is integrin-dependent, whereas Lasp's function in actin cone migration does not depend on integrins. The investigation presented in Chapter 3 will focus on integrin-independent aspects of Lasp's role in remodeling the actin cytoskeleton in striated muscle.

CHAPTER 3

The nebulin family member Lasp regulates thin filament length in *Drosophila* myofibrils

3.1 Abstract

Lasp is the only member of the nebulin family in *Drosophila*, which comprises nebulin, nebulette, NRAP, Lasp-1 and Lasp-2 in vertebrates. Lasp binds filamentous actin through its two nebulin repeats, and is required for actin cone migration in the testis. Here we show that $Lasp^{1}$ mutants exhibit impaired crawling, climbing and flying ability. Lasp localizes to Z lines of larval body wall muscles. While sarcomere ultrastructure is not visibly affected in $Lasp^{1}$ mutants, both absence and overexpression of Lasp results in misregulation of sarcomere and thin filament length. In contrast to nebulin, Lasp can regulate thin filament length with only two nebulin repeats.

3.2 Introduction

Cytoskeletal remodeling is at the core of most developmental and physiological changes in metazoan animals. The actin cytoskeleton plays a central role in cell morphology, motility, division, signaling, muscle contractility, and in many other processes. The properties of filamentous actin, that is assembly, disassembly, stabilization, and bundling, are regulated by a wide range of actin binding proteins, resulting in many different structures in differentiated cells (Ayscough, 1998; Chhabra *et al*., 2007; Winder and Ayscough, 2005).

One group of actin-binding proteins is the nebulin family, which comprises nebulin, nebulette, N-RAP, Lasp-1 and Lasp-2/LIM-nebulette (Zieseniss *et al*., 2008). Each member of the family contains from 2 to 185 actin-binding nebulin repeats. Nebulin, the founding member of the family, is a giant molecular protein with a C-terminal SH3 domain and 185 nebulin repeats spanning the entire length of vertebrate thin filaments in the sarcomere. Nebulin depletion by RNAi in rat cardiomyocytes causes longer thin filaments than usual, whereas nebulin depletion by RNAi or in knock-out mice reduces thin filament length, prompting the notion that nebulin acts as a molecular ruler to specify the final length of thin filaments in vertebrates (Bang *et al*., 2006; McElhinny *et al*., 2005; Pappas *et al*., 2008; Witt *et al.*, 2006). Recently, nebulin was shown to bind to and regulate the capping protein CapZ at the Z line (Pappas *et al*., 2008).

Nebulette is smaller than nebulin and cannot span the entire length of thin filaments, but contains like nebulin a C-terminal SH3 domain. Its expression is restricted

to cardiac muscles, where it is involved in heart development and myofibril assembly (Arimura *et al*., 2000; Moncman and Wang, 2002). N-RAP is yet smaller, contains 45 nebulin repeats and an N-terminal LIM domain, and has been implicated in myofibril assembly (Carroll *et al*., 2004; Carroll *et al*., 2001; Dhume *et al*., 2006).

Finally, Lasp-1 and Lasp-2 are unique, because they have both the N-terminal LIM domain and the C-terminal SH3 domain, but only 2 or 3 nebulin repeats, respectively (Grunewald and Butt, 2008). They are both ubiquitously expressed and localize to focal adhesions as well as the cortical cytoskeleton (Chew *et al*., 2002; Lin *et al*., 2004). They are also expressed in striated muscle, where Lasp-1 localizes to the Z line and A band, whereas Lasp-2 localizes only to Z lines. Lasp-2 also interacts directly with α-actinin, which may mediate its Z line localization (Zieseniss *et al*., 2008).

Here we show that *Lasp* mutants have impaired muscle function, and that both Lasp overexpression and its mutation result in misregulated thin filament length.

3.3 Results

3.3.1 *Lasp* mutants have impaired crawling, climbing, and flying ability

We created a null mutation in *Drosophila* Lasp by deleting the second exon including 31 amino acids of the LIM domain, which we refer to as $Lasp^{1}$ (Lee *et al.*, 2008). These flies are homozygous viable, but we noted that they are not as agile as wild type *Drosophila*. We first assayed the crawling ability of third instar larvae in wild type

and *Lasp*^{1} mutants, counting the number of their body wall contractions per minute. *Lasp*^{1} mutant larvae crawl much slower than the wild type as their body wall contracts less per minute (Fig. 3.3.1A). Then we assayed the climbing ability of adult flies, measuring the time they require to climb a certain distance inside a cylinder. *Lasp*^{1} mutant flies are considerably worse climbers (Fig. 3.3.1B). We also checked if this phenotype is progressive by assaying the climbing ability of 5-d and 14-d old flies and we see that old flies show the same ability gap to wild type as young flies, showing that this phenotype is not progressive. We also quantified their flying abilities with a flight test cylinder (Koana and Hotta, 1978). Wild type flies stick to the very top of the cylinder, indicating their perfect flying ability. In contrast, *Lasp*^{1} mutant flies stick preferentially to the lower portions of the cylinder, demonstrating that their flight is compromised. Flying ability slightly diminished with age compared to wild type (Fig. 3.3.1C).

