Drosophila Swiprosin-1/EFHD2 accumulates at the prefusion complex stage during *Drosophila* myoblast fusion

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Accepted 30 May 2011
Journal of Cell Science 124, 3266–3278

doi: 10.1242/jcs.083907

Summary

In the *Drosophila* embryo, transient cell adhesion during myoblast fusion is known to lead to the formation of fusion-restricted myogenic-adhesive structures (FuRMASs). Here, we report that within these FuRMASs, a *Drosophila* homologue of human and mouse swiprosins (EF-hand-domain-containing proteins) is expressed, which we named *Drosophila* Swiprosin-1 (*Drosophila* Swip-1). *Drosophila* Swip-1 is highly conserved and is closely related to the calcium-binding proteins swiprosin-1 and swiprosin-2 that have a role in the immune system in humans and mice. Our study shows that *Drosophila* Swip-1 is also expressed in corresponding cells of the *Drosophila* immune system. During myoblast fusion, *Drosophila* Swip-1 accumulates transiently in the foci of fusion-competent myoblasts (FCMs). Both the EF-hand and the coiled-coil domain of *Drosophila* Swip-1 are required to localise the protein to these foci. The formation of *Drosophila* Swip-1 foci requires successful cell adhesion between FCMs and founder cells (FCs) or growing myotubes. Moreover, *Drosophila* Swip-1 foci were found to increase in number in $sing^{22}$ mutants, which arrest myoblast fusion after prefusion complex formation. By contrast, *Drosophila* Swip-1 foci are not significantly enriched in $blow^{2}$ and $kette^{J4\text{-}48}$ mutants, which stop myogenesis beyond the prefusion complex stage but before plasma membrane merging. Therefore, we hypothesise that *Drosophila* Swip-1 participates in the breakdown of the prefusion complex during the progression of myoblast fusion.

Key words: *Drosophila* Swip-1, CG10641, Calcium, Myogenesis, Immunological synapse, FuRMAS, Singles bar, Sticks-and-stones, SL2 cells, Actin foci

Introduction

Membrane fusion is a fundamental feature found in many processes such as vesicle fusion with plasma membranes, fusion of intracellular vesicles, viral fusion and cell–cell fusion, e.g. epithelial fusion and sperm–egg fusion (Martens and McMahon, 2008; Sapir et al., 2008). In this study, we focus on cell–cell fusion during myogenesis in *Drosophila*.

In higher organisms, myotubes are multinuclear and arise by myoblast fusion during development, as well as during postnatal growth and repair. In *Drosophila*, founder cells (FCs) determine the final identity of larval muscles, whereas fusion-competent myoblasts (FCMs) fuse to them until the final characteristic number of nuclei is reached (Abmayr et al., 2008; Chen and Olson, 2004; Maqbool and Jagla, 2007). Fusion in the somatic mesoderm progresses in two temporal phases within approximately 5.5 hours, with the individual fusion event taking only a matter of minutes (Richardson et al., 2007). These fusion events are characterised by transient F-actin plugs or foci at the contact sites of myoblasts (Richardson et al., 2008b). Studies in *Drosophila* have provided key insights into the underlying mechanisms and key components involved in cell adhesion, signalling, local F-actin accumulation and branching by activation of the Arp2/3 complex (Haralalka and Abmayr, 2010; Önel and Renkawitz-Pohl, 2009; Rochlin et al., 2010).

Heterologous cell adhesion is mediated by immunoglobulin (Ig) transmembrane cellular receptors. Dumbfounded/Kin of Irre (Duf/Kirre) is known to enable FCs and growing myotubes to adhere to FMCs and acts in functional redundancy to Roughest/Irregular chiasma C (Rst/IrreC) (Ruiz-Gómez et al., 2000; Strünkelnberg et al., 2001). In addition, the FCM-specific protein Sticks-and-stones (Sns) also enables cell adherence between FCMs with FCs or growing myotubes and can act in partial redundancy with Hibris (Shelton et al., 2009). Duf/Kirre, Rst/IrreC and Sns were shown to form a ring-shaped structure at the contact sites of FCs or growing myotubes and FCMs, and to establish the fusion-restricted myogenic-adhesive structure (FuRMAS) (Kesper et al., 2007). After successful cell adhesion, F-actin and its regulators accumulate as plugs or foci in the centre of this structure (Kesper et al., 2007; Richardson et al., 2007). Furthermore, prefusion complex formation, exocytosis of its vesicles and membrane breakdown have all been proposed to occur at the centre of the FuRMASs (Önel and Renkawitz-Pohl, 2009). Interestingly, FuRMASs share many features with the immunological synapse (IS), podosomes and invadopodia, such as transient adhesive rings, as well as F-actin accumulation and branching (Kesper et al., 2007; Önel and Renkawitz-Pohl, 2009). Finally, recent ultrastructural analyses have revealed that podosome-like structures are present in FCMs that are fused with FCs or growing myotube (Sens et al., 2010).

Membrane-associated, ultrastructural features during myoblast fusion

Importantly, myoblast fusion has been studied intensively at the ultrastructural level in wild-type (Doberstein et al., 1997) and

mutant embryos (Berger et al., 2008; Gildor et al., 2009; Kim et al., 2007; Massarwa et al., 2007; Schröter et al., 2004; Schäfer et al., 2007). Of particular interest are the membrane-associated structures, because fusion is a membrane-merging process. First, prefusion complexes are formed by accumulation of electron-dense vesicles at opposing membranes between FCs or growing myotubes and FCMs. At the individual contact site, these pairs of vesicles are distributed over an area of 1 μm^2. Occasionally, electron-dense, desmosome-like plaques are observed in the opposing membranes; however, their nature and function is currently unclear. The opposing membranes vesiculate into many membrane remnants (Doberstein et al., 1997), thereby generating cytoplasmic continuities over 4 μm wide. Subsequently, the fusing FCMs are integrated into the growing myotube.

The progression from prefusion complex to membrane breakdown

The electron-dense vesicles of the prefusion complex are proposed to exocytose fusion-relevant molecules (Doberstein et al., 1997). To date, only one protein has been characterised that might be needed to enable progression from prefusion complex to exocytosis of these vesicles. This protein was named Singles bar (Sing), a multipass transmembrane protein with a MARVEL domain, which is expressed in both FCs and FCMs (Estrada et al., 2007). However, the subcellular localisation of Sing is still unknown.

Point mutation in homozygous $sing^{22}$ mutant embryos is known to lead to a missense mutation (A46V) in translation and to an accumulation of prefusion complexes (Estrada et al., 2007). In *blown fuse* ($blow^2$) loss-of-function mutant embryos, the prefusion complexes are assembled and dissolved, but the opposing plasma membranes do not vesiculate (Doberstein et al., 1997; Estrada et al., 2007). Blow is solely expressed in FCMs (Schröter et al., 2006) and accumulates with F-actin plugs or foci after successful cell adhesion (Kesper et al., 2007). Genetically, *blow* interacts with *kette*, which suggests a function in F-actin regulation (Gildor et al., 2009; Schröter et al., 2004). Moreover, in $kette^{J4\text{-}48}$ loss-of-function mutant embryos, plasma membranes remain intact (Doberstein et al., 1997; Schröter et al., 2004). Other mutants for proteins that are predicted to be relevant for actin regulation stop development at later stages during fusion. To date, there are contradictory data for the gene encoding the WASP-interacting protein (Wip), also known as Verprolin. Studies on the $solitary^{S1946}$ allele of *wip* revealed that Sltr might be involved in targeting and coating vesicles that are required for myoblast fusion; however, the membranes of FCMs and FCs or growing myotubes, which were brought in close proximity for fusion, were found to remain intact (Kim et al., 2007). By contrast, the loss-of-function allele wip^{30D} leads to fusion arrest with vesiculating membranes (Massarwa et al., 2007) and Arp2/3 activation is necessary to obtain complete cytoplasmic continuity (Berger et al., 2008). However, Arp2/3 seems not to be relevant for exocytosis of the electron-dense vesicles or for membrane merging (Önel and Renkawitz-Pohl, 2009; Önel, 2009).

FuRMAS, a transient adhesion-signalling centre, is comparable to the immunological synapse

In a topological model, we proposed that the prefusion complex is surrounded by a ring of cell-adhesion molecules: Duf/Kirre on the site of FCs or growing muscles and Sns on the site of the FCMs. Thereby, a transient FuRMAS signalling centre is established; a structure that is proposed to trigger myoblast fusion (Kesper et al., 2007; Önel and Renkawitz-Pohl, 2009). The prefusion complex resembles the accumulation of synaptic vesicles on the site of the axon at the neuromuscular synapse. For the neuromuscular synapse, exocytosis is known to be triggered in the presence of calcium (Schweizer and Ryan, 2006). In mouse cell culture (C2C12 cells), intracellular calcium increases before fusion and can induce myoblast fusion (Horsley and Pavlath, 2004).

Interestingly, the FuRMASs and the IS share many common features (Önel and Renkawitz-Pohl, 2009). For example, they both contain adhesion molecules and actin regulators are required to produce F-actin plugs or foci at the centre of the respective structures. Moreover, IS formation leads to increased cytoplasmic calcium concentration in T-cells (Kummerow et al., 2009). Therefore, there might be a comparable process during myoblast fusion in *Drosophila*. To add weight to this notion, calcium is known to be bound by EF-hand-domain-containing proteins (Gifford et al., 2007) including swiprosins (Vega et al., 2008; Dütting et al., 2011).

