Mechanisms of Development 138 (2015) 336–348

Contents lists available at ScienceDirect

Mechanisms of Development

journal homepage: www.elsevier.com/locate/mod

Stage-specific control of niche positioning and integrity in the *Drosophila* testis

Lisa Schardt [a,b], Janina-Jacqueline Ander [a], Ingrid Lohmann [a,*], Fani Papagiannouli [a,*]

[a] Centre for Organismal Studies (COS) Heidelberg, Cell Networks — Cluster of Excellence, University of Heidelberg, D-69120, Germany
[b] Deutsches Krebsforschungszentrum (DKFZ), D-69120, Germany

ARTICLE INFO

Article history:
Received 7 May 2015
Received in revised form 23 June 2015
Accepted 24 July 2015
Available online 28 July 2015

Keywords:
Abd-B
Drosophila testis
Integrin
Talin
Niche positioning
Hox genes

ABSTRACT

A fundamental question is how complex structures are maintained after their initial specification. Stem cells reside in a specialized microenvironment, called niche, which provides essential signals controlling stem cell behavior. We addressed this question by studying the *Drosophila* male stem cell niche, called the hub. Once specified, the hub cells need to maintain their position and architectural integrity through embryonic, larval and pupal stages of testis organogenesis and during adult life. The *Hox* gene *Abd-B*, in addition to its described role in male embryonic gonads, maintains the architecture and positioning of the larval hub from the germline by affecting integrin localization in the neighboring somatic cyst cells. We find that the AbdB-Boss/Sev cascade affects integrin independent of Talin, while genetic interactions depict integrin as the central downstream player in this system. Focal adhesion and integrin-adaptor proteins within the somatic stem cells and cyst cells, such as Paxillin, Pinch and Vav, also contribute to proper hub integrity and positioning. During adult stages, hub positioning is controlled by Abd-B activity in the outer acto-myosin sheath, while Abd-B expression in adult spermatocytes exerts no effect on hub positioning and integrin localization. Our data point at a cell- and stage-specific function of Abd-B and suggest that the occurrence of new cell types and cell interactions in the course of testis organogenesis made it necessary to adapt the whole system by reusing the same players for male stem cell niche positioning and integrity in an alternative manner.

1. Introduction

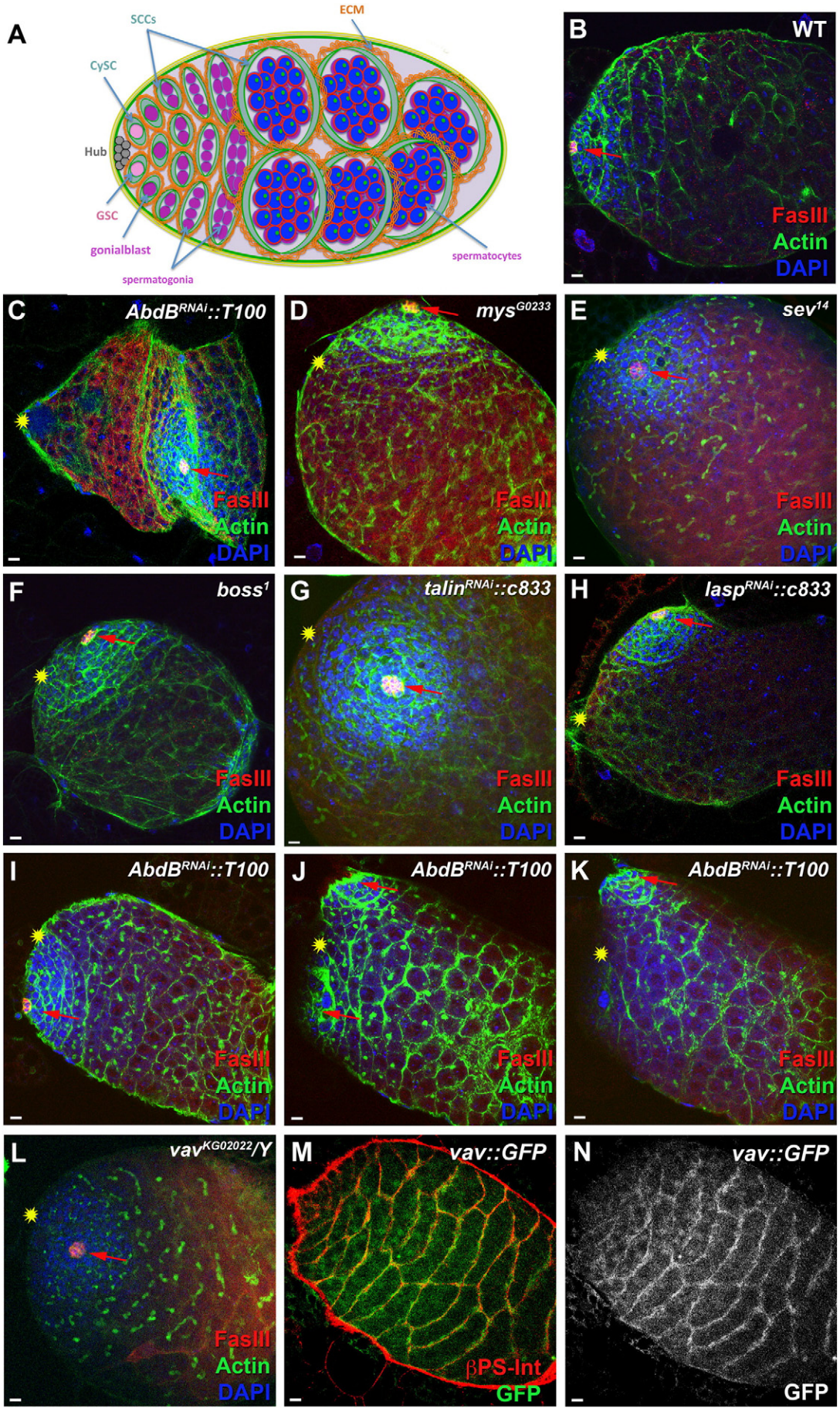
In all adult tissues harboring stem cells, the niche has a critical function as an organizer, which recruits the stem cells and provides the microenvironment that supports stem cell identity. Organogenesis of the *Drosophila* testis, initiated by the coalescence of germ cells and somatic gonadal cells late in embryogenesis, proceeds throughout embryonic and larval stages, and culminates in a second wave of organ shaping in pupal stages to reach maturation in adult stages. The *Drosophila* male stem cell niche, called the hub, is a cluster of non-dividing cells specified in the anterior most somatic gonadal cells already before the gonads coalesce (Boyle and DiNardo, 1995; DeFalco et al., 2008; DeFalco et al., 2004; Dinardo et al., 2011; Hatini et al., 2005; Le Bras and Van Doren, 2006).

The first signs of testis organogenesis are detected in late embryogenesis, once the specified hub cells recruit the anterior-most germ cells to become the germline stem cells (GSCs) (Sheng et al., 2009). A testis with a mature stem cell niche and all pre-meiotic cell types is detected at 3rd instar larvae (L3) (Fig. 1A). The *Drosophila* testis contains two types of stem cells arranged in stereotypic manner: the germline stem cells (GSCs) and the somatic cyst stem cells (CySCs). Each GSC is flanked by two CySCs and both stem cell identities are sustained through their association with the hub. Upon asymmetric cell division, each GSC produces a new GSC attached to the hub and a distally located gonialblast. The CySCs also divide with an asymmetric outcome, to generate a CySC remaining associated with the hub and a distally located post-mitotic daughter somatic cyst cell (SCC) (Fuller and Spradling, 2007). CySCs and SCCs are collectively called here cyst cells. Two SCCs enclose each gonialblast thereby forming a cyst that is "sealed" outside by the extracellular matrix (ECM) (Fig. 1A) (Papagiannouli et al., 2014). The gonialblast divides mitotically four more times to give rise to 16 interconnected spermatogonial cells, which then undergo pre-meiotic DNA replication and become spermatocytes. The cyst cells co-differentiate with the germ cells they enclose, grow enormously in size, elongate and accompany them throughout their differentiation steps up to individualization and sperm production in the adult testis

Abbreviations: Abd-B, Abdominal-B; CySCs, somatic cyst stem cells; ECM, extracellular matrix; GSCs, germ-line stem cells; L, larval stage; SC, spermatogonial cysts; SCCs, somatic cyst cells; wt, wild type.

* Corresponding authors at: Centre for Organismal Studies (COS), University of Heidelberg, Im Neuenheimer Feld 230, D-69120 Heidelberg, Germany.
E-mail addresses: ilohmann@flydev.org (I. Lohmann), fani.papagiannouli@cos.uni-heidelberg.de (F. Papagiannouli).

http://dx.doi.org/10.1016/j.mod.2015.07.009


A
SCCs
ECM
CySC
Hub
GSC
gonialblast
spermatogonia
spermatocytes
B
WT
FasIII
Actin
DAPI
C
AbdB^RNAi::T100
D
mys^G0233
E
sev^14
F
boss^1
G
talin^RNAi::c833
H
lasp^RNAi::c833
I
AbdB^RNAi::T100
J
AbdB^RNAi::T100
K
AbdB^RNAi::T100
L
vav^KG02022/Y
M
vav::GFP
βPS-Int
GFP
N
vav::GFP
GFP

(Fuller, 1993). Spermatocytes turn on the transcription program required for terminal differentiation and undergo meiotic divisions. During pupal stages, testis morphogenesis is completed with the addition of the acto-myosin sheath originating from the genital disc (Kozopas et al., 1998), which consists of multinucleated smooth muscles resembling the vertebrate smooth muscles (Susic-Jung et al., 2012).

A critical question is how the activity of the niche, after its initial specification, is secured during the successive stages of testis organogenesis. Integrin-mediated adhesion is important for maintaining the correct position of embryonic hub cells during gonad morphogenesis. In the absence of integrin-mediated adhesion, the hub cells still form a cluster, but instead of remaining at the gonad-anterior they migrate to the middle part of the developing gonad (Tanentzapf et al., 2007). In adult testes, disruption of integrin-mediated adhesion is observed by knocking down *talin*/*rhea*, a gene coding for an essential integrin-binding and cytoskeletal protein (Cram and Schwarzbauer, 2004; Tanentzapf et al., 2006). This results in a gradual loss of hub cells, a phenotype that becomes more severe as adult males age (Tanentzapf et al., 2006). Consequently, signals that normally emanate from the hub to instruct stem cell renewal are reduced or absent, resulting in a progressive loss of GSCs (Tanentzapf et al., 2007). A similar hub displacement phenotype is observed by depleting adult testes of Lasp (Lee et al., 2008), an actin-binding protein, which in vertebrates interacts genetically with integrin (Suyama et al., 2009; Traenka et al., 2009). In a few cases, loss of Lasp leads to hub integrity defects in which the hub cell arrangement is disturbed or double hubs are observed. The Bride of Sevenless (Boss)/Sevenless (Sev) signaling pathway also plays an important role in hub positioning and integrity in the *Drosophila* male gonads by preventing ectopic niche differentiation in the posterior gonadal somatic cells. Upstream of this cascade, Abd-B activates *sev* in the posterior somatic gonadal cells (Kitadate et al., 2007). Consistent with this observation, weak *Abd-B* mutant alleles result in hub expansion and hub integrity defects in embryonic gonads (Le Bras and Van Doren, 2006). At the same time, the epidermal growth factor receptor (EGFR) signaling represses hub formation in the rest of the somatic gonadal cells (Kitadate and Kobayashi, 2010).