3.3.2 Lasp localizes to Z lines in third instar larval body wall muscles

The impaired muscle function we observed could be caused by defects in myofibril assembly, and we therefore determine if Lasp is expressed in muscles and where it localizes. Lasp localizes to the myotendinous junction and a repetitive line pattern in larval body wall muscles (Fig. 3.3.2). To see if these lines correspond to the Z lines, we analyzed Lasp distribution in relation to α-actinin, a well-known marker of Z lines. Lasp and α-actinin fully colocalize at myotendinous junctions and Z lines, confirming the Z line localization of Lasp (Fig. 3.3.2).

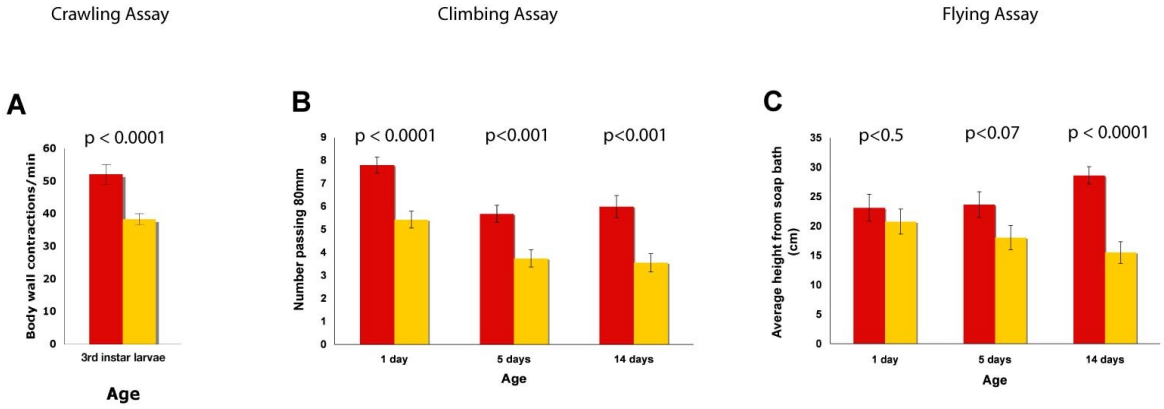


Figure 3.3.1. *Lasp*^{1} mutants cannot crawl, climb, or fly properly.
(A) Crawling assay. Average body wall contractions per minute at which larvae crawl in a petri dish are indicated. (B) Climbing assay. Avearge number of flies crossing the 80mm line in a 100ml cylinder within 10 s are indicated. (C) Flying assay. Flies were dropped into a cylinder. Average height at which flies alight for the first time is indicated. Red= Oregon R; yellow= *Lasp*^{1}. Standard error of the mean is shown. P-value is shown.

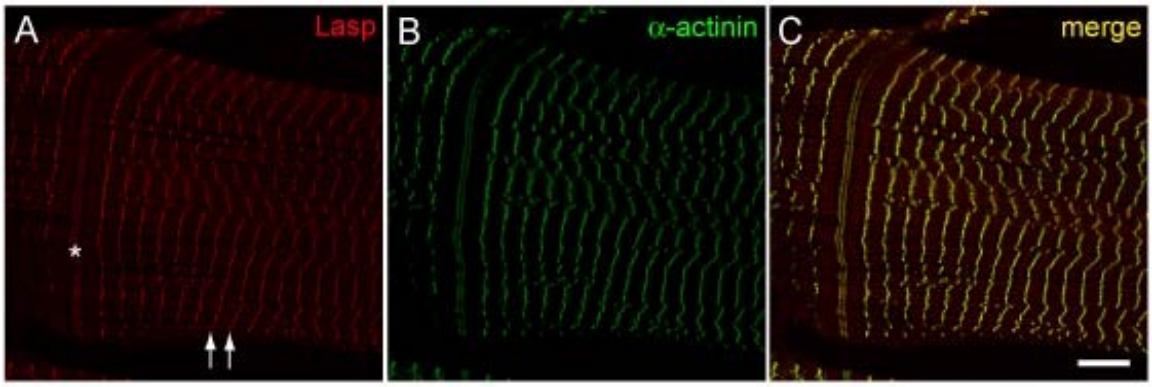


Figure 3.3.2. Lasp localized to Z lines.
(A) Anti-Lasp antibody staining of third instar larval body wall muscle. (B) Anti-α-actinin antibody staining. (C) Merge. Asterisk, myotendinous junction; arrows, Z lines. Bar, 20μm.