Mouse swiprosin-1/EFHD2 is an EF-hand and coiled-coil containing protein, which was identified in lipid rafts of mouse B-cells (Avramidou et al., 2007; Mielenz et al., 2005). Furthermore, Swiprosin-1/EFHD2 is thought to control B-cell-receptor signalling in mouse (Kroczek et al., 2010) and cytokine expression in human mast cells (Ramesh et al., 2009; Thylur et al., 2009), and it is also known to be present in human cytotoxic lymphocytes (Vuadens et al., 2004).

Here, we present our analysis of *Drosophila* Swip-1 distribution during myoblast fusion in wild-type embryos and show selective accumulation in FuRMASs on the site of FCMs. Our results show that *Drosophila* Swip-1 does not accumulate in *sticks-and-stones* (*sns*) mutants. Therefore, we predict that *Drosophila* Swip-1 accumulation depends on Sns-mediated cell adhesion and also probably signalling. Importantly, *Drosophila* Swip-1 foci were found to accumulate in *singles bar* ($sing^{22}$) mutants; a phenomenon not observed in *blown fuse* ($blow^2$) and *kette* ($kette^{J4\text{-}48}$) mutants. We therefore hypothesise that *Drosophila* Swip-1 is one of the molecules that either directly or indirectly regulates calcium-dependent exocytosis of the electron-dense vesicles of the prefusion complex.

Results

CG10641 encodes a protein with two EF-hand domains and one coiled-coil domain and is related to mammalian swiprosin proteins

As a result of the many shared features between FuRMASs and ISs, we asked whether calcium-binding proteins might be involved at a distinct time point during myoblast fusion. Therefore, we took a data-mining approach and searched databases (e.g. FlyBase) for putative calcium-binding proteins that are expressed preferentially in the mesoderm. This search revealed the *Drosophila* gene *CG10641* (Tweedie et al., 2009). CG10641 appears to have a role in myogenesis, as indicated first by a screen for putative Twist targets (Furlong et al., 2001) and then in a screen for transcripts enriched in FCMs or FCs (Estrada et al., 2006).

CG10641 is predicted to encode a 217 amino acid cytosolic protein and similarly to the human and mouse swiprosins, it also has two EF-hand domains and one coiled-coil domain at the C-

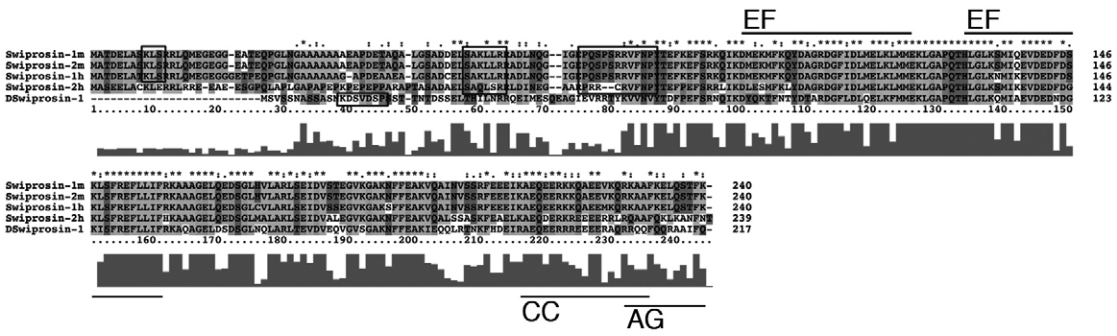

Fig. 1. Swiprosin proteins are evolutionary highly conserved in mouse, human and *Drosophila*. An amino acid alignment with Clustalw (http://www.ebi.ac.uk/Tools/Clustalwz/index) of swiprosin-1/EFHD2 and swiprosin-2/EFHD1 from mouse (m) and human (h), and the *Drosophila* orthologue CG10641 (DSwiprosin-1) shows high conservation between these three species. Especially conserved are the two EF-hand (EF) and one coiled-coil (CC) domains. EF-hand domains are known to bind calcium (Gifford et al., 2007), whereas the coiled-coil domain might allow homo- or heterodimerisation (Lupas, 1996). Furthermore, the four predicted SH3-binding sites are marked in boxes and the peptide used as an antigen to generate anti-*Drosophila* Swip-1 antibody is marked in the C-terminal region as AG.

terminus (Fig. 1). Furthermore, CG10641 also contains one SH3 domain recognition motif at the N-terminus – three of which characterise the N-terminal region of mammalian swiprosins. Interestingly, mammalian Swip-1/EFhd2 is known to bind calcium – at least in vitro (Vega et al., 2008). Thus, in many aspects, the *Drosophila* protein is highly conserved with mammalian swiprosins (Fig. 1), and therefore, we named the CG10641 protein *Drosophila* Swip-1 in accordance with Kroczek and colleagues (Kroczek et al., 2010).

Expression in the immune system is a conserved feature between *Drosophila* and mammalian swiprosin

Estrada and co-workers (Estrada et al., 2006) showed that *CC10641*/*Drosophila Swip-1* is transcribed at the extended germ-band stage shortly after the determination of FCs and FCMs in the visceral and somatic mesoderm, as well as in hemocytes of the head mesoderm at this stage. In this study, we verified these transcription data and analysed the detailed transcript distribution of *Drosophila Swip-1* during myoblast fusion (supplementary material Fig. S1). We found that *Drosophila* Swip-1 transcripts are not maternally contributed to any significant extent, and that transcripts are first detectable at the beginning of gastrulation in the mesoderm and then persist in the somatic and visceral mesoderm until the completion of myogenesis (supplementary material Fig. S1).

To analyse the subcellular distribution of *Drosophila* Swip-1 during embryogenesis, we raised an antibody in rabbits against 18 amino acids from the C-terminal region of *Drosophila* Swip-1 (see Fig. 1 and the Materials and Methods). At the extended germ band stage, *Drosophila* Swip-1 was visible in the somatic and visceral mesoderm (Fig. 2A). During myoblast fusion, *Drosophila* Swip-1 was visible in many foci within the somatic mesoderm (Fig. 2B,C; Fig. 3A–C). Towards the end of embryogenesis, only a few *Drosophila* Swip-1 foci were visible in the somatic myoblasts and *Drosophila* Swip-1-positive stripes were detected at the muscle ends (Fig. 2D). In the pharynx, *Drosophila* Swip-1 accumulated at the contact sites of the individual muscles (Fig. 2F). A ventral view visualised *Drosophila* Swip-1 expression in the nervous system (Fig. 2E); this expression was observed from stage 13 onwards. At higher magnification, it was evident that at stage 16, *Drosophila* Swip-1 accumulates at the ends of the muscles towards their epidermal attachment sites (Fig. 2G, arrows). *Drosophila* Swip-1 localised closer to the epidermal attachment sites, which were marked by βPS integrin (for review see Brown, 1993), compared with the majority of microtubules, which were arranged in parallel to the length of the muscles (Fig. 2H,I).

In addition, *Drosophila* Swip-1 expression was also detectable in hemocytes in the head region (Fig. 2B, arrow), as well as in macrophage-like hemocytes in the region between the epidermis and the nervous system, where it was concentrated in distinct areas (Fig. 2J, arrow). β3-tubulin is known to be expressed in the macrophage-like hemocytes (Leiss et al., 1988). Therefore, we used antibodies against *Drosophila* Swip-1 and β3-tubulin in double-labelling studies and found that *Drosophila* Swip-1 does not overlap with the majority of microtubules (Fig. 2K). Taken together, we conclude that the role of swiprosin in the immune system is a conserved feature between *Drosophila* and mammals and that *Drosophila* Swip-1 is involved in further physiological and developmental processes.

Western blot analysis revealed a band at 25 kDa in extracts of embryos, larvae, pupae and hemocyte-like SL2 cells, which appeared as a double band in embryos, larvae and pupae (Fig. 2L, arrow). In contrast to vertebrates (for review see Dütting et al., 2011), there is no evidence for splice variants (Tweedie et al., 2009) and therefore, the additional band might indicate that *Drosophila* Swip-1 can carry a secondary modification in *Drosophila*. Indeed, vertebrate Swip-1 is known to be phosphorylated (Dütting et al., 2011). Finally, the specificity of our *Drosophila* Swip-1 antibody was demonstrated by transfecting SL2 cells with *Drosophila* Swip-1 constructs including or lacking the antigen (supplementary material Fig. S2).