Our recent work revealed a new role of the posterior *Hox* gene *Abd-B* in niche positioning and integrity during larval stages. Sustained activity of Abd-B in pre-meiotic germline spermatocytes of larval testes is required to secure niche positioning and integrity by regulating integrin localization in the neighboring SCCs (Papagiannouli et al., 2014). Identification of direct Abd-B target genes revealed that Abd-B mediates its effects by controlling the activity of the Sev ligand Boss via its direct targets *src42A* and *sec63*. Therefore, the switch of Abd-B expression from the embryonic male-specific somatic cells to the larval spermatocyte germ cells correlates with a change in the Abd-B dependent mechanism of hub positioning between embryonic and larval stages. This results in a dramatic reduction of the pre-meiotic cell types in adult testes, which emphasizes the importance of niche positioning and integrity throughout testis organogenesis.

This work provides new insights into the cell-type and stage-specific regulation of niche architecture, in terms of both positioning and integrity. Analysis of the genetic interactions of Abd-B with integrin and focal adhesion proteins in the larval testis revealed that male stem cell niche positioning and integrity are regulated by a number of factors within the cyst cells, which link integrin to cytoskeletal adaptor proteins. Moreover, AbdB-Boss/Sev germline-to-soma communication, required for larval hub positioning, affects integrin localization in cyst cells independent of Talin, an integrin binding protein and activator linking integrin to the cytoskeleton in variable ways (Cram and Schwarzbauer, 2004; Franco-Cea et al., 2010). During adult stages, hub positioning is controlled by Abd-B active in cells of the acto-myosin sheath, while *Abd-B* expression in adult spermatocyte cell population exerts no effect on hub positioning and integrin localization. Taken together, our data point to a cell- and stage-specific function of Abd-B in controlling hub architecture and function, since the mechanisms regulating hub positioning at different stages seem to be different despite the employment of similar factors.

2. Results

2.1. Hub positioning and integrity in the Drosophila larval testis, and the role of integrin-related adaptor proteins

In order to prevent the accumulation of cell autonomous and non-autonomous stem cell and spermatogenesis defects, proper function of the stem cell niche has to be secured at subsequent steps of testis organogenesis (Kitadate et al., 2007; Le Bras and Van Doren, 2006; Lee et al., 2008; Papagiannouli et al., 2014; Tanentzapf et al., 2007). Hub positioning and integrity is tightly coupled to adhesion and cell communication, with βPS-Integrin (encoded by the *myospheroid* (*mys*) gene) and the Boss/Sev pathway playing key roles in embryonic gonads (Kitadate et al., 2007; Tanentzapf et al., 2007) as well as in larval (Figs. 1B, D–F, 2A–D) (Papagiannouli et al., 2014) and adult testes (Kitadate et al., 2007; Lee et al., 2008; Papagiannouli et al., 2014; Tanentzapf et al., 2007). A similar role in hub positioning and integrity was shown for Lasp in larval (Fig. 1H) and adult testes (Lee et al., 2008). Talin has been shown previously to be involved in integrin-mediated adhesion and in hub positioning and maintenance in the adult testis (Tanentzapf et al., 2007). Immunostainings revealed Talin localization in CySCs and SCCs of 3rd instar larval testes but not in the hub cells (Fig. 3A, B). Consistent with its described function in the adult testis, 3rd instar larval testes either mutant (data not shown) or RNAi silenced for *talin* (called also *rhea* in *Drosophila*) displayed defects in niche positioning (Figs. 1G, 3C, D) and hub integrity (Fig. 2E, F) in comparison to wild type testes (Figs. 1B, 2A, B). Yet, we have not observed hub loss in *talin* depleted larval testes, as it has been reported for *talin* depleted adult testes (Tanentzapf et al., 2007) (see "Experimental procedures" section for detailed explanation). Throughout this study, normal hub position is expected at the anterior tip of the testis, at the opposite side of the posterior terminal cells, as shown before (Papagiannouli et al., 2014).

The posterior Hox protein Abd-B acts as an upstream regulator of the Boss/Sev pathway in this system, by controlling Sev expression in the embryonic male gonad (Kitadate et al., 2007) and Boss function in the larval testis (Papagiannouli et al., 2014). Cell-type specific knockdown of *Abd-B* in L3 *Drosophila* germline spermatocytes, by *Abd-B^{RNAi}* transgene expression, led to hub mispositioning (Fig. 1C) (Papagiannouli et al., 2014) and hub integrity defects (Fig. 1I–K). In particular, two independent niches, one organized around a FasIII-positively stained hub (Fig. 1I) and one without a FasIII stained hub (Fig. 1J, K), were observed as rare single events. Our previous study has shown that Abd-B controls βPS-Integrin localization in the SCCs by activating the Boss/Sev pathway in L3 spermatocyte cysts

Fig. 1. (A) Diagram of 3rd instar larval testis in *Drosophila*. GSC: germline stem cell, CySC: somatic cyst stem cell, SCC: somatic cyst cell. For simplicity CySCs and SCCs are collectively called cyst cells. Testicular cysts comprise of a pair of cyst cells flanking the germline (GSCs, spermatogonia and spermatocytes). Integrin localization is indicated in green and ECM molecules surrounding the cyst cells in orange. Within the spermatocytes, the red line indicates the nuclear membrane, the green dots resemble Abd-B distribution in the nucleolus and blue represents the nucleus. (B–L) Known and new players affecting hub positioning and architecture in the larval *Drosophila* testis. Stainings of wt, *AbdB^{RNAi}::T100*, *mys^{G0233}*, *sev^{14}*, *boss^{1}*, *talin^{RNAi}::c833*, *Lasp^{RNAi}::c833* and *vav^{KG02022}/Y* testes for FasIII (red) to visualize the hub cells, phalloidin for filamentous Actin (green; marks the hub, CySCs, SCCs and germline fusome) and DAPI for the DNA (blue). Actin labels the hub cells, underlies the membrane of CySCs and SCCs as well as the branched fusomes. (C–L) In all mutant and RNAi knockdowns the hub is not in the expected position (yellow asterisks) but is mispositioned (red arrows). The normal hub position is expected at the anterior tip of the testis, at the opposite side of the posterior terminal cells, as described before (Papagiannouli et al., 2014). (I–K) Cell-type specific RNAi knockdown of *Abd-B* in L3 spermatocytes leads to two niches, from which only one is organized around FasIII-positive cells. (M, N) Vav in wt testis, using a *Vav*-GFP enhancer trap line (green), shows co-localization with βPS-integrin (red), predominantly in SCCs. Subpanel (N) shows the *Vav*-GFP alone. Crosses were set up and progeny were raised at 25 °C, the phenotype was scored at L3. Testis hub is oriented towards the left. Scale bar: 10 μm.

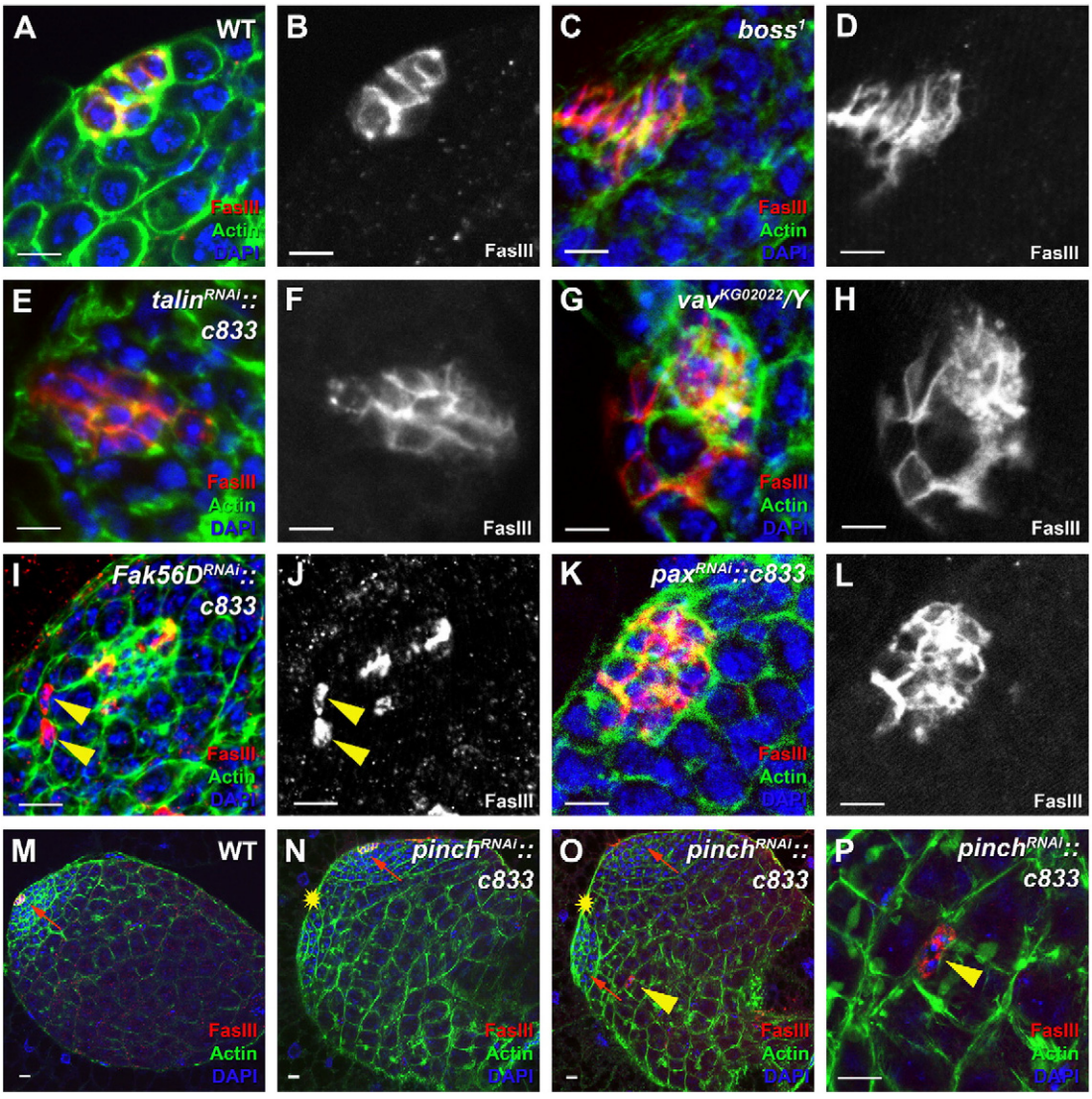


Fig. 2. Critical new players involved in hub shape and integrity in the larval *Drosophila* testis cyst cells. (A–P) FasIII (red), F-Actin (green) and DAPI (blue) stainings of wt, *boss*^{1}, *talin*^{RNAi}::*c833*, *vav*^{KG02022}/Y, *FAK56D*^{RNAi}::*c833*, *Pax*^{RNAi}::*c833*, and *pinch*^{RNAi}::*c833* L3 testes. Subpanels (B), (D), (F), (H), (J) and (L) show only the FasIII staining of (A), (C), (E), (G), (I) and (K) respectively. (C–L) show hub integrity and hub shape defects of *boss*, *talin*, *vav*, *Fak56D* and *Pax* depleted testes in comparison to wt (A, B). (I, J) Fragmented FasIII staining in *Fak56D* depleted testes, with yellow arrowheads pointing at FasIII positive cells outside of the hub region marked by Actin. (M–P) In contrast to the wt, cell-type specific knockdown of *pinch* in cyst cells reveals the presence of two niches, one organized around a FasIII positively stained hub (N) and one without (O and P). FasIII positive, hub-like, cells were found displaced among the spermatocyte cysts (yellow arrowheads in O and P), which speaks for niche integrity defects. (N–P) Show different focal planes of the same testis. (P) is a magnification of (O). Red arrows point at the hub. Yellow asterisks indicate the expected hub position. Crosses were set up and progeny were raised at 25 °C, the phenotype was scored at L3. Testis hub is oriented towards the left. Scale bar: 10 μm.