3.3.3 Sarcomere ultrastructure is generally unperturbed in *Lasp* mutants

To determine if Z line or sarcomere structure is affected in *Lasp* mutants, we analyzed sarcomeric ultrastructure of both indirect flight muscle and third instar larval body wall muscle by transmission electron microscopy. We did not observe any obvious structural defects in electron micrographs of $Lasp^{1}$ indirect flight muscle and third instar larval body wall muscle compared to wild-type. Both the overall morphology as well as the Z line appears very similar (Fig. 3.3.3A and Fig 3.3.3B). However, we noted that sarcomere length appears to be misregulated in *Lasp* mutants.

3.3.4 Sarcomere and thin filament length is reduced in *Lasp* mutant indirect flight muscles

To obtain a global view of sarcomere length, we stained indirect flight muscles with Z line marker Zasp and with phalloidin to visualize thin filaments. In wild type indirect flight muscles, both sarcomere length and thin filament length are very stereotypical (Fig. 3.3.4A, C). In $Lasp^{1}$ mutants, both sarcomere length and thin filament length is reduced (Fig. 3.3.4B, C).

3.3.5 Sarcomere length is altered in Lasp mutant larval muscles and upon OE

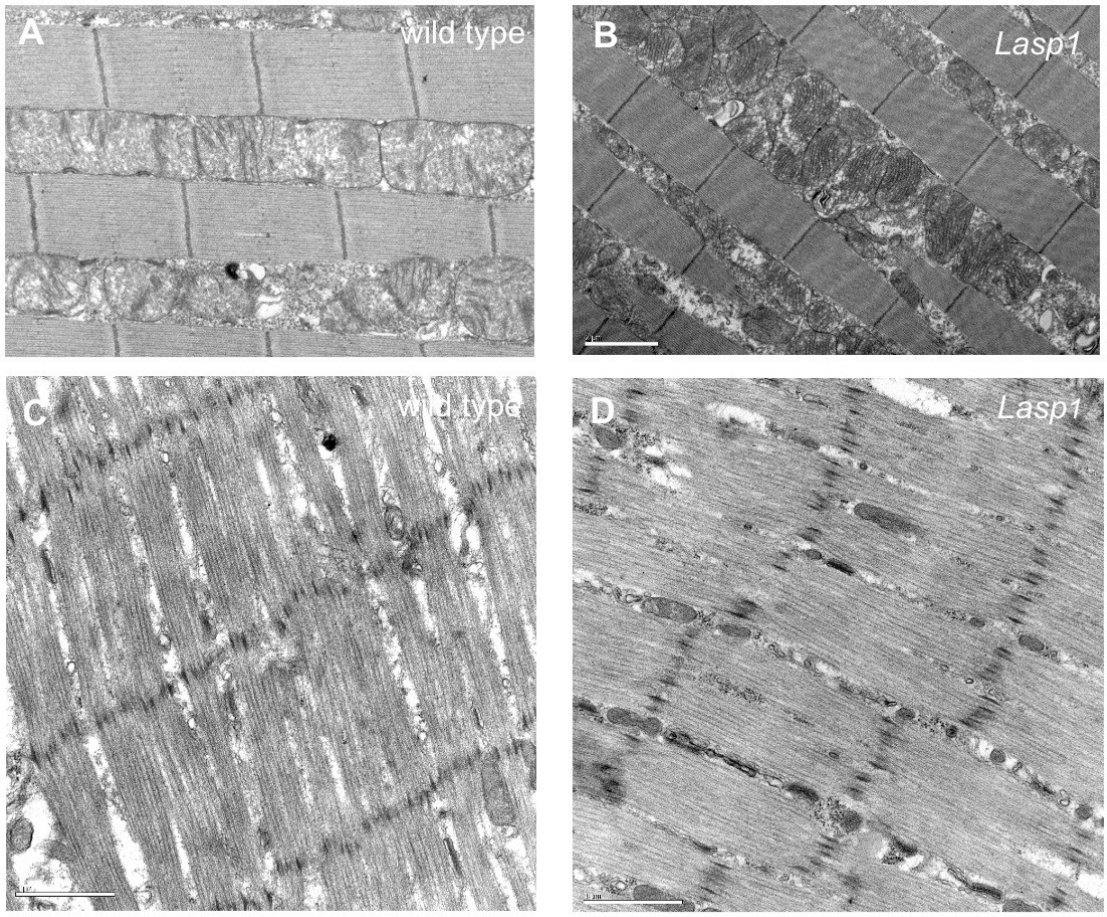


Figure 3.3.3. Sarcomere ultrastructure is not significantly affected in *Lasp*^{1} mutants.

(A) Electron micrographs of Oregon R indirect flight muscle. (B) Electron micrographs of *Lasp*^{1} indirect flight muscle. (C) Electron micrographs of Oregon R third instar larval muscle. (D) Elctron micrographs of *Lasp*^{1} third instar larval muscle. Bars, 2 µm (A, B); 200nm (C, D).