Drosophila Swip-1 is mainly visible in FCMs during myoblast fusion and accumulates transiently in foci at the contact sites between FCs or growing myotubes and FCMs

As mentioned above, during fusion-relevant stages, *Drosophila* Swip-1 was detected in numerous foci within the somatic mesoderm. The pattern of these foci varied from segment to segment and from stage to stage (Fig. 3A–C, arrowheads). Thus, not all myoblasts showed these *Drosophila* Swip-1 foci at the same time. Late in myogenesis, at stage 15, only a few fusion events took place, and thus, fewer *Drosophila* Swip-1 foci were present and these were restricted to the FCMs that were in contact

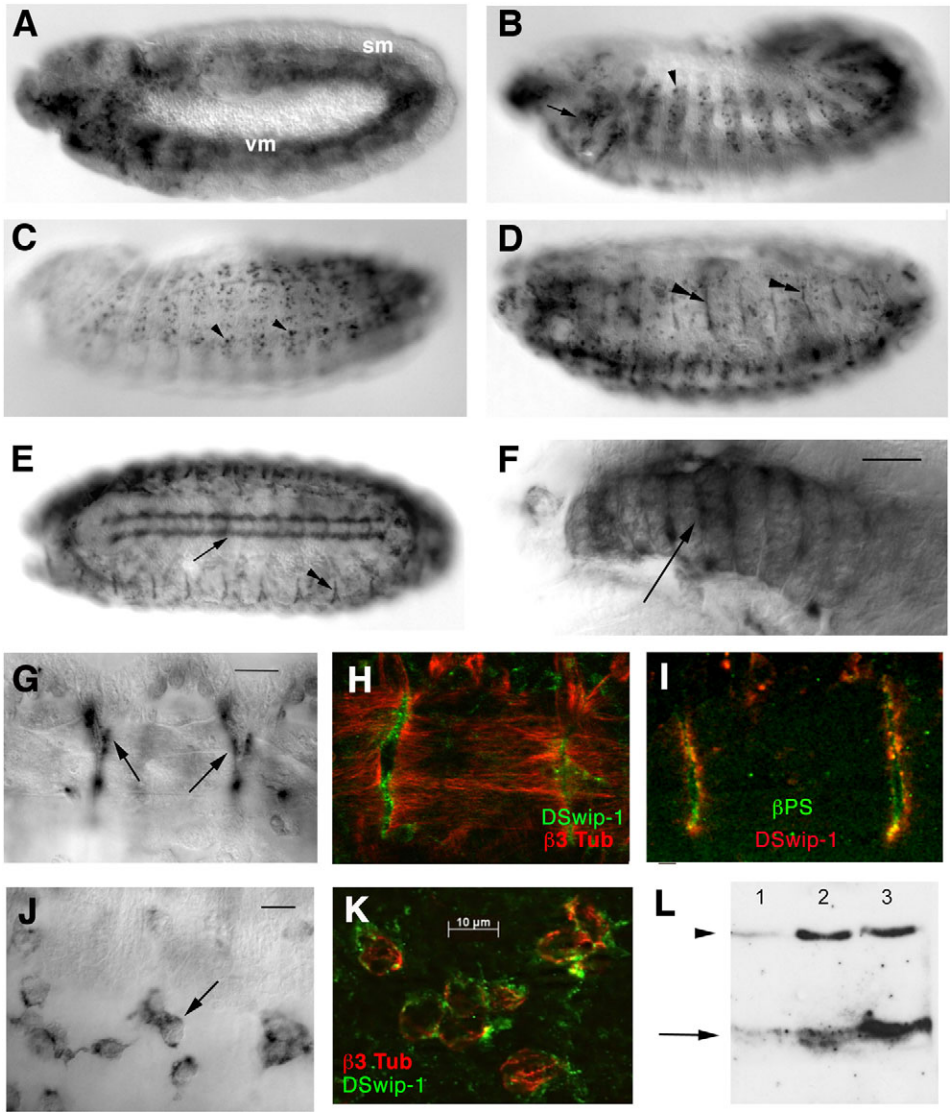

Fig. 2. *Drosophila* Swip-1 is expressed during muscle development. (**A**) *Drosophila* Swip-1 (DSwip-1) protein is visible at the extended germ-band stage in the somatic (sm) and visceral (vm) mesoderm. (**B**,**C**) During germ-band retraction, *Drosophila* Swip-1 expression is detectable in the hemocytes in the head region (arrow), as well as in foci in the somatic mesoderm (arrowheads). (**D**,**E**) At late fusion-relevant stages, *Drosophila* Swip-1 is visible in a few foci within the somatic mesoderm. Additionally, the protein is detectable in stripes, which mark the attachment sites at the end of embryogenesis (double arrowheads). (E) In a ventral view, *Drosophila* Swip-1 expression is visible in the nervous system (arrow). (**F**) *Drosophila* Swip-1 accumulates at the contact sites of the muscles of the pharynx towards their epidermal attachment site (arrow). (**G**) Additionally, *Drosophila* Swip-1 accumulates at the attachment sites (arrows). (**H**,**I**) Double labelling shows that *Drosophila* Swip-1 localises closer to the epidermal attachment sites (I: marked with βPS integrin antibody) than to the microtubules (H: marked with β3-tubulin antibody). (**J**) Between the epidermis and the central nervous system, macrophage-like hemocytes express *Drosophila* Swip-1 (arrow). (**K**) Double labelling with the β3-tubulin antibody shows that *Drosophila* Swip-1 seems not to overlap with the microtubles of the cytoskeleton. (**L**) Western blot analyses reveal that in addition to *Drosophila* Swip-1 expression (arrow) in the embryo (lane 1), the endogenous protein (25 kDa) is also expressed in larvae (lane 2) and in pupae (lane 3). Detection of actin (45 kDa) serves as a control (arrowhead). Scale bars: 10 μm.

with a growing myotube (Fig. 3C, arrowheads). These observations were confirmed by visualisation of *Drosophila* Swip-1 foci in the *rP298-LacZ* enhancer trap line (Nose et al., 1992), where nuclei of FCs or growing myotube were labelled (Fig. 3Da–c, optical sections following one FCM (arrow) with respect to the growing myotube). These analyses revealed that *Drosophila* Swip-1 is not expressed in FCs and growing muscles, but is restricted to FCMs. In FCMs without contact to a FC or growing myotube *Drosophila* Swip-1 was distributed throughout the cytoplasm at a low level. Because *Drosophila* Swip-1 was

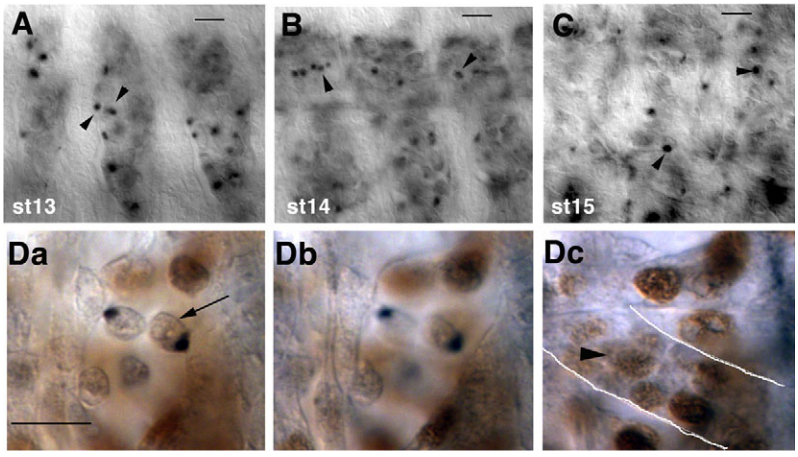

Fig. 3. *Drosophila* Swip-1 shows FCM-specific expression during fusion-relevant stages in the somatic mesoderm. (**A**–**C**) Anti-*Drosophila* Swip-1 (DSwip-1) staining on wild-type embryos (lateral view) shows expression at FCMs in the somatic mesoderm during fusion (arrowheads). (**D**) Inner (a), middle (b) and outer (c) optical sections to visualise the arrangement of an FCM (arrow) with respect to the growing muscle to which this particular FCM is attached. The nuclei of the muscles are marked with anti-β-galactosidase (*rP298-LacZ* strain). Scale bar: 10 μm.

barely detectable in the growing myotube, it could be degraded after successful fusion or transported towards the attachment sites.

Loss of *Drosophila* Swip-1 does not interfere with myogenesis

To better determine the exact biological function of *Drosophila Swip-1*, we first analysed P-element insertions upstream of the translation initiation codon of the *Drosophila Swip-1* gene or localised in the intron. All of these P-element line insertions yielded homozygous viable flies that expressed *Drosophila* Swip-1 at a level indistinguishable from that in the wild type and exhibited normal myoblast fusion (see supplementary material Table S1).

We also mobilised the P-element P(SUPor-P)CG10641^{KG08194} and screened 1000 jump-out lines for *Drosophila* Swip-1 expression using PCR. No line was found to have lost *Drosophila* Swip-1 expression during myoblast fusion (data not shown). Once again, no evidence was found that *Drosophila* Swip-1 expression was affected during myoblast fusion.

Therefore, we next tested several *Drosophila Swip-1*-deficient fly lines and deficient fly lines in trans (supplementary material Fig. S3 and Table S2). Note that these deficient fly lines also delete *mind bomb2* (*mib2*) (Carrasco-Rando and Ruiz-Gómez, 2008; Nguyen et al., 2007). The absence of *Drosophila Swip-1* did not enhance the *mib2* phenotype and therefore, we conclude that a loss of *Drosophila Swip-1* causes no obvious defects during myoblast fusion.