(Papagiannouli et al., 2014). However, the molecular function of Abd-B in the adult testis as well as the role of integrin-related and focal adhesion proteins in hub positioning and integrity remains unclear.

Integrins are dynamic cell-surface receptors, composed of one α- and one β-subunit, that provide a link between the ECM and the actin cytoskeleton (Brakebusch and Fassler, 2003; Delon and Brown, 2007; Vicente-Manzanares et al., 2009). The role of the ECM proteins Laminin A (Fristrom et al., 1993; MacKrell et al., 1993) and the Laminin A-type protein Wing blister (Martin et al., 1999) that co-localize with βPS-integrin, in larval niche mispositioning has been previously addressed (Papagiannouli et al., 2014). On the cytoplasmic side of the cell membrane, integrins assemble into large multi-protein complexes composed of integrin-binding adaptor proteins, focal adhesion scaffolding proteins, actin-binding proteins and cell-signaling molecules (Brakebusch and Fassler, 2003; DeMali et al., 2003; Ellis and Tanentzapf, 2010; Grashoff et al., 2004; Vicente-Manzanares et al., 2009). In order to address the potential role of downstream effectors in hub positioning and integrity within the cyst cells, we characterized the function of integrin-related and focal adhesion proteins like Vav, Paxillin, Focal Adhesion Kinase and α-chain Integrins in the *Drosophila* larval testis.

Vav is a guanine–nucleotide-exchange factor, an activator of the Rho-family of proteins (Tybulewicz, 2005) and a known effector of integrin signaling in vertebrates crucial for actin filament organization (Hornstein et al., 2004; Nolz et al., 2005). Our analysis revealed that Vav localized predominantly in SCCs together with βPS-Integrin (Fig. 1M, N). Vav is required for niche positioning and architecture, since 3rd instar larval testes either mutant (Fig. 1L) or RNAi depleted (data not shown) for the *vav* gene displayed defects not only in hub positioning but also in hub shape and integrity (Fig. 2G, H). We also found that αPS2-Integrin localized in cyst cells (CySCs and SCCs) (Fig. S1G, S1H) and RNAi-mediated downregulation of α*PS1-integrin* (also called *multiple edematous wings*/*mew*) and α*PS2-integrin* (also called *inflated*/*if*) activity in cyst cells severely affected hub positioning (Fig. S1A–S1F). This result suggested that βPS-integrin

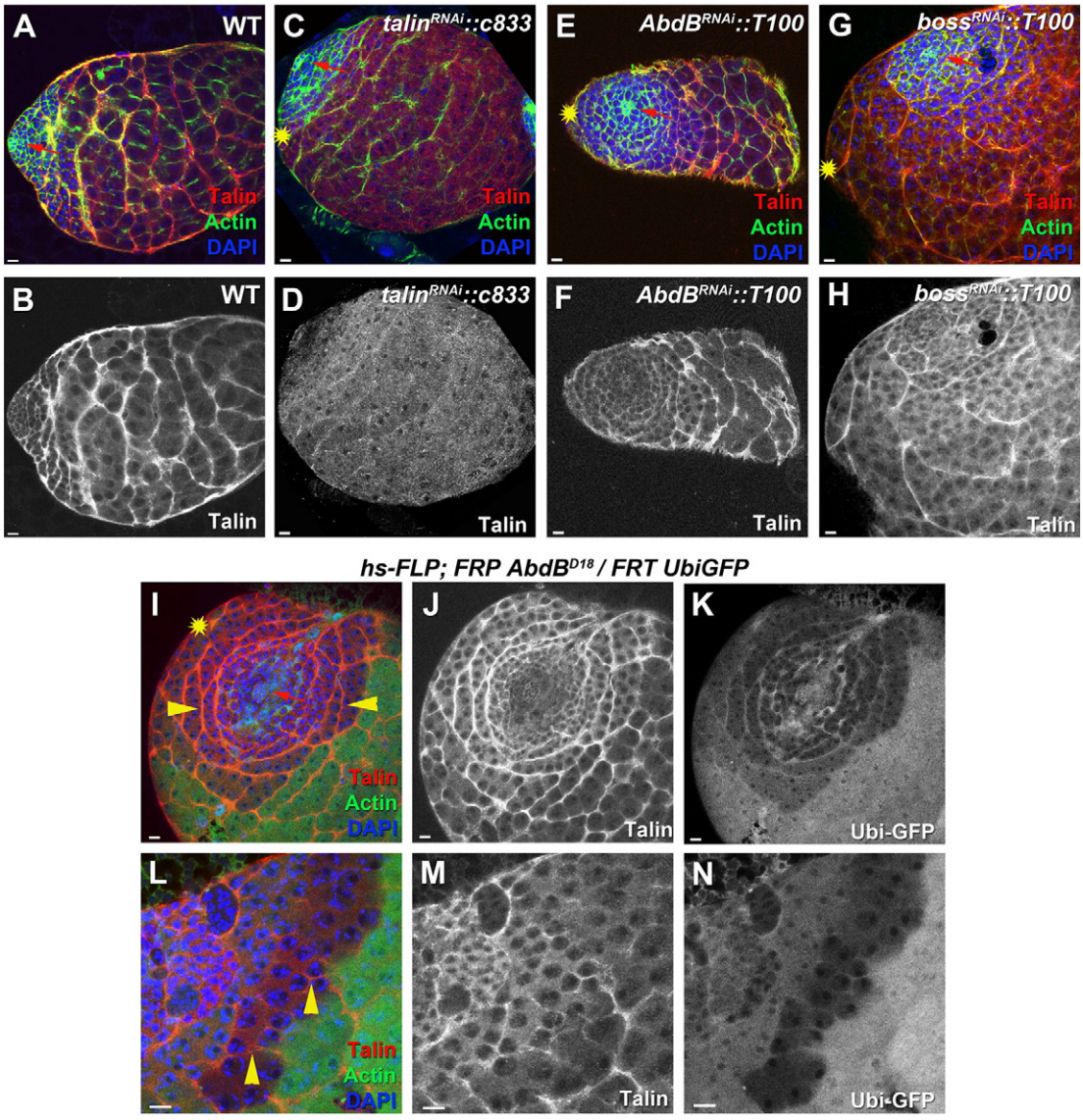


Fig. 3. Abd-B and the Boss/Sev pathway act via a Talin-independent mechanism. (A–N) Talin, Actin and DAPI stainings of wt, *talin*^{RNAi}*::c833*, *Abd-B*^{RNAi}*::T100*, *boss*^{RNAi}*::T100* and *hsFlp122*; *FRT82B-AbdB*^{D18}*/FRT82B-Ubi-GFP* clones of L3 testes. (B), (D), (F), (H), (J) and (M) show only the Talin staining from (A), (C), (E), (G), (I) and (L) respectively. (K) and (N) show separately the Ubi-GFP from (I) and (L) respectively. Talin localizes in CySCs and SCCs throughout the wt testis (A, B) and localization is lost by effective *talin* RNAi knockdown in (C) and (D) followed by hub mispositioning (red arrow). Talin is not affected in *Abd-B* and *boss* depleted spermatocytes (E–H) or in *Abd-B*^{D18} clones (identified by the absence of Ubi-GFP) in L3 testes (I–N), suggesting that Talin is not downstream the AbdB-Boss/Sev cascade and probably acts in a separate pathway. For inducing the *Abd-B*^{D18} clones, (I–N), progeny with the genotype *hsFlp122*; *FRT82B-AbdB*^{D18}*/FRT82B-Ubi-GFP* were heat shocked at 37 °C for 1 h as 1st and 2nd instar larvae and dissected as 3rd instar larvae. Red arrows point at the hub. Yellow asterisks show the expected hub position. The yellow arrowheads in (I) and (L) point at Talin localization between neighboring *Abd-B*^{D18} clones. Testis hub is oriented towards the left. Scale bar: 10 μm.

heterodimerizes with at least two of the five *Drosophila* α1–α5 Integrin chains for maintaining positioning and integrity of the male stem cell niche.

Our analysis showed that hub displacement phenotypes are often associated with hub shape and integrity defects (Fig. 2C–H), hub loss or hub-split giving rise to two niches (Fig. 1I–K) (Papagiannouli et al., 2014), which is in line with previous observations (Kitadate et al., 2007; Lee et al., 2008; Tanentzapf et al., 2007). Interestingly, defects in hub integrity and architecture were also obtained when interfering with the function of other integrin-binding and adaptor molecules (Lo, 2006; Palmer et al., 1999; Pasapera et al., 2010; Wozniak et al., 2004). Cell-type specific RNAi knockdown of *Focal Adhesion Kinase* (*Fak56D*) and *Paxillin* (*Pax*) in cyst cells of L3 testes led to hub shape and integrity defects (Fig. 2I–L) whereas knocking down *pinch* (also called *steamer duck*/*stck*) led to a splitting of the hub resulting in two independent niches [red arrows; Fig. 2M–O] and a FasIII-positive cell cluster located far from the niches [yellow arrowhead; Fig. 2O, P]. Taken together, integrin together with focal adhesion and adaptor proteins on the intracellular side, and ECM on the extracellular side of the cyst cells (CySCs and SCCs) orchestrate multiple adhesion events to position the niche and preserve its architecture.

2.2. The AbdB-Boss/Sev cascade affects integrin and hub architecture in the larval testis in a Talin-independent way

Since Talin is an integrin-binding protein and an essential effector of the integrin-cytoskeleton within the cyst cells (Cram and Schwarzbauer, 2004; Tanentzapf et al., 2006), we investigated whether Talin is part of the AbdB-Boss/Sev-Integrin germline-to-soma cascade (Papagiannouli et al., 2014). To this end, we stained *Abd-B* and *boss* depleted testes with a Talin specific antibody (Fig. 3E–H) and compared to Talin stainings of wild type and *talin*-depleted testes (Fig. 3A–D).

Talin localization was unaffected in *boss* and *Abd-B* depleted testes, indicating that Talin controls hub positioning and integrity independent of Abd-B and the Boss/Sev pathway. This was further confirmed by the unaffected Talin localization in $AbdB^{D18}$ clones in L3 testes (Fig. 3I–N).

In order to elucidate the relationship of "larval niche positioning" genes to integrin, we established double mutant combinations by removing one wild type copy of each gene and analyzed the penetrance of the phenotypes in comparison to the single mutant combinations (Fig. 4 and Table S1). This analysis revealed βPS-Integrin as a central player in larval niche positioning with a 66% penetrance of the hub mispositioning phenotype (Table S1). The enhanced niche mispositioning phenotypes observed in double-mutant combinations of (1) either *Abd-B*, *boss* or *talin* with $\beta PS\text{-}integrin^{mys}$ (Fig. 4H–J) and of (2) either *sev* or *Abd-B* with *talin* (Fig. 4E–G), which was similar to the phenotype penetrance of $\beta PS\text{-}integrin^{mys}$ single mutants (Table S1; Fig. 7), suggested that two separate pathways converge on integrin: one mediating a germline-to-soma signal by the AbdB-Boss/Sev cascade and another one by Talin in the cyst cells (Fig. 7). Double mutant combinations of either *Abd-B*, *boss*, *sev* or *talin* with *vav* (Fig. 4K–N) had an enhanced hub mispositioning phenotype, with a penetrance of 55% similar to single *vav* mutants (Table S1). This further indicated that Vav is genetically interacting with both the AbdB-Boss/Sev and the Talin pathways. Taken together, two independent pathways regulate hub positioning and architecture: one mediated by the AbdB-Boss/Sev pathway acting upstream of integrin and one mediated by Talin. And Integrin, as a downstream effector, controls niche positioning and integrity in the larval testis together with multiple cytoskeletal cyst cell regulators, focal adhesion and adaptor proteins.