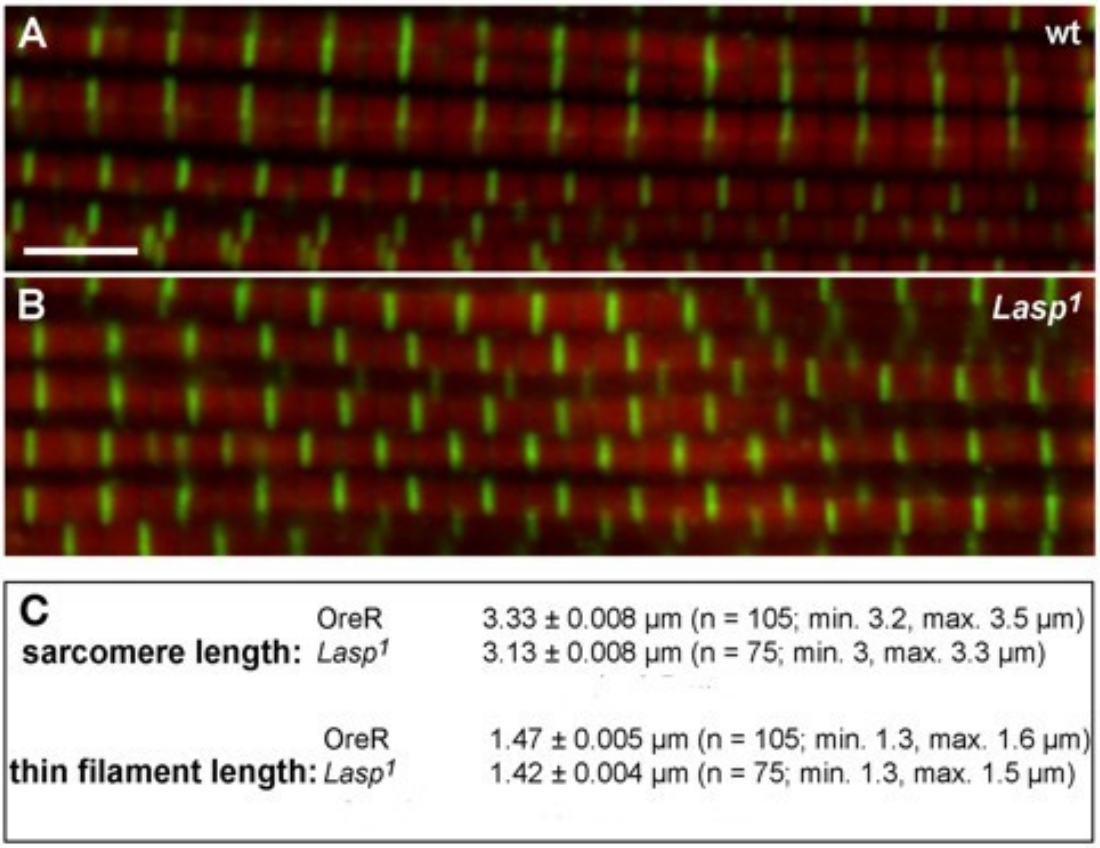


Figure 3.3.4. Sarcomere and thin filament length is reduced in *Lasp*^{1} mutant indirect flight muscles.

(A) Wild type indirect flight muscle double-stained with anti-Zasp antibody (green) and phalloidin-Alexa 594 (red). (B) *Lasp*^{1} mutant indirect flight muscle double-stained with anti-Zasp antibody (green) and phalloidin-Alexa 594 (red). (C) Average sarcomere and thin filament length of indirect flight muscles. Standard error of the mean, sample size, and minimal/maximal values are indicated in C. Bar, 5µm.