In addition, we performed RNAi experiments to reduce the level of *Drosophila Swip-1* specifically in the mesoderm taking RNAi for *blow* as a control (Fig. 4). However, although RNAi nearly completely knocked down *Drosophila* Swip-1 expression in the mesoderm, expression was maintained in the nervous system (Fig. 4A) and the musculature develops similarly to the wild type (Fig. 4B). Visualisation of *Drosophila* Mef2 in all mesodermal nuclei revealed that the number and localisation of the nuclei in these muscles was similar to that in wild-type embryos. For example, the VA2 muscle contained 7 nuclei, whereas in the wild type, this was between 6 and 13 (Beckett and Baylies, 2007) (Fig. 4E,F). We therefore conclude that the absence of *Drosophila* Swip-1 expression does not significantly affect myoblast fusion.

Thus, we asked whether other proteins could compensate for a loss of *Drosophila* Swip-1. There are 70 EF-hand-domain-containing proteins predicted to be encoded by the *Drosophila* genome, of which 63 are proposed to be cytosolic (Chintapalli et al., 2007). We have so far analysed CG2256, Calmodulin, CG31650 and Troponin C (1–4; next paragraph), which contain a similar domain structure to *Drosophila* Swip-1 (supplementary material Table S3).

(1) FlyBase (Chintapalli et al., 2007) predicts a single gene (*CG2256*) on the X-chromosome that encodes a small protein similar to *Drosophila* Swip-1 with two EF-hand domains and one coiled-coil domain. We found no *CG2256* transcripts in the embryo (data not shown). Thus, it is unlikely that CG2256 acts in redundancy to *Drosophila* Swip-1. (2) Calmodulin is the best-characterised cytosolic and highly conserved EF-hand-domain-containing and calcium-binding protein. The *Drosophila* genome contains a single calmodulin gene (*cam*) (Yamanaka et al., 1987), which we analysed. (3) *CG31650* encodes a protein with five EF-hand domains but no coiled-coil domain. The P-element insertion

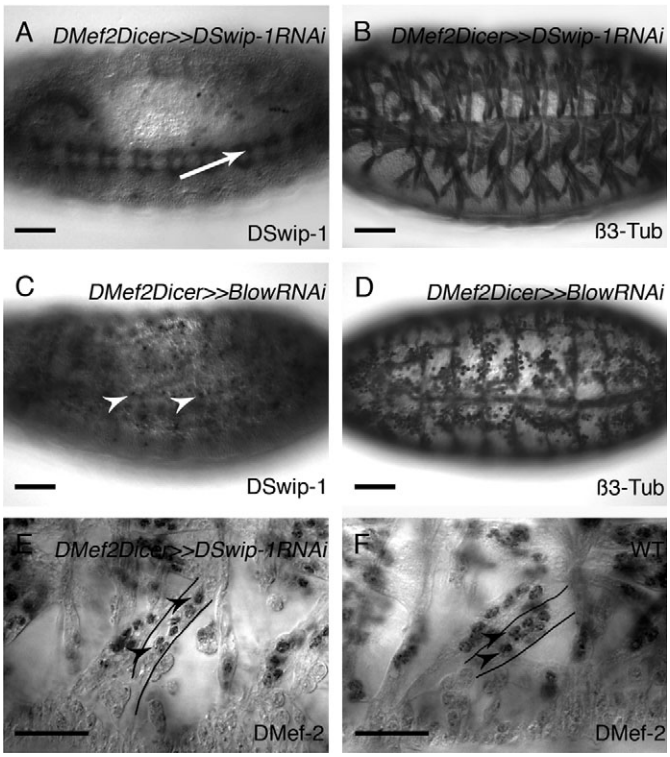

Fig. 4. *Drosophila Swip-1* knockdown does not disturb myoblast fusion. To reduce the level of *Drosophila* Swip-1 (DSwip-1) expression in the mesoderm, RNAi experiments were performed with (**A**,**C**) anti-DSwip-1, (**B**,**D**) anti-β3-tubulin and (**E**,**F**) anti-DMef2. (A) Expression of *Drosophila* Swip-1 was knocked down specifically in the mesoderm but maintained in the nervous system (arrow). (B) However, myogenesis in these embryos appeared typical of the wild type. (C,D) As a positive control, comparable experiments with a *blow* RNAi fly strain were performed. These embryos reveal the specific myoblast fusion phenotype (D) and *Drosophila* Swip-1 expression in the mesoderm (C, arrowheads). (E,F) The number and the positioning of the nuclei in the VA2 muscle of *Drosophila* Swip-1 knockdown embryos resemble that of a wild-type embryo (arrowheads). Scale bars: 100 μm (A–C), 20 μm (E,F).

line *P(SUPor-P)CG31650*^{KG09054} has an integration upstream of the predicted ATG of *CG31650* (Chintapalli et al., 2007). (4) Troponin C is a calcium-binding protein that acts in concert with Tropomyosin in muscle contraction. TroponinC73F (TpnC73F) (Fyrberg et al., 1994) is expressed in the mesoderm at fusion-relevant stages (Herranz et al., 2004); a finding that is corroborated by our in situ hybridisation data (supplementary material Table S3). We examined double mutants and visualised the musculature by observing β3-tubulin distribution. All corresponding mutants showed only the *mib2* phenotype caused by *Df(2L)Exel6042* and thus are concluded not to be potential redundancy partners to *Drosophila* Swip-1 (supplementary material Table S4). Thus, it remains to be clarified whether there are other proteins that can replace or compensate for the function of *Drosophila* Swip-1.

In *Drosophila* SL2 cells, the coiled-coil domain of *Drosophila* Swip-1 is required for localisation to the plasma membrane

We next investigated the role of the *Drosophila* Swip-1 domains for subcellular localisation. For this purpose, we first analysed

the distribution of *Drosophila* Swip-1 in *Drosophila* Schneider cells (Schneider, 1972). We used the subclone SL2 because SL2 cells have many characteristics of hemocytes (Armknecht et al., 2005) and *Drosophila* Swip-1 is known to be expressed in hemocytes, macrophage-like, cells in the embryo. We found that *Drosophila* Swip-1 was endogenously expressed in SL2 cells close to the plasma membrane and in dots close to the nucleus. These Swip-1-positve dots did not overlap with the Golgi marker syntaxin (Fig. 5C).

We next deleted the EF-hand domain and the proline-rich region (DSwip-1ΔEFPX10xMyc), the coiled-coil domain (DSwip-1ΔCC10xMyc) or the N-terminal proline-rich region only (DSwip-1ΔPX10xMyc) (for constructs see Fig. 5A and Table 1) and then tested protein extracts of SL2 cells transfected with these *Drosophila* Swip-1 variants under the control of the UAS/GAL4 system (Brand and Perrimon, 1993) on western blots with anti-Myc antibody. Constructs were expressed at comparable levels and were of the expected size (Fig. 5B). Additionally, we probed these western blots with the antibody against *Drosophila* Swip-1. As expected, we detected the full-length protein and DSwip-1ΔEFPX10xMyc, but not DSwip-1ΔCC10xMyc, which lacks the antigen site used for immunisation of rabbits (supplementary material Fig. S2). Thus, these findings confirm the specificity of our anti-*Drosophila* Swip-1 antibody.

To gain more insight into the role of the individual *Drosophila* Swip-1 protein domains in regard to the subcellular localisation of DSwip-1, we analysed the subcellular distribution of the protein variants in the transfected SL2 cells (Table 1). Interestingly, our immunofluorescence studies revealed that although the full-length DSwip-1-fl10xMyc protein localised to the plasma membrane (Fig. 5D), deletion of the coiled-coil domain (DSwip-1ΔCC10xMyc) led to localisation solely in the cytoplasm (Fig. 5E). In double-labelling studies with the Rab5 antibody (marker for early endosomes), we excluded the possibility that *Drosophila* Swip-1 colocalises with these endosomes. Despite the loss of the PX and EF-hand domains, the truncated DSwip-1ΔEFPX10xMyc protein localised to the plasma membrane (Fig. 5F). In addition, deletion of only the proline-rich (PX) region did not change the subcellular localisation (data not shown). We therefore conclude that the coiled-coil domain is responsible for localisation to the plasma membrane, at least in SL2 cells.

During myoblast fusion the EF-hand and the coiled-coil domains are required to recruit *Drosophila* Swip-1 to foci in FCMs

We established transgenic *Drosophila* lines with DSwip-1-fl10xMyc, DSwip-1ΔCC10xMyc, DSwip-1ΔEFPX10xMyc and DSwip-1ΔPX10xMyc with a modified Gateway-phiC31 system using a landing site at the third chromosome (Bischof et al., 2007). Driving expression with *wg-GAL4* in the *wingless* domains of the epidermis showed that the full-length, as well as the truncated proteins, were synthesised in the embryo (Table 1).