2.3. Abd-B from the acto-myosin sheath controls hub positioning in the adult Drosophila testis

Our previous work has shown that Abd-B depletion in L3 spermatocytes leads to cell non-autonomous spermatogenesis defects in adult testes with a dramatic reduction in all premeiotic cell types (Papagiannouli et al., 2014). As testis organogenesis proceeds with the

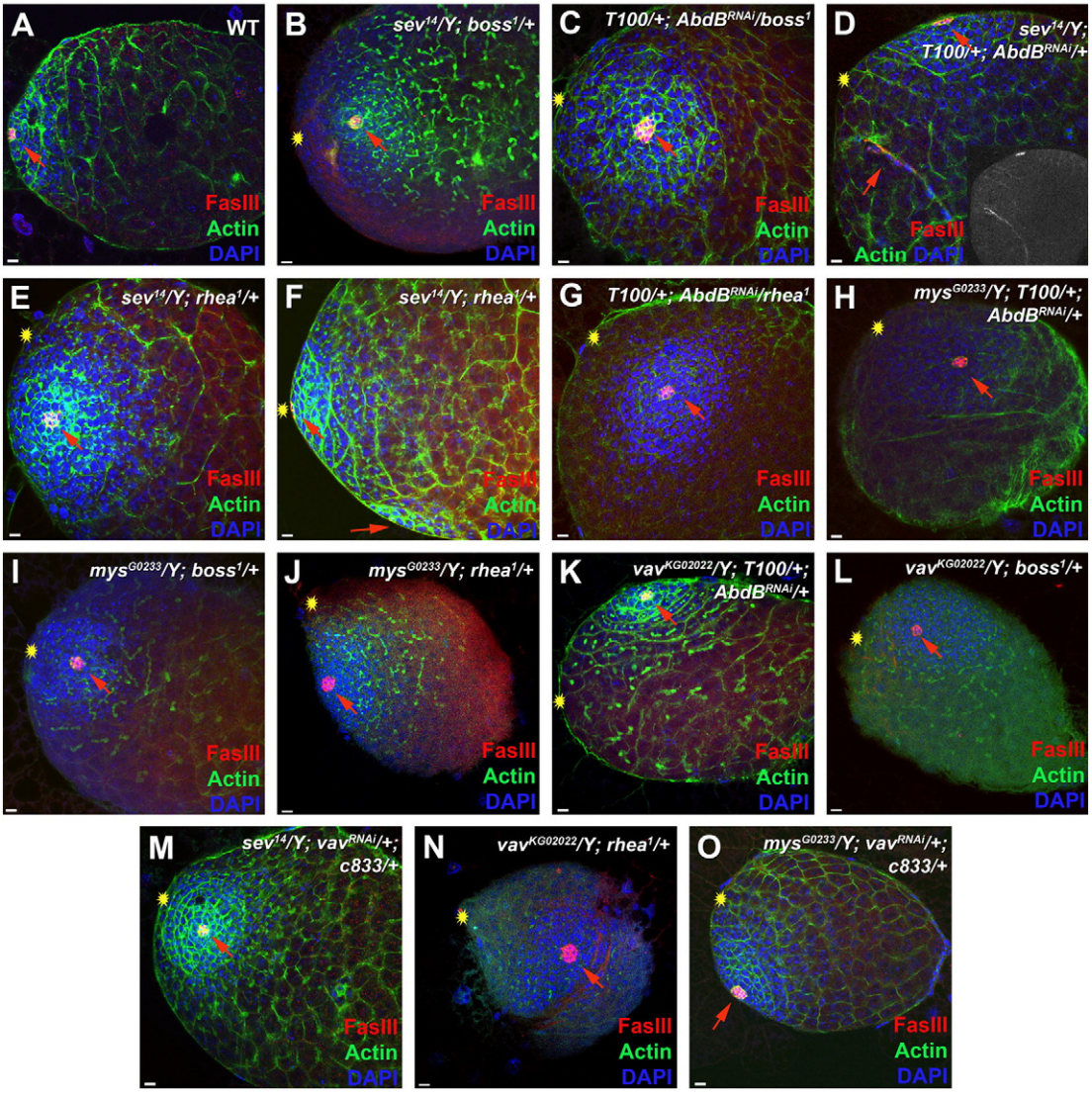


Fig. 4. Talin and the AbdB-Boss/Sev cascade affect integrin and larval hub positioning through independent pathways. (A–O) FasIII, F-Actin and DAPI stainings of L3 testes from wt, single and double mutant or RNAi knockdown combinations of genes affecting hub positioning. Hub mispositioning phenotypes in double mutant combinations of sev^{14} and $AbdB^{RNAi}$ with *talin* ($rhea^1$) (E–G) are as strong as those of $AbdB^{RNAi}$, $boss^1$ and *talin* ($rhea^1$) with $\beta PS\text{-}integrin$ (mys^{G0233}) (H–J), and much stronger than double mutants of sev^{14}-$boss^1$, $boss^1$-$AbdB^{RNAi}$ and sev^{14}-$AbdB^{RNAi}$ (B–D) which belong to the same pathway. Similarly, all double mutant combinations with $vav^{KG02022}$ *or* vav^{RNAi} show the same strong hub mispositioning phenotypes (K–O), suggesting that Vav is genetically interacting with both Talin and the AbdB-Boss/Sev cascade. (D) shows the presence of ectopic FasIII positive cells, with the small inset figure showing the FasIII staining only. (F) shows the presence of two niches, with only one organized around FasIII-positive hub cells. Red arrows point at the hub. Yellow asterisks show the expected hub position. Crosses were set up and progeny were raised at 25 °C, the phenotype was scored at L3. Testis hub is oriented towards the left. Scale bar: 10 μm. (This figure is associated with Table S1).

addition of the acto-myosin sheath, Abd-B is found not only in adult spermatocytes but also in the acto-myosin sheath surrounding the adult testis (Fig. S2E, S2F) (Morillo Prado et al., 2012; Papagiannouli et al., 2014). The outer sheath of the adult testis develops from two cell populations: the pigment cells of the testis and the acto-myosin sheath from the genital disc (Kozopas et al., 1998; Susic-Jung et al., 2012). Analysis of *AbdB*^{D18} clones in adult *Drosophila* spermatocytes revealed that hub positioning and integrin localization were not affected by Abd-B during adult stages (Fig. 5I–L). As Integrin was properly localized in cyst cells even between neighboring *AbdB*^{D18} spermatocyte clones [Fig. 5I (yellow arrowheads) and K] similar to non-clonal regions and to the wild type control testes (Fig. 5A–D), we concluded that integrin localization does not rely on Abd-B during adult stages but most likely requires additional inputs. However, in cases when heat-shock activated *AbdB*^{D18} clones gave rise to defects in the acto-myosin sheath surrounding the testes (Fig. 5E–H and M–P), the adult hub was always mispositioned.

To further confirm the requirement of Abd-B in the acto-myosin sheath for correct hub positioning in adult testes, we performed RNAi *Abd-B* knockdown using the *T100*-GAL4 driver, which also drives expression in the acto-myosin sheath of adult *Drosophila* testes (Fig. S2G, S2H). We observed a disrupted acto-myosin sheath in *T100*::*AbdB*^{RNAi} testes as shown by Actin surface views (Fig. 6D–F). In addition, niche defects were observed ranging from mild hub mispositioning (Fig. 6B) to severe niche shape distortions (Fig. 6C). While the hub lost its anterior anchoring at the testis, disruption of the acto-myosin sheath was associated with secondary effects leading to bigger and irregularly shaped testes. The observed disruption of the outer acto-myosin sheath did not correlate with the age of *Abd-B*-depleted adult testes and was obvious in newly hatched as well as

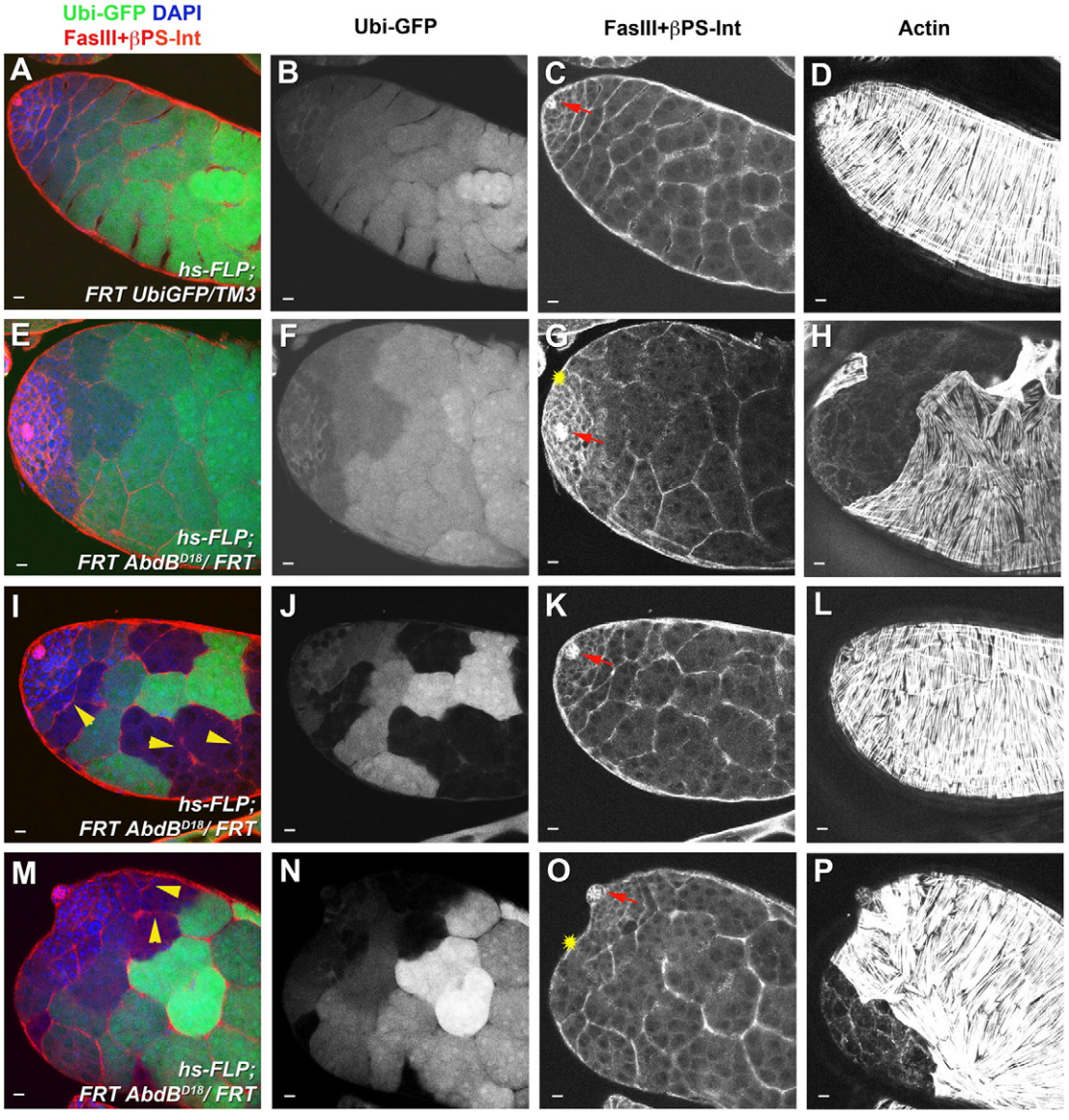


Fig. 5. Abd-B in the adult testis spermatocytes does not affect integrin. Stainings of control *hsFlp122*; *FRT82B-Ubi-GFP/TM3* (A–D) and *hsFlp122*; *FRT82B-AbdB*^{D18}/*FRT82B-Ubi-GFP* (E–P) adult testes, for FasIII + βPS-Integrin (red) and DAPI (blue). *Abd-B*^{D18} clones were identified by the absence of endogenous Ubi-GFP (green). (B), (F), (J), and (N) show only the Ubi-GFP localization. (C), (G), (K), and (O) show separately the FasIII+βPS-Integrin. (D), (H), (L), and (P) are surface views of (A), (E), (I) and (M) respectively, showing phalloidin stainings of filamentous Actin in the adult testis sheath. Yellow arrowheads (I, M) point at βPS-Integrin localization between neighboring *AbdB*^{D18} spermatocyte clones suggesting that Integrin localization is not affected in *Abd-B* depleted adult spermatocytes. (E–H) the acto-myosin sheath is affected in testes containing no *AbdB*^{D18} spermatocyte clones, suggesting that sheath integrity is independent of Abd-B function in adult spermatocytes and that hub positioning is disrupted when the sheath integrity is affected. (I–L) testes containing *AbdB*^{D18} spermatocyte clones show a normal acto-myosin sheath, suggesting that Abd-B function in adult spermatocytes is not related to sheath integrity and anterior hub positioning. For inducing the clones, progeny with the genotype *hsFlp122*; *FRT82B-AbdB*^{D18}/*FRT82B-Ubi-GFP* were heat shocked at 37 °C for 1 h as 1st and 2nd instar larvae and were dissected as 0–2 days old adult males.