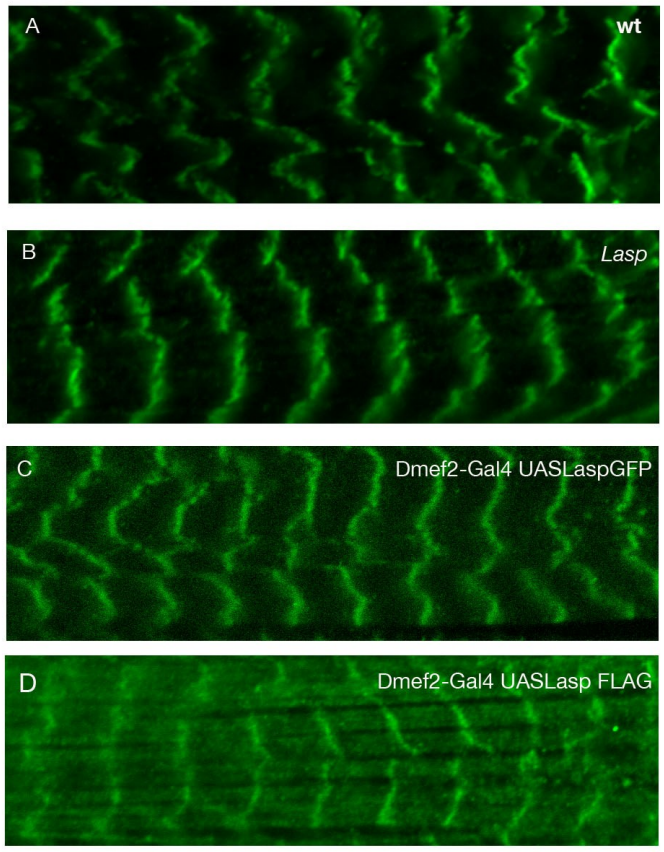
We also measured sarcomere length of larval body wall muscles of wild type and *Lasp*^{1} mutant stained with the Z line marker Zasp. We observe a similar reduction in sarcomere length in body wall muscles of third instar larvae (Fig.3.3.5A, B, E). We finally determined if Lasp overexpression also results in altered sarcomere length. We used 2 transgenes: UAS-LaspGFP and UAS-LaspFlag, which both can rescue male sterility and are therefore functional. Upon expression of both transgenes with Dmef2-Gal4 in muscles, Lasp localizes to Z lines. We observe that sarcomere length upon expression of UAS-LaspGFP with Dmef2-Gal4 is even more reduced than upon expression of UAS-LaspFlag with Dmef2-Gal4 (Fig. 3.3.5C, D, E). We then stained muscles overexpressing these constructs in parallel with anti-Lasp antibody, which shows that UAS-LaspGFP is overexpressed more strongly, argueing that stronger overexpression causes stronger shortening of sarcomere length (Fig. 3.3.6A, B).

3.4 Materials and methods

3.4.1 Crawling, climbing and flying assays

For the crawling assays, third instar larvae were separated from media with 25% sucrose solution, rinsed with distilled water, and then placed individually onto 10-cm agar plates. After 10 min with no handling, the plates were placed under a stereo microscope and the numbers of forward body-wall contractions were counted.

For the climbing assays, males of each genotype were collected and aged by



E **larval body wall muscle sarcomere length:**

OreR	7.37±0.021 µm (n=80)
Lasp	6.81±0.024 µm (n=80; p<0.001)
Lasp OE	6.28±0.013 µm (n=80; p<0.001)
FlagLasp	6.95±0.013 µm (n=80; p<0.001)

Figure 3.3.5. Sarcomere length is altered in *Lasp*^{1} mutant larval muscles and upon OE.

(A)Anti-Zasp antibody staining of third instar wild type larval muscle. (B) Anti-Zasp antibody staining of third instar *Lasp*^{1} mutant larval muscle. (C) Anti-Lasp antibody staining of third instar larval muscle overexpressing UAS-LaspGFP with Dmef2-Gal4. (D) Anti-Lasp antibody stainig of third instar larval muscle overexpressing UAS-LaspFLAG with Dmef2-Gal4. (E) Average sarcomere length of larval body wall muscles. Sample size and p-values are indicated in E. Bar, 5µm.