We next asked whether the full-length protein and the truncated versions localised in FCMs similarly to the endogenous protein. To answer this question, we drove expression ofDSwip-1-fl10xMyc, DSwip-1ΔCC10xMyc, DSwip-1ΔEFPX10xMyc and DSwip-1ΔPX10xMyc selectively in FCMs with the *sns4,5-GAL4* driver line (Stute et al., 2006) and analysed these embryos with respect to subcellular localisation during myoblast fusion. The FCM-specific expression through the *sns4,5-GAL4* driver line was analysed in parallel with standardised conditions. The DSwip-1-fl10xMyc protein localised to characteristic foci on the side of the FCMs when

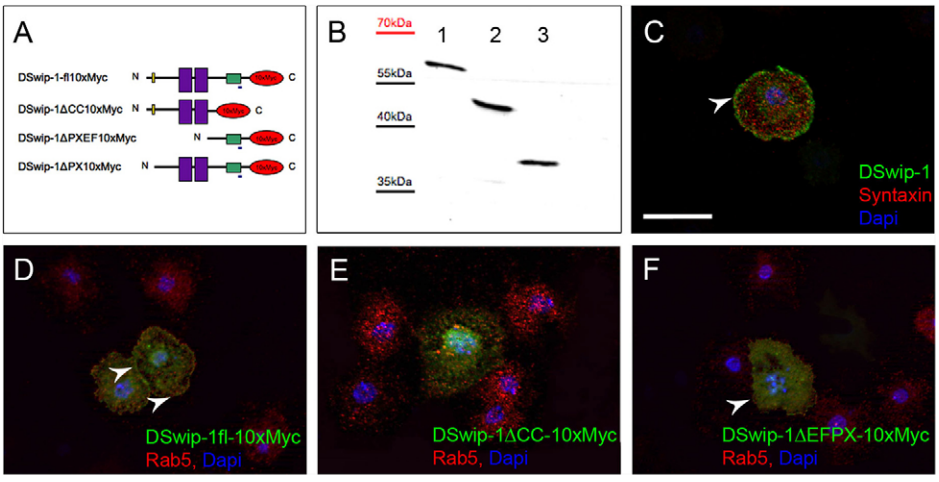

Fig. 5. The coiled-coil domain is essential for localisation of *Drosophila* Swip-1 to the plasma membrane in SL2 cells. (**A**) Deletion constructs of *Drosophila* Swip-1 (DSwip-1) with 10xMyc-tag under the control of the UAS/GAL4 system. A *Drosophila Swip-1* full-length construct serves as a control. The EF-hand domain, the coiled-coil domain and the proline-rich region are shown in purple, green and yellow, respectively, and the 10xMyc-tag is shown in red. The blue bar marks the sequence that is detected by the antibody against *Drosophila* Swip-1. *DSwipDEFPX10xMyc* lacks the EF-hand domain and the predicted proline-rich region, *DSwipDCC10xMyc* the coiled-coil domain and *DSwipDPX10xMyc* the predicted proline-rich region only. (**B**) Protein expression after transfection of SL2 cells. In western blot analyses, 10xMyc-constructs were detected using a Myc antibody. Lane 1: DSwip-1-fl10xMyc; lane 2: DSwipDCC10xMyc; lane 3: DSwipDEFPX10xMyc. (**C**) In SL2 cells, endogenous *Drosophila* Swip-1 localises to the plasma membrane (arrowhead) and in cytoplasmic dots in the periphery of the nucleus, which do not overlap with the Golgi marker Syntaxin. (**D**–**F**) Expression of *Drosophila* Swip-1 versions after transfection into SL2 cells was monitored by anti-Myc staining, early endosomes by Rab5 staining and nuclei were visualised by DAPI. DSwip-1-fl10xMyc (D) and DSwip-1DEFPX10xMyc (F) localise to the plasma membrane (arrowheads), whereas DSwip-1DCC10xMyc (E) is distributed in the cytoplasm. *Drosophila* Swip-1 does not colocalise with Rab5. Scale bar: 20 μm.

Table 1. Analysis of *Drosophila* Swip-1 constructs in SL2 cells and embryos

	Detection by western blot	Localisation in SL2 cells	Detection in embryos	Driving expression in the mesoderm	Localisation in FCMs
DSwip-1-fl10xMyc	+	Cell membrane	+	No muscle phenotype	Characteristic foci
DSwip-1ΔEFPX10xMyc	+	Cell membrane	+	No muscle phenotype	Cytoplasm
DSwip-1ΔCC10xMyc	+	Cytoplasm	+	No muscle phenotype	Cytoplasm
DSwip-1ΔPX10xMyc	+	Cell membrane	+	No muscle phenotype	Characteristic foci

they attached to the FC/growing myotubes (Fig. 6A, arrowheads), whereas DSwip-1-fl10xMyc was not detectable in FCMs without contact to a growing myotube. This reflects the subcellular protein expression pattern known for the endogenous *Drosophila* Swip-1 protein (Fig. 3A–C) and is evidence that the 10xMyc tag does not disturb subcellular localisation. However, the truncated proteins lacking the coiled-coil (DSwip-1ΔCC10xMyc; Fig. 6B) or proline-rich region and EF-hand domain (DSwip-1ΔEFPX10xMyc; Fig. 6C) were expressed in the cytoplasm, but do not concentrate into foci, whereas DSwip-1ΔPX10xMyc was localised in the characteristic foci (supplementary material Fig. S4).

We next addressed the question whether the expression of the full-length or truncated versions of *Drosophila* Swip-1 disturbs myoblast fusion. To answer this question, we drove the *Drosophila* Swip-1 variants with *DMef-GAL4* and *twist* driver lines. The distribution of β3-tubulin revealed that DSwip-1-fl10xMyc, DSwip-1ΔCC10xMyc, DSwip-1ΔEFPX10xMyc and DSwip-1ΔPX10xMyc do not interfere with muscle development (Table 1). We therefore conclude that the truncated versions of *Drosophila* Swip-1 variants do not act as a dominant negative. In the cases of DSwip-1ΔEFPX10xMyc and DSwip-1ΔCC10xMyc, this is presumably due to failure to localise to the plasma membrane.

Drosophila Swip-1 foci are in close proximity to transient F-actin foci

The *Drosophila* Swip-1 foci strongly resemble the known F-actin foci in the FuRMASs (Kesper et al., 2007). Live imaging has shown that these F-actin foci are a transient feature during myoblast fusion (Richardson et al., 2008a; Richardson et al., 2007). We addressed the question whether *Drosophila* Swip-1 foci overlap with F-actin foci. Therefore, we combined the visualisation of F-actin by the Phalloidin-TRITC marker with *Drosophila* Swip-1 distribution and found that *Drosophila* Swip-1 foci and F-actin foci were at different positions from segment to segment (supplementary material Fig. S5). Moreover, we observed that the *Drosophila* Swip-1 foci sometimes overlapped with the F-actin foci and sometimes did not (Fig. 7A–C). We therefore conclude that both molecules are localised in close proximity in the tip of the filopodia in FCMs as far as the resolution of fluorescence microscopy allows (Fig. 7), and that there might be a functional connection between *Drosophila* Swip-1 and F-actin (see the Discussion).

Drosophila Swip-1 recruitment to the FuRMASs on the site of the FCMs depends on successful cell adhesion

We analysed whether successful cell adhesion due to the chemoattracting function of Duf/Kirre and Rst/IrreC and their interaction with Sns is essential for *Drosophila* Swip-1 foci formation (Galetta et al., 2004; Ruiz-Gomez et al., 2000; Strünkelnberg et al., 2001). For this purpose, we analysed embryos homozygous for *Df(1)w67k30/z(1)v(1)*, which do not contain both Duf/Kirre and Rst/IrreC. Here, *Drosophila* Swip-1 was expressed only in the cytoplasm of FCMs; however, in the absence of cell adhesion, no characteristic *Drosophila* Swip-1 foci were formed (data not shown).

Because Sns is the most relevant cell adhesion molecule in FCMs, we analysed the localisation of *Drosophila* Swip-1 in $sns^{20\text{-}2}$ (formerly $rost^{20\text{-}2}$) (Paululat et al., 1995) loss-of-function mutants. Without Sns, *Drosophila* Swip-1 was expressed (visible as diffuse weak staining), but it rarely accumulated in the characteristic foci (Fig. 8B) typical of the wild type (Fig. 8A). Hibris is known to be able to replace Sns, and both proteins act in functional redundancy to some degree (Shelton et al., 2009). Thus, we suspect that this functional redundancy is responsible for the appearance of the few observed *Drosophila* Swip-1 foci. This would be in agreement with the rare fusion events observed in *sns* mutants. Taken together, we conclude that *Drosophila* Swip-1 accumulation in foci depends on successful cell adhesion.

We next performed anti-*Drosophila* Swip-1 staining on $sns^{20\text{-}5}$ mutant embryos (Paululat et al., 1995). $sns^{20\text{-}5}$ encodes a truncated protein of 1163 amino acids that contains the extracellular domain and the transmembrane domain but lacks

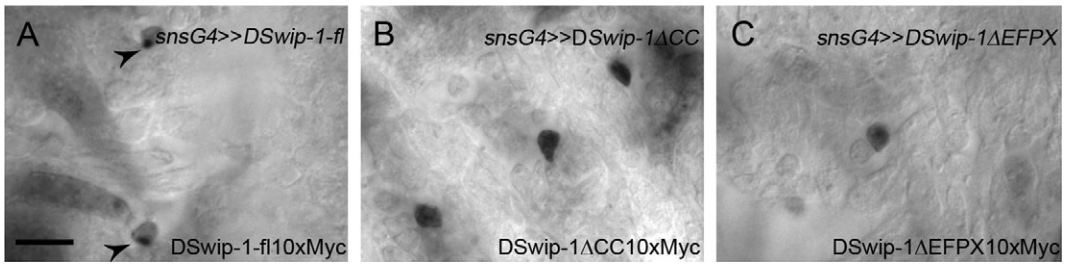

Fig. 6. The EF-hand and the coiled-coil domain are essential for accumulation in foci in FCMs during myoblast fusion. (**A**–**C**) Versions of *Drosophila* Swip-1 (DSwip-1) are ectopically expressed in FCMs in transgenic embryos by the UAS/GAL4 system. (A) In transgenic embryos stained with a Myc antibody, DSwip-1-fl10xMyc localises at the characteristic foci in FCMs (arrowheads). (B,C) The truncated versions DSwip-1DCC10xMyc and DSwip-1DEFPX10xMyc are localised in the cytoplasm of FCMs. Scale bar: 20 μm.