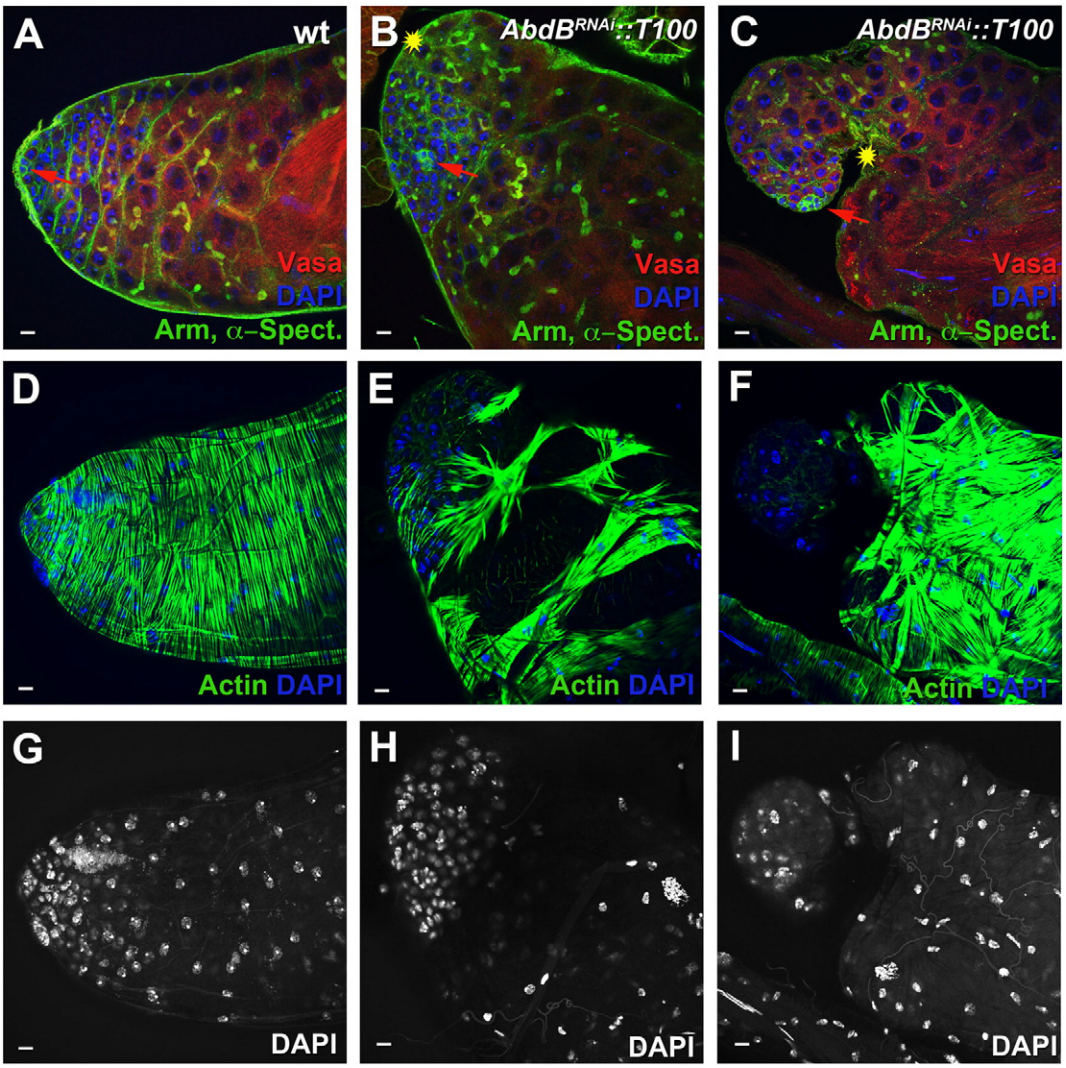


Fig. 6. Abd-B from the acto-myosin sheath controls hub positioning in the adult *Drosophila* testis. Wt (A) and *AbdB^RNAi::T100* (B and C) adult testes stained for α-Spectrin (for the fusome) and Arm (for the hub, CySCs and SCCs), Vasa for the germline and DAPI (blue). (D–F) Surface views from (A–C) respectively, showing the acto-myosin sheath stained with phalloidin for the filamentous Actin and DAPI. (G–I) show only the DAPI in the acto-myosin sheath cells from (D–F). Abd-B knockdowns in adult testes with acto-myosin sheath disruption (E, F) correlated with hub mispositioning phenotypes (B, C) (for penetrance of the acto-myosin sheath disruption phenotype see "Experimental procedures" section). Crosses were set up and progeny were raised at 25 °C. Testis hub is oriented towards the left. Bar: 10 μm.

in aged adult testes. Similar to previous observations (Papagiannouli et al., 2014), the penetrance of this phenotype was variable depending on the Abd-B RNAi fly line used (see "Experimental procedures" section). On the other hand, *Abd-B* knockdown in adult spermatocytes had no effect on hub positioning (Fig. S2). This suggested that during adult stages additional mechanisms secure the correct positioning of the hub, which is of outmost importance for a normal niche function. These data highlight also the stage- and cell-type specific function of Abd-B in *Drosophila* niche positioning throughout testis organogenesis.

3. Discussion

The niche is a vital component of all stem cell systems. From its initial specification throughout adult life, the niche preserves stem cell identity thereby building a dynamic system ensuring sustained tissue homeostasis, cell renewal and balanced response of the organism to challenges such as injury or disease. The *Drosophila* male stem cell niche, called the hub, is a cluster of non-dividing cells at the anterior of the *Drosophila* testis, which is already specified before the germ cells and somatic gonadal cells coalesce to form the male gonad (Boyle and DiNardo, 1995; DeFalco et al., 2008; DeFalco et al., 2004; Dinardo et al., 2011; Hatini et al., 2005; Le Bras and Van Doren, 2006). Testis organogenesis begins as soon as the hub cells recruit the anterior-most germ cells to become the GSCs (Sheng et al., 2009) and continues during larval stages with the formation of a mature stem cell niche and all pre-meiotic cell types in 3rd instar larvae. Testis organogenesis is completed during pupal stages with the addition of the acto-myosin sheath originating from the genital disc (Kozopas et al., 1998; Susic-Jung et al., 2012) that fuses the testis to the seminal vesicle and the genitalia. Our work provides new insights into the mechanisms critical for establishing and maintaining hub architecture and positioning during *Drosophila* testis organogenesis. In particular, we reveal that a network of cytoskeletal components within the SCCs as well as the cell-type and stage specific activity of Abd-B are required to maintain the position and integrity of the male stem cell niche.

3.1. A cell-type and stage-specific role of Abd-B in controlling hub positioning and integrity

Several studies including this work have shown that positioning and integrity of the hub requires the function of Integrin, Talin and the Boss/Sev pathway during embryonic, larval and adult stages of testis

organogenesis (Kitadate et al., 2007; Lee et al., 2008; Papagiannouli et al., 2014; Tanentzapf et al., 2007). Interestingly, these players exert their function always in the same cell lineage even at different developmental stages: Boss in the germ cells (embryonic pole cells and larval germline), and Integrin, Talin, Sev and Lasp in the somatic cell population (somatic gonadal cells and cyst cells). However, the mechanisms regulating hub positioning at the different developmental stages seem to be different despite the employment of similar factors and critically depend on important master regulators, like Abd-B, which functions in a cell-type and stage-specific manner. In embryonic gonads Abd-B is expressed in the somatic lineage, the male-specific somatic gonadal precursors, and regulates *sev* expression in this cell population, whereas Boss signals from the pole cells to the Sev expressing cells to ensure that the niche develops only in the anterior region of the gonad (Kitadate et al., 2007). Hub expansion and integrity defects are also observed in embryonic gonads of weak *Abd-B* mutant alleles (Le Bras and Van Doren, 2006). Independently, it has been shown that Integrin is required in the somatic cells of the embryonic gonads for anterior positioning of the hub by anchoring the hub to the ECM (Tanentzapf et al., 2007). On the other hand, in larval testes Abd-B regulates this pathway in the germline spermatocytes by ensuring Boss function and recycling via its direct targets *sec63* and *src42A*, and thereby controls integrin localization in the neighboring SCCs (Papagiannouli et al., 2014). This expression switch of Abd-B from the somatic to the germline lineage highlights the difference on the mechanism of Abd-B-dependent hub positioning between embryonic and larval stages. During adult stages when testis morphogenesis is completed with the addition of the acto-myosin sheath originating from the genital disc (Kozopas et al., 1998; Susic-Jung et al., 2012), hub positioning and integrity are still regulated by Integrin (Lee et al., 2008) however in an Abd-B independent way (Papagiannouli and Lohmann, 2015; Papagiannouli et al., 2014). Along this line, we have shown that Abd-B also controls hub positioning in adult testes, however not by exerting its function in the testis spermatocytes but through its activity in the acto-myosin sheath, which surrounds the adult testis. Thus, Abd-B switches its expression from the embryonic male-specific somatic cells to the larval spermatocyte germ cells and the adult acto-myosin sheath, which correlates with a change in the Abd-B dependent mechanisms of hub positioning during subsequent steps of testis organogenesis (Papagiannouli and Lohmann, 2015).