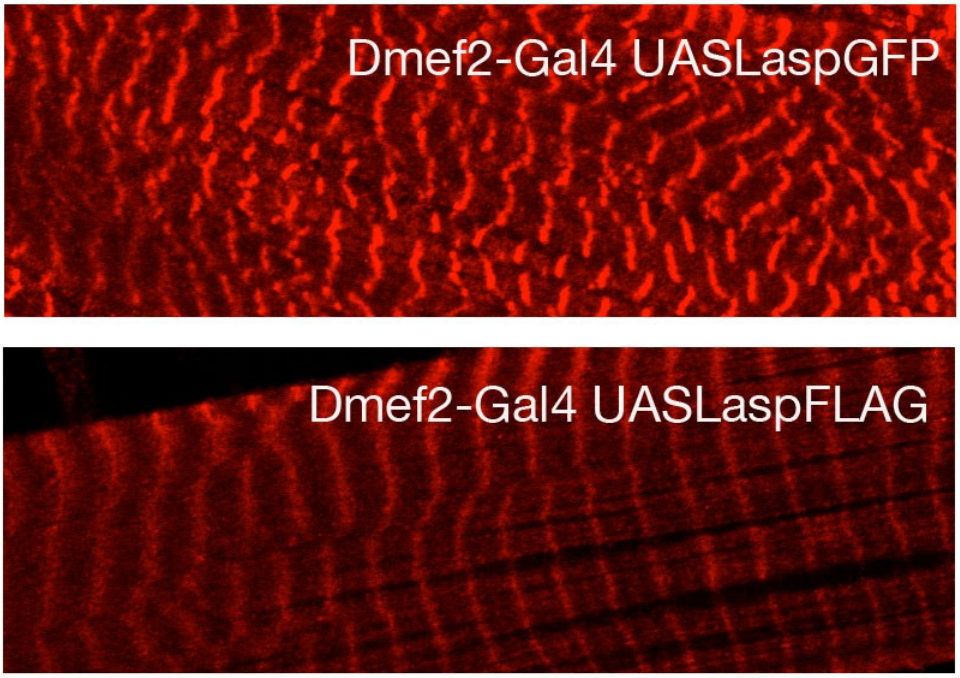


Figure 3.3.6. Stronger overexpression causes stronger shortening of sarcomere length.
(A) Anti-Lasp antibody staining of third instar larval muscles overexpressing UAS-LaspGFP with Dmef2-Gal4. (B) Anti-Lasp antibody staining of third instar larval muscles overexpressing UAS-LaspFLAG with Dmef2-Gal4.

switching them to new food every 2 days. On assay days 10 flies were separated into new vials and allowed to recover for a couple of hours. They were then tapped into a 100 ml graduated cylinder, and assayed for their negative geotaxis response by tapping the cylinder. Flies of up to 14 days of age were assayed by counting the number of flies passing the 80 mm line from the base of the cylinder within 10 s. Each set of 10 flies was assayed twice.

For the flying assays, on assay days 20 flies were separated before being tapped into a paper cylinder coated with Tangle-Trap fly glue (Distributions Solida) with a soap bath at the bottom. The distance from the soap bath was recorded for each fly.

3.4.2 Electron microscopy

For electron microscopy of IFMs, flies were dissected by removing the head and the abdomen. The entire thorax was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 at 4° C overnight. After rinsing with 0.1 M sodium cacodylate buffer for 40 min the next day, thoraces were cut in half sagittally and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h on ice and rinsed with 0.1 M sodium cacodylate buffer for 40 min at 4° C. They were stained with 2% tannic acid in 0.1 M sodium cacodylate buffer for 1 h at 4° C and rinsed with distilled water for 40 min at 4° C. Sections were restained with 2% uranyl acetate for 1 h at 4° C and washed in distilled water for 45 min at 4° C, followed by dehydration in acetone at room temperature. Samples were embedded in epoxy resin (EPON-815; Electron Microscopy Sciences) and cut with an Ultracut AV (Reichert). The specimens were then stained in

7% uranyl acetate in absolute methanol for 4 min followed by incubation in Reynolds' lead citrate for 4 min and examined on a transmission electron microscope (Tecnai 12; FEI).

For electron microscopy of larval muscles, third instar larvae were washed thoroughly with distilled water and placed on ice in order to immobilize the larvae. Then a larva was placed in a drop of cold HL3 buffer (70 mM NaCl, 5 mM KCl, 20 mM MgCl2, 10 mM NaHCO3, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES; pH 7.2) or relaxing buffer and fixed with dissection pins on a rubber block. Larvae were dissected along the dorsal line using Vannas Spring Scissors (0.05 mm blade diameter; Fine Science Tools). Further steps were performed as described above for IFMs.

3.4.3 Immunohistochemistry and statistical analysis

We used the following primary antibodies for immunofluorescent stainings of IFMs: rabbit anti-Zasp (1:400) (Jani and Schöck, 2007), mouse anti-α-actinin (1:10; provided by J. Saide) (Saide et al., 1989), and rabbit anti-Lasp (1:2000; provided by A. Ephrussi). Fluorescently labeled secondary antibodies of the Alexa Series (Invitrogen) were used at 1:300. Filamentous actin was visualized using Alexa 594-labelled phalloidin (1:200; Invitrogen).

Fly thoraces were separated from male flies in relaxing buffer (Ringers plus 1 mM EGTA) and fixed in 4% formaldehyde in relaxing buffer. Dorsolongitudinal indirect flight muscles were then dissected out in PBS containing 0.3% Triton X-100 (PBT). After washing IFMs in PBT for 1 h, muscles were preincubated in PBT containing 1%

normal goat serum (PBTN), followed by overnight incubation in the primary antibody at room temperature, which was diluted in PBTN containing 0.1% BSA. After 1 h wash in PBT and preincubation in PBTN for 1 h, IFMs were incubated with secondary antibody for overnight at room temperature. IFMs were then washed in PBT and embedded in Prolong gold antifade solution (Invitrogen).

For larval muscle staining, third-instar larvae were dissected as described previously. Dissected larvae were rinsed with HL3 buffer twice and fixed with 100% methanol for 2 min. Images were obtained on a Zeiss LSM 510 Meta confocal microscope using a 63x 1.4 NA Plan Apo oil immersion objective. Sarcomere and thin filament lengths were measured using ImageJ 1.37v software. The data was compiled into Excel to calculate averages and standard errors.