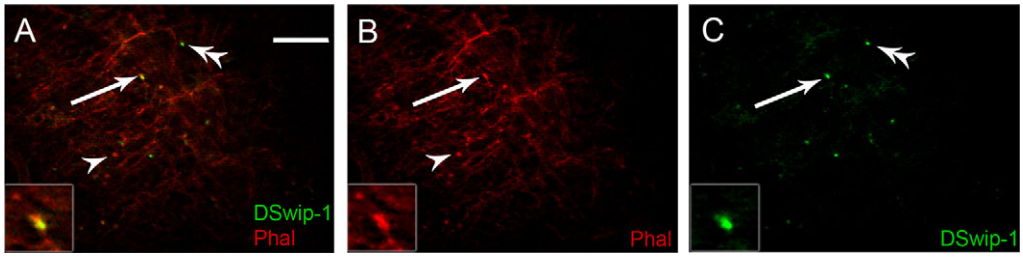

Fig. 7. *Drosophila* Swip-1 foci are often in close proximity to F-actin foci in the somatic mesoderm. (**A**–**C**) Staining with the *Drosophila* Swip-1 antibody (green, C) and Phalloidin-TRITC marker (red, B) show that *Drosophila* Swip-1 (DSwip-1) is often localised in close proximity to F-actin in the tip of the filopodia of the FCMs during fusion-relevant stages (arrows). In addition, some *Drosophila* Swip-1 foci (double arrowheads) do not overlap with the F-actin foci and some F-actin foci (arrowhead) do not overlap with *Drosophila* Swip-1 foci. Scale bar: 20 µm.

the majority of the intracellular domain (Bour et al., 2000). In $sns^{20\text{-}5}$ mutants, *Drosophila* Swip-1 did not accumulate into characteristic foci (Fig. 8C). Thus, we conclude that the accumulation of *Drosophila* Swip-1 in foci depends on Sns-mediated cell adhesion and probably signalling.

Drosophila Swip-1 foci accumulate in *singles bar* mutants, but not in *blow* and *kette* mutant embryos

To obtain further insight into the role of *Drosophila* Swip-1 during myoblast fusion, we analysed *Drosophila* Swip-1 distribution in fusion mutants. We cannot present quantification of the *Drosophila* Swip-1 foci accumulation in wild-type and mutants because this would require knowing the situation in the wild type at a given time and in an individual segment. Moreover, *Drosophila* Swip-1 (Fig. 9A,C,F) and F-actin (supplementary material Fig. S5) foci vary highly in number and position during fusion. This is in contrast to muscle identity conferring factors such as nuclear markers that allow fusion efficiency to be monitored (Richardson et al., 2007). We therefore analysed the distribution of *Drosophila* Swip-1 in a number of fusion mutants with the aim of correlating its expression pattern to the known status of myoblast fusion at the ultrastructural level (for mutant fly strains used see Table 2). In this respect, it is of prime interest to analyse mutants in which cell adhesion was successful, but where the opposing membranes remain intact. This is the case for *singles bar* mutants ($sing^{22}$) where prefusion complexes accumulate as well as for *blown fuse* ($blow^{2}$) and *kette* ($kette^{J4\text{-}48}$) mutants where the prefusion complex is dissolved but plasma membranes remain intact (Doberstein et al., 1997; Estrada et al., 2007). Of further interest are mutants that stop myoblast fusion when the membranes between FCMs and the FC or growing myotube vesiculate. One example of this is wip^{30D}, which lacks the WASP-interacting protein (Massarwa et al., 2007). In our study, we analysed *arp3* ($arp3^{schwächling}$) (Berger et al., 2008) mutant embryos, which stop myogenesis after membrane vesiculation, removal of the membrane remnants and the formation of cytoplasmic continuity (Önel and Renkawitz-Pohl, 2009; Önel et al., 2011; Önel and Renkawitz-Pohl, 2009). Furthermore, the expression of *Drosophila* Swip-1 in both $sing^{22}$ and $blow^{2}$ mutant embryos appears to be different compared with that in the wild type, whereas in the remaining mutants tested, *Drosophila* Swip-1 expression resembled that in the wild type (Fig. 9). For example, in contrast to wild-type embryos (Fig. 9A,C,F), in $sing^{22}$ mutants, many FCMs contacted an individual FC or growing myotube at the same time (Fig. 9B,D,G), and all these FCMs contained *Drosophila* Swip-1 foci (Fig. 9D,G). In $blow^{2}$ mutant embryos, fewer *Drosophila* Swip-1 foci accumulated compared with $sing^{22}$ mutant embryos, and they appeared smaller and more diffuse (Fig. 9E,H). In $kette^{J4\text{-}48}$ mutant embryos, *Drosophila* Swip-1 distribution resembled that in the wild type (Fig. 9I). Moreover, wip^{30D} and $arp3^{schwächling}$ both lacked a protein that regulates F-actin branching, but also had a *Drosophila* Swip-1 distribution that resembled that in the wild type (Fig. 9J,K). Taken together, these data clearly show that *Drosophila* Swip-1 only accumulated significantly in $sing^{22}$ mutants, and therefore, we hypothesise that *Drosophila* Swip-1 participates in regulating vesicle exocytosis from the prefusion complex.

Discussion

Drosophila Swip-1 is the *Drosophila* homologue of human and murine swiprosins

The CG10641-encoded protein *Drosophila* Swip-1 is homologous to mammalian swiprosins, EFHD1 and EFHD2. *Drosophila* Swip-1 and mammalian EFHD1 and EFHD2 share two EF-hand domains and one coiled-coil domain. The mouse proteins have been described to localise to lipid rafts in B-cells and to be involved in different processes such as swiprosin-dependent calcium influx and efflux in B-cells of the adaptive immune system (for a review, see Dütting et al., 2011). Additionally, cytokine secretion in human mast cells is thought to be guided by swip-1/EFHD2 (Ramesh et al., 2009). Hemocytes, macrophage-like cells of the invertebrate innate immune system, are known to transcribe CG10641 (Estrada et al.,

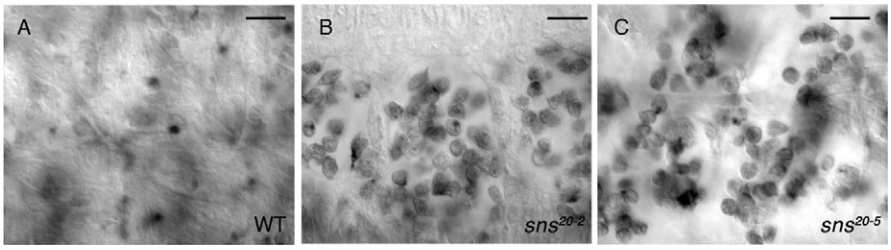

Fig. 8. *Drosophila* Swip-1 rarely forms foci in FCMs of *sns* mutants. (**A**) In wild-type embryos, *Drosophila* Swip-1 (DSwip-1) protein is localised in characteristic foci at the FCMs in the somatic mesoderm. (**B**) In $sns^{20\text{-}2}$ loss-of-function mutants lacking the transmembrane domain, *Drosophila* Swip-1 is localised in the cytoplasm of the FCM and rarely concentrated. (**C**) $sns^{20\text{-}5}$ mutants lacking the intracellular domain also show rare *Drosophila* Swip-1 foci in FCMs. Scale bars: 10 µm.