Comparable to the cascade uncovered in the *Drosophila* testis, the central morphogenetic function of Abd-B is also illustrated in the development of the genital disc (Coutelis et al., 2013; Foronda et al., 2006). Both sexes of *Drosophila* have a single genital disc formed by the primordial A8, A9 and A10 abdominal segments. The female genitalia develop from the A8 and the male from the A9 segments. In the embryonic as well as in the larval genital discs, the isoform Abd-B^m is expressed in A8 for the development of female derivatives and the isoform Abd-B^r in A9 shapes the male genitalia. Cross-regulatory interactions between abd-A, Abd-B^m and Abd-B^r shape the internal female genitalia, so that Abd-B maintains *abd-A* transcription in contrast to the embryonic epidermis where Abd-B represses *abd-A* (Foronda et al., 2006). Moreover, Abd-B^m is needed for the external female genitalia and Abd-B^r for the male genitalia. Interestingly, in the male A9, some *Abd-B* mutant clones transform part of the genitalia into leg or antenna (Foronda et al., 2006). Finally, the Abd-B^m isoform regulates the clockwise (dextral) looping of male genitalia, a typical left/right asymmetry feature mediated by its direct target *myosinID* (Coutelis et al., 2013). Therefore, Abd-B isoforms control morphogenesis of female and male genitalia in a cell- and stage-specific way. Similar to what is already described, the disrupted adult acto-myosin sheath phenotype observed by RNAi-mediated knockdown of *Abd-B* in this study correlated often with external genitalia defects such as partial transformation of male external genitalia to leg or antenna and external genitalia looping defects (data not shown). Males with strong *Abd-B* RNAi knockdowns seemed to be sterile due to the defects at the external genitalia, while escapers with normal genitalia were fertile (see also "Experimental procedures" section). All the examples from *Drosophila* discussed here, emphasize the micromanager role of Abd-B, which through complex genetic interactions guides morphogenetic events as diverse as male stem cell niche positioning and maintenance, posterior spiracle organogenesis and genitalia shaping. This raises the possibility of organogenesis being a common feature of Abd-B and likely a common property of Hox proteins in general.

3.2. The role of integrin-mediated adhesion in the somatic lineage of the Drosophila larval testis

When integrins cluster at sites of matrix contact, they support the assembly of cell–matrix junctions. Cytoplasmic partners recruited into these junctions include among others Talin, Paxillin, Pinch and Focal Adhesion Kinase (FAK) that link integrins to the polymerized actin fibers and control a variety of signaling pathways regulated by the interplay with the ECM (Giepmans and van Ijzendoorn, 2009; Zamir and Geiger, 2001). In *Drosophila*, Fak56D acts in integrin-mediated signaling pathways in the wing and in multiple morphogenetic processes (Palmer et al., 1999), while Talin plays a decisive role in the recruitment of Pinch and Paxillin at sites of integrin adhesion (Zervas et al., 2011). In muscle and wing epithelial cells, the evolutionarily conserved LIM protein Pinch colocalizes with βPS-Integrin at sites of actin filament anchorage, and acts as part of the integrin-dependent signaling complex (Clark et al., 2003). The guanyl–nucleotide exchange factor (GEF) Vav, a master regulator of cytoskeletal and actin filament reorganization, transmits the integrin signaling and controls integrin clustering at vertebrate immunological synapses (DeMali et al., 2003; Hornstein et al., 2004). In *Drosophila*, Vav acts as an activator of Rac GTPases in S2 cells, axon growth and guidance, and in adult testis cyst cells (Hornstein et al., 2003; Malartre et al., 2010; Sarkar et al., 2007).

Integrin is a critical downstream component of hub positioning and integrity in cyst cells (CySCs and SCCs) of the larval testis, and similar effects are observed by knocking down ECM proteins in cyst cells surrounding the larval testicular cysts (Papagiannouli et al., 2014). Here, we provide evidence that a network of integrin-related adaptor proteins within the cyst cells acts in hub positioning and integrity of the larval testis. These factors include Talin, Vav and the focal adhesion proteins Fak56D, Paxillin and Pinch. One of the emerging questions is how these players from the cyst cells act on stem cell niche positioning and integrity outside of the hub region. Our current hypothesis is that integrins, ECM components, focal adhesion proteins and actin filaments (Fig. 1A) together build a dynamic scaffolding network thereby regulating male stem cell niche positioning and integrity in a cell non-autonomous manner (Papagiannouli and Lohmann, 2015; Papagiannouli et al., 2014). As the role of integrins and their binding proteins in mechanosensitive adhesion is well established (Colombelli et al., 2009; Hirata et al., 2008; Maruthamuthu et al., 2011; Myers et al., 2011), we hypothesize that integrin-mediated adhesion in cyst cells generates tensional forces through interaction with the cytoskeleton and ECM to maintain the testicular cysts and overall testis rigidity, which is required for keeping the hub at the anterior part of the testis outside the integrin expression region. This is achieved by a two-step process: first, local signaling from the differentiating germline and the neighboring cyst cells forming a functional testicular cyst, ensures proper integrin localization in the cyst cells (Fig. 1A). Subsequently, integrin interacts with actin, the cytoskeleton and the ECM that provides the testis with the rigidity required for keeping the hub at the anterior part of the testis. Since the testis during larval stages lacks the acto-myosin sheath that surrounds the outer testis basal lamina and adheres the hub cells in the anterior of the adult testis (Hardy et al., 1979), the larval testis requires the rigidity provided by the ECM proteins surrounding the testicular cysts (throughout the testis in early and late cyst cells) to prevent the hub from losing its anterior positioning. The Hox transcription factor Abd-B is a crucial player in

this network, since it provides critical inputs for the main components of this regulatory cascade.

Furthermore, we show that the AbdB-Boss/Sev cascade that mediates the germline-to-soma signaling within the testicular cysts acts independent of Talin, which cooperates with integrin within the SCCs in an independent way. This is supported from the observation (1) that Talin remains unaffected in *Abd-B* and *boss* depleted testes, and (2) that knocking down *talin* with either *Abd-B*, *boss* or *sev* results in enhanced hub positioning and integrity phenotype, which reaches the penetrance of loss of integrin. Therefore, niche positioning and integrity in the *Drosophila* larval testis is a dynamic process that relies in a combination of upstream regulators, signaling pathways and a network of downstream tethering molecules within the larval cyst cells. Our quantifications show that a single copy of the *rhea* gene (in heterozygous condition $rhea^1/+$) does not produce the same numbers of larval hub positioning phenotype as the wild type testes and also not a stoichiometric result compared to the $rhea^1$ homozygotes: 6% in wt flies versus 38% in the heterozygous $rhea^1$ mutant testes and 41% in homozygous *rhea* mutant testes. This might be a surprising result since *rhea* is a recessive gene, and one would expect a wt hub mispositioning phenotype or a stoichiometric one in respect to the homozygous ones (41%/2 = 20,5%). Similar results were also obtained between $boss^1$ heterozygous and homozygous mutant testes (data not shown). However, several observations from other researchers show that this is the case with several integrin-associated proteins: gene dosage affects protein stoichiometry in a variable way (non-stoichiometric) and gene dosage of one component may even result in compensatory increase of other components (Chountala et al., 2012; Inoue, 2011). This underlines the complexity and robustness of the integrin protein network, and reflects the complex epistatic effects among genetically interacting players. Even more, several components such as Talin have been shown to also have integrin-independent functions (Becam et al., 2005). All these provide a valid explanation to the slight variation or non-stoichiometric quantification results we obtained by calculating the larval "hub mispositioning" phenotype in our genetic interaction analysis (Fig. 7). Yet, getting more insights into the mechanisms of epistatic relations among the integrin-interacting network components is beyond the focus of this study.

3.3. Active maintenance of male stem cell niche function during testis organogenesis

It becomes obvious that proper niche function needs to be actively maintained in order to avoid the accumulation of cell autonomous and non-autonomous effects in the *Drosophila* testis (Kitadate et al., 2007; Le Bras and Van Doren, 2006; Lee et al., 2008; Papagiannouli et al., 2014; Tanentzapf et al., 2007). The employment of a combination of critical players and pathways that continuously provide inputs to secure

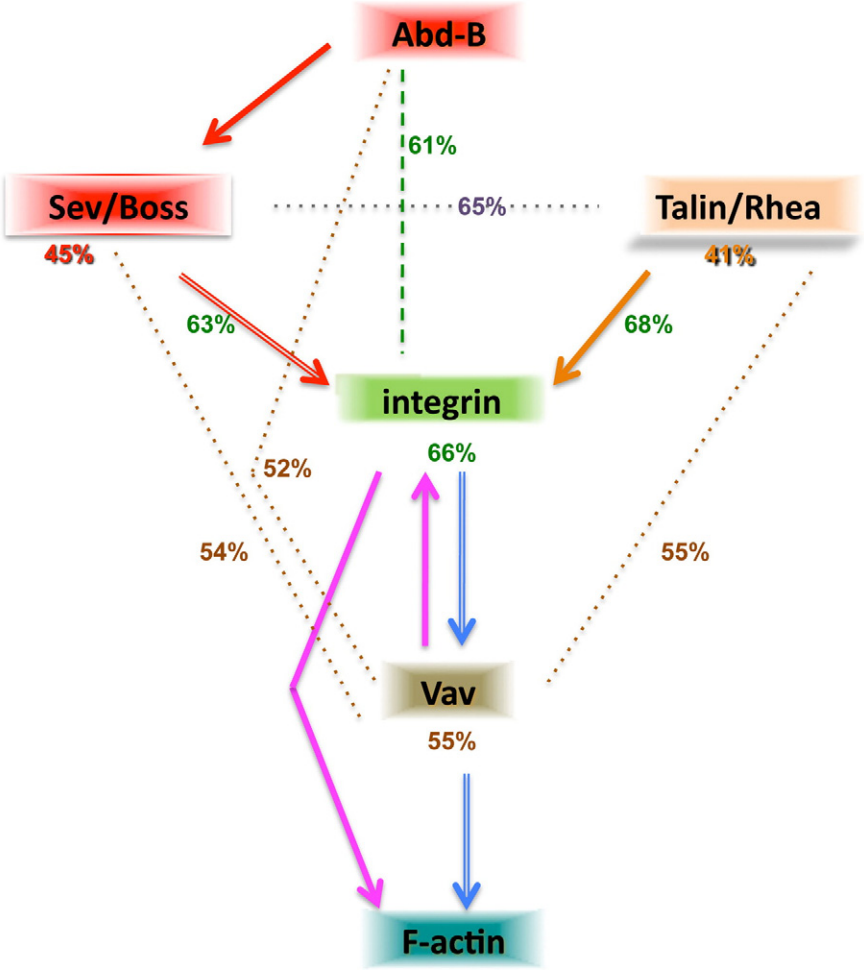


Fig. 7. Schematic diagram of genetic interactions among the key players involved in larval male niche positioning. The % reflects the penetrance of the mutant phenotypes in single and double mutant combinations (across dashed lines) resulting from Table S1. Arrows show the pathways and relations among the players investigated in this study. Pink and blue arrows indicate the two possible ways of interaction between Integrin and Vav, as it is currently unclear whether Vav acts upstream or downstream of Integrin. The variation in the penetrance of the hub mispositioning phenotype among the different mutant combinations is probably due to epistatic interactions, which cannot be excluded as e.g. Talin has been shown to have also Integrin-independent functions (Becam et al., 2005). The role of Actin in hub positioning downstream of Integrin has been shown previously (Papagiannouli et al., 2014). (This diagram is associated with Table S1).

niche positioning and architecture, and protect the function of the niche, confirms the vital importance for the organism to preserve adult germline stem cell function and spermatogenesis in order to produce healthy gametes and progeny (Papagiannouli and Lohmann, 2015). Abd-B acts as precise cell-type and stage-specific upstream micromanager, the Boss/Sev signaling mediates germline–soma communication while integrin-mediated adhesion provides a network of downstream cyst cell specific factors. All these factors coordinate their action towards a functional male stem cell niche, which is required for the testis to functionally mature, to become part of the male genitalia and to be able to produce healthy gametes. Since the main players analyzed here are conserved in other model systems and higher organisms (Cobb and Duboule, 2005; de Rooij et al., 2008; Ellis and Tanentzapf, 2010; Kanatsu-Shinohara et al., 2008; Papagiannouli and Lohmann, 2012; Rijli et al., 1995; Schreiber et al., 2009), the mechanisms we discovered in the *Drosophila* testis could provide paradigms for regulatory strategies that can be exported in other stem cell systems and tissue contexts.