3.4.4 Fly stocks and genetics

The following fly stocks were used: *Lasp*^{1}*, Dmef2-Gal4, UASLasp-GFP, UASLasp-Flag*. The following recombinants were made by standard genetic crosses: *Dmef2-Gal4 UASLasp-GFP; Dmef2-Gal4 UASLasp-Flag; Dmef2-Gal4 Lasp*^{1}*; UASLasp-GFP Lasp*^{1}*; Lasp*^{1} *UASLasp-Flag.*

3.4.5 Molecular Biology

To generate UASLasp-Flag construct, full length *Lasp* cDNA (AT23571) was

amplified by primers CACCATGAATAAAACCTGTGCCCCGT and TATAACCGCCTGCTCCACGTA and cloned into the Gateway pENTR/D-TOPO vector (Invitrogen). The product was transformed into competent *E.coli* cells, and a positive transformant grown on plate with antibiotic for ampicillin was selected after one day incubation. This product was then recombined into UASp from the *Drosophila* Gateway vector collection (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors,html).

To generate Lasp-His tag fusion for antibody production, Lasp pEntry vector made by the previous member Soojin were used. Recombination between the entry clone and the Gateway pBAD-DEST49 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-NTA beads (Qiagen) according to the manufacturer's instructions. Protein concentration was determined using BioRad protein reagent. The injection was done by the Animal Services platform at the NRC-BRI, using a standard 80 day protocol.

Chapter4

General Discussion

4.1 Summary

The work described in Chapter 2 shows we have generated a *Lasp* null mutant, $Lasp^{1}$, which is homozygous viable, but male sterile. Our data show $Lasp^{1}$ has two distinct phenotypes in *Drosophila* testis: hub cell mislocalization and failure of actin cone function during spermatid individualization. The first phenotype demonstrates that in *Lasp* mutants, hub cells fail to localize to the apical tip of the testis indicating that Lasp is required for an integrin-dependent pathway in hub cells to anchor the stem cell niche. The second phenotype illustrates that in *Lasp* mutants, actin cones are perturbed resulting in improper spermatid individualization.

Chapter 3 focuses on the function of Lasp in the sarcomeres of striated muscle. Our results show $Lasp^{1}$ mutants do not crawl, climb or fly as well as wild type flies. Coimmunostaining of larval muscles with Lasp and α-actinin showed that Lasp localizes to Z lines. We then measured sarcomere and thin filament length in third instar larval muscles and of indirect flight muscles in adult flies, and observed that both sarcomere and thin filament length is reduced in *Lasp* mutants. Thus, our data demonstrate that Lasp, a protein with two nebulin repeats, regulates thin filament length in *Drosophila* myofibril assembly.

4.2 Identification of Lasp function in the *Drosophila* testis

From our investigation, Lasp functions in anchoring the *Drosophila* male stem cell niche and in mediating spermatid individualization.

In Chapter 2, I first demonstrated that Lasp is expressed in hub cells and that in $Lasp^{1}$ testes the stem cell niche is not anchored to the apical tip. This observation is very similar to the previous study of Talin (Tanentzapf *et al*., 2007). Talin is a protein that associates with integrins and is expressed in somatic cells as well as βPS integrin that is also found in somatic cells of the testis. In the same study, it has been shown that the stem cell niche directly interacts with the extracellular matrix (ECM), and the hub cell mislocalization was suggested to be caused by the loss of integrin adhesion function by depleting Talin in vivo. A Talin RNAi transgene expressed in somatic cells displays hub cell mislocalization in 3-day old adult testis. Similar to the Talin study, $Lasp^{1}$ also displays hub cell mislocalization in 3-day old adult testis. Thus, we suggest that Lasp plays a role in localizing hub cells to the apical tip of the testis.

I also showed that actin cones are perturbed causing spermatid individualization defects in $Lasp^{1}$ mutant testis. Our data show that Lasp colocalizes with filamentous actin in actin cones, and actin cones are only weakly visible in the assembly stage of actin during individualization and barely visible in the migrating stage. We suggest that Lasp may tether actin to the plasma membrane of actin cones during individualization. In addition, the phenotype of $Lasp^{1}$ is similar to Myosin VI mutant phenotype (Noguchi *et al*., 2006). Myosin VI is also present in actin cones and its mutant (jar^{1}) fails to individualize spermatids (Noguchi *et al*., 2006).

4.3 Lasp regulates thin filament length in *Drosophila* myofibrils

Our data identify a novel regulator of thin filament regulation in *Drosophila* Lasp, because both overexpression and its mutation change sarcomeric thin filament length.