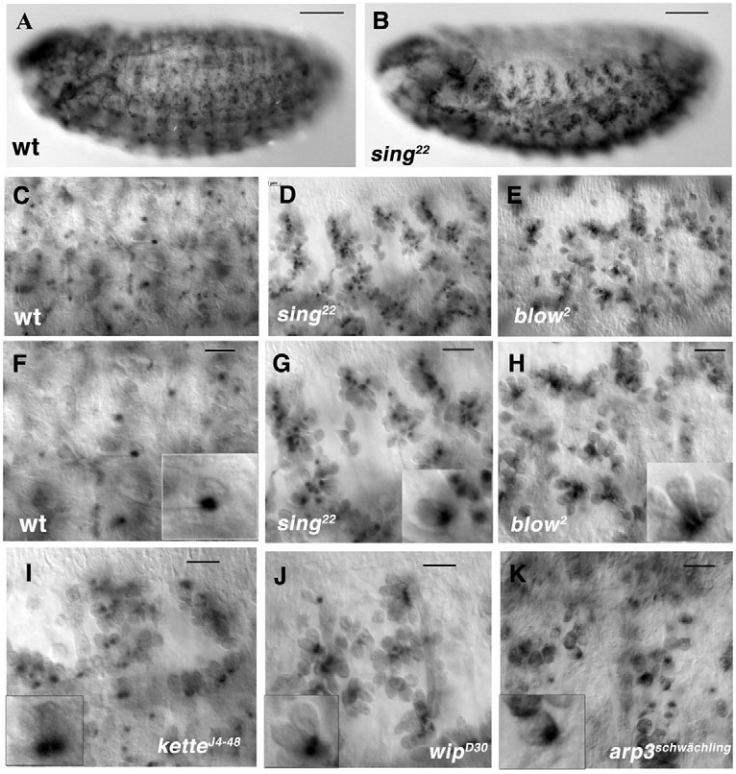

Fig. 9. *Drosophila* Swip-1 foci accumulate in *sing*^{22}. Analysis of *Drosophila* Swip-1 (DSwip-1) distribution in fusion mutants. *Drosophila* Swip-1 distribution is shown in (**A**,**B**) whole embryos, (**C**–**E**) an overview over some segments, (**F**–**K**) higher magnifications and (**G**–**K**) as the order of arrest of myoblast fusion in the respective mutants. (A) *Drosophila* Swip-1 in wild-type embryos localises transiently in characteristic foci in FCMs. (B) By contrast, *sing*^{22} mutant embryos show accumulation of *Drosophila* Swip-1 foci. (C–E) An overview over some segments shows that *Drosophila* Swip-1 distribution in wild-type embryos appears more transient than in *sing*^{22} and *blow*^{2} mutant embryos. (F) *Drosophila* Swip-1 expression in a wild-type embryo reveals specific transient foci. (G) By contrast, *sing*^{22} mutant embryos exhibit an accumulation of foci. (H) *blow*^{2} mutants do not accumulate *Drosophila* Swip-1 foci. Here, the foci appear slightly smaller and more diffuse. (I) *kette*$^{J4\text{-}48}$ embryos show a wild-type *Drosophila* Swip-1 foci distribution. (J,K) *wip*^{D30} and *arp3*^{Schwächling} also exhibit no change in *Drosophila* Swip-1 foci distribution. Scale bars: 100 μm (A,B), 10 μm (F–K).

2006; Kroczek et al., 2010) and our study found a polar distribution of *Drosophila* Swip-1 in the macrophage-like hemocytes of the embryo. Taken together, these findings suggest a conserved function of swiprosin in the innate immune system of invertebrates and vertebrates.

In *Drosophila* SL2 cells, the coiled-coil domain of *Drosophila* Swip-1 is required for localisation to the plasma membrane whereas in embryos, the EF-hand domains are also necessary

Drosophila Swip-1 is transcribed in SL2 cells (Henikoff et al., 2009) – cells that have a hemocyte-like character (Armknecht et al., 2005). In this study, we show that *Drosophila* Swip-1 is associated with the plasma membrane in SL2 cells and that this localisation depends on the coiled-coil domain. We conclude that the behaviour of *Drosophila* Swip-1 in SL2 cells, where only loss of the coiled-coil domain leads to failure to localise to the plasma membrane, is different from the situation in the embryo, where the EF-hand domains are also essential for *Drosophila* Swip-1 localisation in FCMs. These findings strongly indicate that *Drosophila* Swip-1 is under a different control mechanism for membrane localisation in hemocytes compared with FCMs.

Myoblast fusion is not affected in *Drosophila* Swip-1 mutants, implying functional redundancy

Surprisingly, myoblast fusion takes place in *Drosophila* Swip-1 mutants implying functional redundancy with – most likely – another calcium-binding protein. Interestingly, functional redundancy is known for proteins involved in vesicle exocytosis in the nervous system of mice. Here, synaptophysin, a major synaptic vesicle protein, contains a MARVEL domain and is proposed to have a role in SNARE assembly into the fusion core complex, fusion pore formation and neurotransmitter release (Hübner et al., 2002). Syp is also known to be essential for Ca^{2+}-induced neurotransmitter release in vitro. However, single-gene knockout of *Syp* in mice revealed no obvious phenotype (McMahon et al., 1996; McMahon and Monroe, 1996), despite the many proposed roles for this protein (Valtorta et al., 2004). This finding is a strong indicator that other proteins are compensating for the role of synaptophysin – a scenario that we also propose is the case for *Drosophila* Swip-1 during myoblast fusion.

Examples of functional redundancy for myogenesis in *Drosophila* are already known. For example, the Ig domain cell adhesion molecules Duf/Kirre and Rst/IrreC (Strünkelnberg et al., 2001), as well as the small GTPases Rac1 and Rac2 (Hakeda-Suzuki et al., 2002; Ng et al., 2002) can completely replace each other. The maternal contribution of the actin regulator WASP can completely rescue a zygotic loss-of-function mutant (Schäfer et al., 2007). Moreover, there is also partial functional redundancy between Sns and Hibris (Hakeda-Suzuki et al., 2002; Ng et al., 2002; Shelton et al., 2009). Because over 60 cytosolic calcium-binding proteins have been predicted for *Drosophila* (Chintapalli et al., 2007), it is a major challenge to identify the exact protein(s) that can act in functional redundancy to *Drosophila* Swip-1.

Drosophila Swip-1 accumulates in foci in FCMs during myoblast fusion independently of actin regulators Kette, Wip and Arp3

The aim of this study was to better determine the function of *Drosophila* Swip-1 during myoblast fusion. We found that *Drosophila* Swip-1 accumulates transiently in the FuRMASs on the site of the FCM and that this accumulation is dependent on

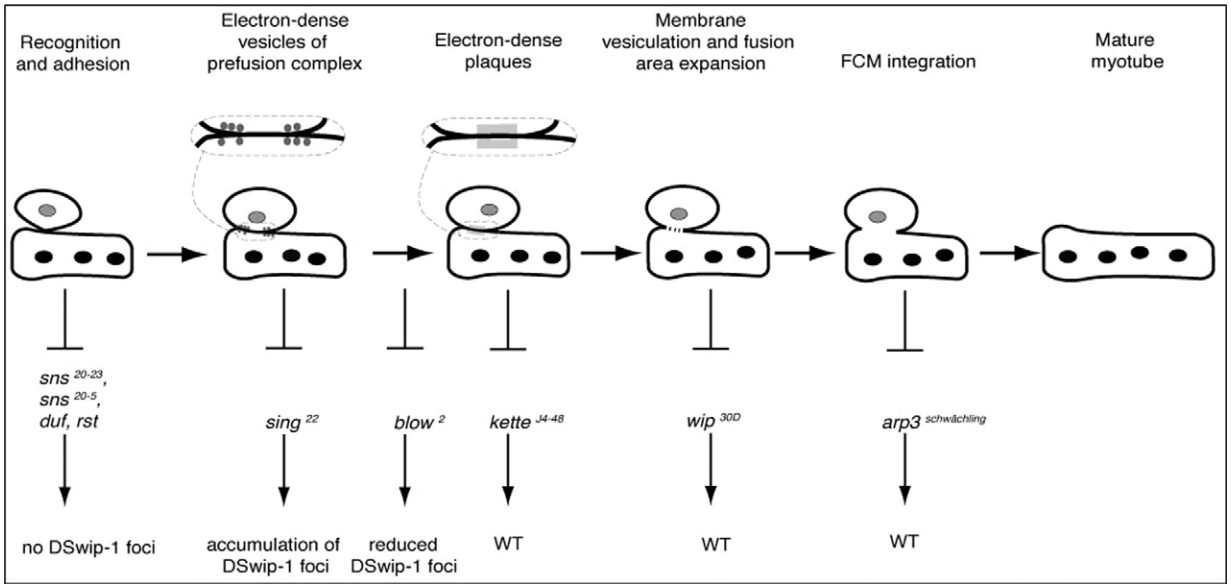

Fig. 10. *Drosophila* Swip-1 might participate as a regulator during exocytosis of electron-dense vesicles of the prefusion complex. The ultrastructural features of myoblast fusion are schematically drawn in the order of progression of fusion. Arrest of fusion mutants with respect to ultrastructure is indicated [modified after published results (Önel et al., 2011)]. The behaviour of *Drosophila* Swip-1 (DSwip-1) foci in individual fusion mutants is indicated. WT indicates wild-type *Drosophila* Swip-1 foci distribution. In *sns* and *duf/rst* mutants, no *Drosophila* Swip-1 foci are formed. *sing*^{22} mutants show accumulating *Drosophila* Swip-1 foci. Slightly smaller and more diffuse *Drosophila* Swip-1 foci appear in *blow*^{2} mutant embryos, whereas in the remaining fusion mutants, *Drosophila* Swip-1 foci resemble the wild type. Therefore, we hypothesise that calcium-binding proteins such as *Drosophila* Swip-1 are regulators participating in exocytosis of vesicles of the prefusion complex.

the transmembrane proteins Sns, Duf/Kirre and Rst/IrreC. Thus, we propose that Sns-mediated cell adhesion and probably signalling triggers the accumulation of *Drosophila* Swip-1 into the foci in the FCM.