4. Experimental procedures

4.1. Fly stocks and husbandry

Oregon R was used as a wild type stock. The following stocks were obtained from Bloomington Stock Center Indiana: *UAS-AbdB-RNAi$^{TRiP.JF02309}$*, *UAS-AbdB-RNAi$^{TRiP.GLV21012}$*, *UAS-Lasp-RNAi$^{TRiP.JF02075}$*, *βPS-integrin-RNAi$^{TRiP.JF02819}$* (also known as *mys*; *myospheroid*), *UAS-pinch-RNAi$^{TRiP.JF01096}$* (also known as *stck*; *steamer duck*), *UAS-Pax-RNAi$^{TRiP.JF03111}$* (*Pax*; *Paxillin*), *UAS-Fak56D-RNAi$^{TRiP.JF02484}$* (*Fak56D*; *Focal Adhesion Kinase 56D*), *UAS-αPS1-integrin-RNAi$^{TRiP.JF02694}$* (also known as *mew*; *multiple edematous wings*), *UAS-αPS2-integrin-RNAi$^{TRiP.JF02695}$* (also known as *if*; *inflated*), *Pvav^{KG02022}*, *sev^{14}/Y* (*sev^{14}* is a truncated *sev* cDNA giving rough eyes in hetero- & homo-zygous), *boss^{1}*, *mys^{G0233}/FM7c*, *rhea^{1}-FRT80B/TM6B*, *Tb* (also known as *talin*), *c833-GAL4* (drives expression in hub, CySCs and SCCs) (Hrdlicka et al., 2002; Papagiannouli and Mechler, 2009; Papagiannouli et al., 2014), *T100-GAL4* (drives expression in the germline including early spermatocytes and the acto-myosin sheath of adult testes) (Hrdlicka et al., 2002; Papagiannouli et al., 2014), *hsFlp122*; *Pr^{1}/TM6B*, *Tb*, *and FRT82B-Ubi-GFP*. The following stocks used in this study were obtained from the Vienna *Drosophila* RNAi Center (VDRC) Austria: *UAS-AbdB-RNAi^{v12024}/TM3*, *UAS-Lasp-RNAi^{v21500}*, *UAS-vav-RNAi^{v103820}*, *UAS-vav-RNAi^{v6243}*, *UAS-boss-RNAi^{v4366}*, *UAS-talin-RNAi^{v40399}*, and *UAS-talin-RNAi^{v40400}*. The following stocks of the FlyTrap project were obtained from Lynn Cooley's and Allan C. Spradling's lab: *Vav-GFP* (#P01810; enhancer trap) (Kelso et al., 2004; Quinones-Coello et al., 2007). The *UAS-nlsGFP* fly stock was a gift of Bruce Edgar.

All *UAS-gene^{RNAi}* stocks are referred to in the text as *gene^{RNAi}* for simplicity reasons. Knockdowns were performed using the *UAS-GAL4* system (Brand and Perrimon, 1993) by combining the *UAS-RNAi* fly lines with cell-type specific GAL4 drivers described above. Crosses were set up and progeny were raised at 25 °C, the phenotype was scored either at 3rd instar larvae or adult testes of newly hatched males (if not indicated differently).

The *FRT82B-AbdB^{D18}* fly line was obtained from Ernesto Sánchez-Herrero (Foronda et al., 2006). For inducing the mosaic clones, progeny with the genotype *hsFlp122*; *FRT82B-AbdB^{D18}/FRT82B-Ubi-GFP* were heat shocked at 37 °C for 1 h as 1st and 2nd instar larvae and dissected either the day after as 3rd instar larvae or as 0–2 days old adult males.

Other fly stocks used in this study are described in FlyBase. According to the FlyBase (www.flybase.org), *rhea^{1}* homozygous mutants are "lethal — all die before end of embryonic stage" and "some die during embryonic stage". Our observation is that there are viable homozygous *rhea^{1}* larvae, which survive up to the beginning of puparium formation and are clearly non-Tb (while other *rhea* alleles, like the *rhea^{MI00296}* are homozygous embryonic lethal). In our experiments, and the analysis of the larval hub mispositioning, we have performed our experiments both with the *talin^{RNAi}* line (knockdown in the cyst cells with the *c833*-GAL4 driver) and the *rhea^{1}* mutant allele, and we confirmed the phenotype in both cases. By knocking down *talin* in the cyst cells (*talin^{RNAi}::c833-GAL4*), we did not get the strong phenotype showed for *talin* depleted adult *Drosophila* testes (Tanentzapf et al., 2007), since we do not observe loss of hub in the *talin* depleted larval testes. We believe that the reason for this is the fact that *talin* knockdown is active longer and leads to a more robust phenotype in the adult testes (Tanentzapf et al., 2007). A similar effect regarding the strength and penetrance of the RNAi knockdown phenotypes has been observed in *Abd-B* and *boss* depleted testes in a previous work (Papagiannouli et al., 2014), since knocking down these genes (1) with different GAL4 drivers, (2) using different *UAS-RNAi* lines, (3) in a timely controlled way in combination with *Gal80^{ts}*, and (4) by raising the flies in different temperatures (25 °C vs. 29 °C) leads to a variability in the quantification of the phenotype strength [(Papagiannouli et al., 2014); Supplementary data]. Therefore, for calculating the penetrance of the hub mispositioning phenotype and the genetic interactions among *mys*, *talin*, *vav* and *boss*, we used the mutant alleles of these genes and quantified the phenotype by removing one dosage of each gene (*mys*, *vav*, *boss*, *rhea*, *sev*) in different combinations (Table S1).

4.2. Immunofluorescence staining and microscopy

Whole mount testes were dissected in PBS, fixed for 30 min in 8% formaldehyde, rinsed in 1% PBX (1% Triton-100x in PBS) and blocked in 5% Bovine Serum Albumin in 1% PBX. Testes were incubated with primary antibodies over-night at 4 °C and the following day with the secondary antibodies for 2 h at room temperature in the dark (Papagiannouli et al., 2014). For testes immunostaining in the presence of GFP, 1% PBT (1% Tween-20 in PBS) was used instead of 1% PBX in all steps.

The monoclonal antibodies used in this study: anti-Armadillo N7A1 (1/10; mouse), anti-FasIII (1/100), anti-eya10H6 (1/100; mouse), anti-α-spectrin-3A9 (1/100; mouse), anti-Vasa (1/10; rat), anti-αPS2-integrin-CF.2C7 (1/10; mouse), anti-βPS-integrin-CF.6G11 (1/10; mouse), anti-Talin-AA2A & anti-Talin-E16B (mouse; 1/20; recommended use as a cocktail of monoclonal antibodies AA2A plus E16B) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Filamentous Actin (F-actin) was stained with Alexa Fluor phalloidin 488, 546 or 647 (1/300, Life Technologies, Karlsruhe, Germany) and DNA with DAPI (Invitrogen). The rabbit anti-GFP primary antibody was from Life Technologies (A11122). The following secondary antibodies were used: Cy5-conjugated goat anti-mouse IgG, Cy3-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rat IgG (Jackson Immunochemistry, PA).

Confocal images were obtained using a Leica system TCS SP2 (1024 × 1024 pix, 238 μm image frame) (ZMBH, University of Heidelberg) and a Leica system TCS SPE (1024 × 1024 pix, 174 μm image frame) (COS, University of Heidelberg). Pictures were finally processed with Adobe Photoshop 7.0.

4.3. Penetrance of the acto-myosin phenotype in Abd-B knockdowns of adult Drosophila testes

The disrupted acto-myosin sheath phenotype, observed in adult *Abd-B* testes knockdowns, was severe and highly penetrant (90%) using the *AbdB-RNAi^{v12024}* fly line, and mild and less penetrant (20%) with the *AbdB-RNAi^{JF02309}* fly line. The penetrance was calculated by comparing the number of testes with acto-myosin sheath defects to the total number of testes checked. The disrupted adult acto-myosin sheath phenotype correlated often with previously described defects in the external genitalia (Coutelis et al., 2013; Foronda et al., 2006). Interestingly, homozygous *T100*; *AbdB-RNAi ^{v12024}* males were sterile, but the sterility was due to defects at the external genitalia, since escapers with normal genitalia were fertile. Similarly, the few

homozygous *T100*; *AbdB-RNAi*^{JF02309} adult males with external genitalia defects were sterile while those with normal external genitalia were fertile.

Acknowledgments

We would like to thank the *Drosophila* community for providing us generously with fly stocks and antibodies, in particular Ernesto Sánchez-Herrero, the Developmental Studies Hybridoma Bank (Iowa State University), Vienna *Drosophila* RNAi Center (VDRC) and Bloomington *Drosophila* Stock Center. We also thank all the members of the Ingrid Lohmann lab for critically reading the manuscript. We apologize to all whose work was not sited due to space limitations. This work was supported by the DFG/SFB 873.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mod.2015.07.009.