Lasp localizes to the third instar larval body wall muscles, and this localization correlates well with its counterparts Lasp-1 and Lasp-2 in vertebrate muscle (Panaviene and Moncman, 2007; Zieseniss *et al*., 2008). Surprisingly, we could not detect endogenous Lasp localizing to myofibrils in indirect flight muscles. We thus assume that Lasp either localizes only very weakly, or transiently to myofibrils in indirect flight muscles.

The reduction in thin filament length we observe in *Lasp* mutant mucles is very similar to what is found in nebulin-deficient mice. Likewise, Lasp as well as *nebulin* mutants are not required for initial myofibril assembly as seen by electron microscopy. However, Nebulin plays a bigger role in myofibril maintenance, because older nebulin knock-out mice display severe muscle weakness and nemaline myopathy (Bang *et al*., 2006; Witt *et al*., 2006). This renews the question of how nebulin repeat proteins regulate thin filament length. Our data show overexpressed Lasp localizes to Z lines and misregulates thin filament length, this suggests that Lasp may regulate monomer addition and thereby thin filament length to the barbed end of thin filaments at the Z line. Barbed-end filament elongation is the standard mechanism in non-muscle cells (Pollard, 2007). However, the mechanisms for thin filament elongation in muscle cells described so far occur at the pointed end through tropomodulin (Bai *et al*., 2007; Mardahl-Dumesnil and Fowler, 2001; Littlefield *et al*., 2001). Lasp however, localizes to the Z line, where the

barbed ends of thin filaments are inserted. Vertebrate Lasp-2 can directly bind to α-actinin (Zieseniss *et a*l., 2008), which, if conserved, could explain Lasp localization to Z lines. The carboxyl-terminal nebulin repeats of nebulin bind to the barbed end-capping protein CapZ, which plays a role in proper termination of barbed ends within the Z line, even though it does not affect the capping activity of CapZ in an in vitro assay (Pappas *et al*., 2008). It is therefore conceivable that Lasp binds simultaneously to α-actinin and CapZ, thereby modulating thin filament length at the Z line.

4.4 Characterization of myofibril assembly in *Lasp* mutants

As thin filament length is reduced in *Lasp* mutants, future studies should investigate how Lasp regulates thin filament elongation. The C-terminal nebulin repeats of nebulin were recently shown to bind to CapZ and to recruit CapZ to Z lines (Pappas et al., 2008). Vertebrate Lasp-2 can also bind to α-actinin (Zeiseniss et al., 2008). Future work will test the hypothesis that Lasp regulates monomer addition to the barbed end of thin filaments at the Z line through regulation of the capping protein CapZ. Further insight on this topic can be gained by conducting genetic interaction experiments with our *Lasp* mutant and homozygous viable *CapZ* mutants (Hopmann and Miller, 2003). *CapZ* mutants show an increase in F-actin in bristles, perhaps because they are stabilized or elongated in the absence of capping. We would therefore expect that Lasp antagonizes CapZ function. One could test if by reducing *CapZ* dosage in the background of homozygous *Lasp* mutants would suppress thin filament shortening. One could also test

by antibody staining if CapZ localization at the Z line is affected in *Lasp* mutants versus wild type. Cytochalasin D binds with high affinity to the barbed ends of actin, and can therefore be used to assess the position of barbed ends (Pappas *et al*., 2008). Fluorescently labeled monomeric G-actin can be injected into live muscles to assess actin turnover at the barbed and pointed ends (Pappas *et al*., 2008). Both methods can be used to determine if barbed ends at the Z line look differently in *Lasp* mutants, and to see if actin incorporation at the barbed end is increased in the absence of Lasp. Similar experiments can be conducted in muscles ectopically overexpressing Lasp. These experiments will establish what kind of role Lasp plays at the barbed end of thin filaments.

4.5 Analyzation of actin turnover in myofibril *Lasp* mutants by live imaging

Myofibril assembly has only rarely been analyzed by live imaging in tissue culture cells (Littlefield *et al*., 2001), but never in model organisms. *Drosophila* has six different actin isoforms that were fused to GFP and show differential localization, when expressed in muscles (Röper *et al*., 2005). In particular, actin88F is found in thin filaments but excluded from the Z line, whereas actin79B is largely restricted to the Z line (Röper *et al*., 2005). These transgenes are therefore uniquely suited to analyze actin turnover in *Lasp* mutants. Future work can be started by recombining UAS-actin79B, UAS-actin88F, and Dmef2-Gal4 to *Lasp* mutants to express these actin isoforms in a

Lasp mutant background. Dmef2-Gal4 drives expression in all muscles from embryonic to adult stages. Fluorescence recovery after photobleaching (FRAP) can then be employed to assess actin filament turnover and thereby monomer addition. These experiments will provide a large amount of information on turnover and assembly of myofibrils in wild type and *Lasp* mutants.

In conclusion, analysis of *Drosophila* Lasp has demonstrated several functional aspects that may provide insights on the mechanisms of human diseases.

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