References

Becam, I.E., Tanentzapf, G., Lepesant, J.A., Brown, N.H., Huynh, J.R., 2005. Integrin-independent repression of cadherin transcription by talin during axis formation in *Drosophila*. Nat. Cell Biol. 7, 510–516.
Boyle, M., DiNardo, S., 1995. Specification, migration and assembly of the somatic cells of the *Drosophila* gonad. Development 121, 1815–1825.
Brakebusch, C., Fassler, R., 2003. The integrin-actin connection, an eternal love affair. EMBO J. 22, 2324–2333.
Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.
Chountala, M., Vakaloglou, K.M., Zervas, C.G., 2012. Parvin overexpression uncovers tissue-specific genetic pathways and disrupts F-actin to induce apoptosis in the developing epithelia in *Drosophila*. PLoS One 7, e47355.
Clark, K.A., McGrail, M., Beckerle, M.C., 2003. Analysis of PINCH function in *Drosophila* demonstrates its requirement in integrin-dependent cellular processes. Development 130, 2611–2621.
Cobb, J., Duboule, D., 2005. Comparative analysis of genes downstream of the Hoxd cluster in developing digits and external genitalia. Development 132, 3055–3067.
Colombelli, J., Besser, A., Kress, H., Reynaud, E.G., Girard, P., Caussinus, E., Haselmann, U., Small, J.V., Schwarz, U.S., Stelzer, E.H., 2009. Mechanosensing in actin stress fibers revealed by a close correlation between force and protein localization. J. Cell Sci. 122, 1665–1679.
Coutelis, J.B., Geminard, C., Speder, P., Suzanne, M., Petzoldt, A.G., Noselli, S., 2013. *Drosophila* left/right asymmetry establishment is controlled by the Hox gene abdominal-B. Dev. Cell 24, 89–97.
Cram, E.J., Schwarzbauer, J.E., 2004. The talin wags the dog: new insights into integrin activation. Trends Cell Biol. 14, 55–57.
de Rooij, D.G., Repping, S., van Pelt, A.M., 2008. Role for adhesion molecules in the spermatogonial stem cell niche. Cell Stem Cell 3, 467–468.
DeFalco, T., Le Bras, S., Van Doren, M., 2004. Abdominal-B is essential for proper sexually dimorphic development of the *Drosophila* gonad. Mech. Dev. 121, 1323–1333.
DeFalco, T., Camara, N., Le Bras, S., Van Doren, M., 2008. Nonautonomous sex determination controls sexually dimorphic development of the *Drosophila* gonad. Dev. Cell 14, 275–286.
Delon, I., Brown, N.H., 2007. Integrins and the actin cytoskeleton. Curr. Opin. Cell Biol. 19, 43–50.
DeMali, K.A., Wennerberg, K., Burridge, K., 2003. Integrin signaling to the actin cytoskeleton. Curr. Opin. Cell Biol. 15, 572–582.
Dinardo, S., Okegbe, T., Wingert, L., Freilich, S., Terry, N., 2011. Lines and bowl affect the specification of cyst stem cells and niche cells in the *Drosophila* testis. Development 138, 1687–1696.
Ellis, S.J., Tanentzapf, G., 2010. Integrin-mediated adhesion and stem–cell–niche interactions. Cell Tissue Res. 339, 121–130.
Foronda, D., Estrada, B., de Navas, L., Sanchez-Herrero, E., 2006. Requirement of abdominal-A and abdominal-B in the developing genitalia of *Drosophila* breaks the posterior downregulation rule. Development 133, 117–127.
Franco-Cea, A., Ellis, S.J., Fairchild, M.J., Yuan, L., Cheung, T.Y., Tanentzapf, G., 2010. Distinct developmental roles for direct and indirect talin-mediated linkage to actin. Dev. Biol. 345, 64–77.
Fristrom, D., Wilcox, M., Fristrom, J., 1993. The distribution of PS integrins, laminin A and F-actin during key stages in *Drosophila* wing development. Development 117, 509–523.
Fuller, M.T., 1993. Spermatogenesis. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Fuller, M.T., Spradling, A.C., 2007. Male and female *Drosophila* germline stem cells: two versions of immortality. Science 316, 402–404.
Giepmans, B.N., van Ijzendoorn, S.C., 2009. Epithelial cell–cell junctions and plasma membrane domains. Biochim. Biophys. Acta 1788, 820–831.
Grashoff, C., Thievessen, I., Lorenz, K., Ussar, S., Fassler, R., 2004. Integrin-linked kinase: integrin's mysterious partner. Curr. Opin. Cell Biol. 16, 565–571.
Hardy, R.W., Tokuyasu, K.T., Lindsley, D.L., Garavito, M., 1979. The germinal proliferation center in the testis of *Drosophila melanogaster*. J. Ultrastruct. Res. 69, 180–190.
Hatini, V., Green, R.B., Lengyel, J.A., Bray, S.J., Dinardo, S., 2005. The drumstick/lines/bowl regulatory pathway links antagonistic hedgehog and wingless signaling inputs to epidermal cell differentiation. Genes Dev. 19, 709–718.
Hirata, H., Tatsumi, H., Sokabe, M., 2008. Zyxin emerges as a key player in the mechanotransduction at cell adhesive structures. Commun. Integr. Biol. 1, 192–195.
Hornstein, I., Mortin, M.A., Katzav, S., 2003. DroVav, the *Drosophila melanogaster* homologue of the mammalian Vav proteins, serves as a signal transducer protein in the Rac and DER pathways. Oncogene 22, 6774–6784.
Hornstein, I., Alcover, A., Katzav, S., 2004. Vav proteins, masters of the world of cytoskeleton organization. Cell. Signal. 16, 1–11.
Hrdlicka, L., Gibson, M., Kiger, A., Micchelli, C., Schober, M., Schock, F., Perrimon, N., 2002. Analysis of twenty-four Gal4 lines in *Drosophila melanogaster*. Genesis 34, 51–57.
Inoue, Y.B.N.H., 2011. Building a complex protein network linking the membrane to the cytoskeleton: impact of gene dosage on protein stoichiometry within the integrin adhesion complex in vivo. Annual *Drosophila* Research Conference, San Diego, CA, USA.
Kanatsu-Shinohara, M., Takehashi, M., Takashima, S., Lee, J., Morimoto, H., Chuma, S., Raducanu, A., Nakatsuji, N., Fassler, R., Shinohara, T., 2008. Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. Cell Stem Cell 3, 533–542.
Kelso, R.J., Buszczak, M., Quinones, A.T., Castiblanco, C., Mazzalupo, S., Cooley, L., 2004. Flytrap, a database documenting a GFP protein-trap insertion screen in *Drosophila melanogaster*. Nucleic Acids Res. 32, D418–D420.
Kitadate, Y., Kobayashi, S., 2010. Notch and Egfr signaling act antagonistically to regulate germ-line stem cell niche formation in *Drosophila* male embryonic gonads. Proc. Natl. Acad. Sci. U. S. A. 107, 14241–14246.
Kitadate, Y., Shigenobu, S., Arita, K., Kobayashi, S., 2007. Boss/Sev signaling from germline to soma restricts germline–stem–cell–niche formation in the anterior region of *Drosophila* male gonads. Dev. Cell 13, 151–159.
Kozopas, K.M., Samos, C.H., Nusse, R., 1998. DWnt-2, a *Drosophila* Wnt gene required for the development of the male reproductive tract, specifies a sexually dimorphic cell fate. Genes Dev. 12, 1155–1165.
Le Bras, S., Van Doren, M., 2006. Development of the male germline stem cell niche in *Drosophila*. Dev. Biol. 294, 92–103.
Lee, S., Zhou, L., Kim, J., Kalbfleisch, S., Schock, F., 2008. Lasp anchors the *Drosophila* male stem cell niche and mediates spermatid individualization. Mech. Dev. 125, 768–776.
Lo, S.H., 2006. Focal adhesions: what's new inside. Dev. Biol. 294, 280–291.
MacKrell, A.J., Kusche-Gullberg, M., Garrison, K., Fessler, J.H., 1993. Novel *Drosophila* laminin A chain reveals structural relationships between laminin subunits. FASEB J. 7, 375–381.
Malartre, M., Ayaz, D., Amador, F.F., Martin-Bermudo, M.D., 2010. The guanine exchange factor vav controls axon growth and guidance during *Drosophila* development. J. Neurosci. 30, 2257–2267.
Martin, D., Zusman, S., Li, X., Williams, E.L., Khare, N., DaRocha, S., Chiquet-Ehrismann, R., Baumgartner, S., 1999. Wing blister, a new *Drosophila* laminin alpha chain required for cell adhesion and migration during embryonic and imaginal development. J. Cell Biol. 145, 191–201.
Maruthamuthu, V., Sabass, B., Schwarz, U.S., Gardel, M.L., 2011. Cell–ECM traction force modulates endogenous tension at cell–cell contacts. Proc. Natl. Acad. Sci. U. S. A. 108, 4708–4713.
Morillo Prado, J.R., Chen, X., Fuller, M.T., 2012. Polycomb group genes Psc and Su(z)2 maintain somatic stem cell identity and activity in *Drosophila*. PLoS One 7, e52892.
Myers, K.A., Applegate, K.T., Danuser, G., Fischer, R.S., Waterman, C.M., 2011. Distinct ECM mechanosensing pathways regulate microtubule dynamics to control endothelial cell branching morphogenesis. J. Cell Biol. 192, 321–334.
Nolz, J.C., Gomez, T.S., Billadeau, D.D., 2005. The Ezh2 methyltransferase complex: actin up in the cytosol. Trends Cell Biol. 15, 514–517.
Palmer, R.H., Fessler, L.I., Edeen, P.T., Madigan, S.J., McKeown, M., Hunter, T., 1999. DFak56 is a novel *Drosophila melanogaster* focal adhesion kinase. J. Biol. Chem. 274, 35621–35629.
Papagiannouli, F., Lohmann, I., 2012. Shaping the niche: lessons from the *Drosophila* testis and other model systems. Biotechnol. J. 7 (6), 723–736.
Papagiannouli, F., Lohmann, I., 2015. Stage-specific control of stem cell niche architecture in the *Drosophila* testis by the posterior Hox gene Abd-B. Comput. Struct. Biotechnol. J. 13, 122–130.
Papagiannouli, F., Mechler, B.M., 2009. Discs large regulates somatic cyst cell survival and expansion in *Drosophila* testis. Cell Res. 19, 1139–1149.
Papagiannouli, F., Schardt, L., Grajcarek, J., Ha, N., Lohmann, I., 2014. The hox gene abd-B controls stem cell niche function in the *Drosophila* testis. Dev. Cell 28, 189–202.
Pasapera, A.M., Schneider, I.C., Rericha, E., Schlaepfer, D.D., Waterman, C.M., 2010. Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. J. Cell Biol. 188, 877–890.
Quinones-Coello, A.T., Petrella, L.N., Ayers, K., Melillo, A., Mazzalupo, S., Hudson, A.M., Wang, S., Castiblanco, C., Buszczak, M., Hoskins, R.A., Cooley, L., 2007. Exploring strategies for protein trapping in *Drosophila*. Genetics 175, 1089–1104.
Rijli, F.M., Matyas, R., Pellegrini, M., Dierich, A., Gruss, P., Dolle, P., Chambon, P., 1995. Cryptorchidism and homeotic transformations of spinal nerves and vertebrae in Hoxa-10 mutant mice. Proc. Natl. Acad. Sci. U. S. A. 92, 8185–8189.
Sarkar, A., Parikh, N., Hearn, S.A., Fuller, M.T., Tazuke, S.I., Schulz, C., 2007. Antagonistic roles of Rac and Rho in organizing the germ cell microenvironment. Curr. Biol. 17, 1253–1258.

Schreiber, T.D., Steinl, C., Essl, M., Abele, H., Geiger, K., Muller, C.A., Aicher, W.K., Klein, G., 2009. The integrin alpha9beta1 on hematopoietic stem and progenitor cells: involvement in cell adhesion, proliferation and differentiation. Haematologica 94, 1493–1501.

Sheng, X.R., Posenau, T., Gumulak-Smith, J.J., Matunis, E., Van Doren, M., Wawersik, M., 2009. Jak-STAT regulation of male germline stem cell establishment during *Drosophila* embryogenesis. Dev. Biol. 334 (2), 335–344.

Susic-Jung, L., Hornbruch-Freitag, C., Kuckwa, J., Rexer, K.H., Lammel, U., Renkawitz-Pohl, R., 2012. Multinucleated smooth muscles and mononucleated as well as multinucleated striated muscles develop during establishment of the male reproductive organs of *Drosophila melanogaster*. Dev. Biol. 370, 86–97.

Suyama, R., Jenny, A., Curado, S., Pellis-van Berkel, W., Ephrussi, A., 2009. The actin-binding protein Lasp promotes Oskar accumulation at the posterior pole of the *Drosophila* embryo. Development 136, 95–105.

Tanentzapf, G., Martin-Bermudo, M.D., Hicks, M.S., Brown, N.H., 2006. Multiple factors contribute to integrin-talin interactions in vivo. J. Cell Sci. 119, 1632–1644.

Tanentzapf, G., Devenport, D., Godt, D., Brown, N.H., 2007. Integrin-dependent anchoring of a stem-cell niche. Nat. Cell Biol. 9, 1413–1418.

Traenka, J., Hauck, C.R., Lewandrowski, U., Sickmann, A., Gambaryan, S., Thalheimer, P., Butt, E., 2009. Integrin-dependent translocation of LASP-1 to the cytoskeleton of activated platelets correlates with LASP-1 phosphorylation at tyrosine 171 by Src-kinase. Thromb. Haemost. 102, 520–528.

Tybulewicz, V.L., 2005. Vav-family proteins in T-cell signalling. Curr. Opin. Immunol. 17, 267–274.

Vicente-Manzanares, M., Choi, C.K., Horwitz, A.R., 2009. Integrins in cell migration—the actin connection. J. Cell Sci. 122, 199–206.

Wozniak, M.A., Modzelewska, K., Kwong, L., Keely, P.J., 2004. Focal adhesion regulation of cell behavior. Biochim. Biophys. Acta 1692, 103–119.

Zamir, E., Geiger, B., 2001. Molecular complexity and dynamics of cell–matrix adhesions. J. Cell Sci. 114, 3583–3590.

Zervas, C.G., Psarra, E., Williams, V., Solomon, E., Vakaloglou, K.M., Brown, N.H., 2011. A central multifunctional role of integrin-linked kinase at muscle attachment sites. J. Cell Sci. 124, 1316–1327.