During myoblast fusion, F-actin accumulates transiently in foci within the FuRMASs (for a review, see Önel et al., 2011). Similarly, *Drosophila* Swip-1 also appears to accumulate transiently in foci; however, these foci are found exclusively in FCMs. Interestingly, the FCM-specific protein Blow, a putative regulator of F-actin and Kette (Kesper et al., 2007; Richardson et al., 2007; Schröter et al., 2006) also accumulates in a similar manner to Swip-1. In human mast cells, EFHD2/swiprosin-1 was shown to colocalise to the actin-cytoskeleton (Kroczek et al., 2010; Ramesh et al., 2009). This raises the question of whether *Drosophila* Swip-1 is a regulator of F-actin on the side of the FCMs. Therefore, we analysed further suitable mutants. Mutations in genes that encode actin regulators such as Kette, Wip and Arp3 do not affect *Drosophila* Swip-1 localisation. By contrast, F-actin foci are not dissolved in *kette*$^{4-48}$ and *arp3* mutants (Richardson et al., 2007) and in the *sltr* allele of *wip*, an abnormally high expression of actin was observed (Kim et al., 2007). Thus, we propose that *Drosophila* Swip-1 is not directly involved in F-actin regulation in connection with Kette, Wip and Arp3, but rather acts in a parallel process such as regulation of vesicle exocytosis (see below).

Drosophila Swip-1 might act during exocytosis of electron-dense vesicles

Regulated exocytosis is characteristic of neurotransmitter-carrying vesicles (Schweizer and Ryan, 2006; Wojcik and Brose, 2007), secretion by cells of the adaptive immune system (Benado et al., 2009) and exocytosis of acrosomal vesicles during fertilisation (Ackermann et al., 2009). Regulated exocytosis has also been proposed to take place during myoblast fusion in *Drosophila* (Doberstein et al., 1997; Estrada et al., 2007). Moreover, Vega and colleagues (Vega et al., 2008) showed that

Table 2. Fusion mutants used to determine the function of *Drosophila* Swip-1

	Structure of encoded protein (EMBL database)	Stop during myoblast fusion	Ref.
sns$^{20-2}$	Ig transmembrane cellular receptor	Stop after recognition and adhesion between FCM and FC/growing myotube	Bour et al., 2000; Paululat et al., 1995
sns$^{20-5}$	Ig transmembrane cellular receptor	Stop after recognition and adhesion between FCM and FC/growing myotube	Bour et al., 2000; Paululat et al., 1995
sing^{22}	Multipass transmembrane protein with a Marvel domain	Stop during prefusion complex formation	Estrada et al., 2007
blow^{2}	Cytoplasmic protein with PH domain	Stop during prefusion complex formation	Doberstein et al., 1997
kette^{J4-48}	Cytoplasmic protein, component of Scar complex	Stop during electron-dense plaque formation	Schröter et al., 2004
wip^{30D}	Cytoplasmic protein with WH2 domain for interaction with WASp	Stop during vesiculation of membranes of FCM and growing myotube	Massarwa et al., 2007
arp3^{Schwächling}	Cytoplasmic protein, subunit of Arp2/3 complex	Stop after membrane vesiculation, after a small fusion pore is made	Berger et al., 2008

the highly conserved mammalian swiprosin-1/EFHD2 exhibits calcium-binding activity. Because swiprosins are known to be calcium-binding proteins, and because calcium has often been described to trigger exocytosis (Barclay et al., 2005; Burgoyne and Morgan, 2003), we asked whether *Drosophila* Swip-1 might be a regulator of exocytosis of the vesicles of the prefusion complex and/or during plasma membrane fusion.

To address this question, we correlated *Drosophila* Swip-1 distribution in fusion mutants to the known arrest of myoblast fusion at the ultrastructural level (Fig. 10, Table 2). From ultrastructural analyses, it is known that after recognition and adhesion between FCM and the growing myotube, electron-dense vesicles accumulate at opposing membranes and participate in the formation of the prefusion complex (Fig. 10). After resolving this complex, electron-dense plaques are established. In the following steps, these plaques dissolve, the opposing membranes become vesiculated and the fusion pore expands to integrate the FCM into the growing myotube to build the mature muscle (Önel and Renkawitz-Pohl, 2009; Önel et al., 2011).

In this study, we have shown that *Drosophila* Swip-1 is transiently expressed during myoblast fusion and becomes concentrated in foci at FCMs – a process that is dependent on successful cell adhesion as well on Sns signalling. Interestingly, we also found that *Drosophila* Swip-1 accumulates in $sing^{22}$ mutants. $sing^{22}$ mutants are known to arrest fusion after establishing the prefusion complexes, and show no resolution of these complexes (Estrada et al., 2007). Therefore, we suggest that the calcium-binding protein *Drosophila* Swip-1 might directly or indirectly trigger exocytosis of these electron-dense vesicles (Fig. 10). We further propose cooperation between Sing and *Drosophila* Swip-1 in a regulatory cascade.

Estrada and colleagues (Estrada et al., 2007) hypothesised that Sing might mediate fusion of the electron-dense vesicles with the plasma membrane because Sing contains a MARVAL domain (Estrada et al., 2007). These domains are implicated in vesicle trafficking and cholesterol-rich membrane opposition events (Sánchez-Pulido et al., 2002). Our analyses of $blow^{2}$ and $kette^{J4\text{-}48}$ mutants has added substantial weight to this hypothesis, because: (1) the prefusion complexes are dissolved in these mutants; (2) the plasma membranes are not vesiculated; and (3) *Drosophila* Swip-1 foci are not accumulated above wild-type level (Fig. 10).

We also asked whether *Drosophila* Swip-1 might be needed in further fusion-relevant steps such as vesiculation of the plasma membranes. Our analyses of $blow^{2}$ and $kette^{J4\text{-}48}$ mutants revealed that failure of plasma membrane merging does not lead to an accumulation of *Drosophila* Swip-1 foci. Thus, we propose that *Drosophila* Swip-1 is specific for prefusion complex resolution (Fig. 10) and hypothesise that calcium-binding proteins such as *Drosophila* Swip-1 are regulators participating in exocytosis of vesicles of the prefusion complex.

Materials and Methods

Fly stocks and genetics

Flies were grown on standard medium at 25℃. Balancers and chromosome markers are as described in FlyBase (Tweedie et al., 2009) unless otherwise specified. Stocks were grown under standard conditions. w^{1118} (BL6326) were taken as a wild-type strain. If/CyOhglacZ strain (kindly provided by Markus Affolter, Basel, Switzerland) was used to rebalance the stocks with a blue balancer.

The following strains were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN); *Df(2)Exel6042*, *Df(2)*ED1202 and Df*(2L)Exel8039*. The following fusion mutants were used: $sns^{20\text{-}2}$/CyOhglacZ (Paululat et al., 1995), $sns^{20\text{-}5}$/CyOhglacZ (Paululat et al., 1995), $blow^{2}$/CyOhglacZ (Doberstein et al., 1997), $sing^{22}$/Fm7, twiGAL4-UAS-2EGFP (kindly provided by Alan Michelson, National Heart, Lung and Blood Institute, Bethesda, MD); $kette^{J4\text{-}48}$/Sb, TDlz (Hummel et al., 2000; Schröter et al., 2004), wip^{30D} (kindly provided by Eyal D. Schejter, Weizmann Institute, Rehovot, Israel) and $arp3^{Schwächling}$/SbTDlz (Berger et al., 2008).

We used meiotic recombination in females to generate flies that carry *Df(2L)Exel6042* and cam^{n339} or *Df(2L)Exel6042* and $CG31650^{KG09054}$ together on the second chromosome, respectively. For generating the double mutants *Df(2L)Exel6042*; $tpnC73F^{MB03946}$, we used the double blue balancer CyOhglacZ/Sp; Tm2/Sb, TDlz (laboratory of Renate Renkawitz-Pohl, Philipps-Universitaet Marburg, Marburg, Germany). Except for the double blue balancer strain, flies were obtained from the Bloomington *Drosophila* Stock Center. As mesoderm-specific driver lines, we used *DMef2-GAL4* (Bloomington Drosophila Stock Center), *twist-GAL4* (TG-X and SG24, kindly provided by Alan Michelson) and *sns4,5-GAL4* (Stute et al., 2006). To determine cell-type specificity, we used the enhancer trap line *rp298-lacZ* (Nose et al., 1992).

RNAi experiments

For generating fly stains, which carried a construct to silence *Drosophila Swip-1* by RNAi, we crossed different fly lines with the mesoderm-specific driver line *DMef2-GAL4* (BL25756, Bloomington Drosophila Stock Center, USA), which drives GAL4 expression specifically in the somatic mesoderm and expresses Dicer additionally. For *Drosophila* Swip-1-specific RNAi induction we used fly lines named v31307, v31308 and BL31585, which knock down *Drosophila Swip-1*. As a positive control we used v10177, a fly strain, in which *blow* is silenced (Dietzl et al., 2007). Crossings were done on 30℃, embryos were fixated and immunohistochemistry with antibodies against β3-tubulin and *Drosophila* Swip-1 was done as described below.

Immunostaining and Phalloidin labelling of *Drosophila* embryos

For immunohistochemistry, embryos were collected from juice agar plates, rinsed with TNX (0.7% NaCl and 0.01% Triton X-100) and dechorionised with 50% Klorix. After fixation for 15 minutes in 4% F-PBS (4% formaldehyde with PBS: 0.13 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4), embryos were devitellinised by shaking in 1:1 methanol and heptane. After rehydration in PBT (PBS, 0.5% Tween-20), embryos were incubated overnight at 4℃ with primary antibodies in PBT at the following concentrations: 1:4000 anti-DSwip-1 (raised in rabbits; Pineda Antibody Service, Berlin), 1:10,000 anti-β3-tubulin (rabbit) (Leiss et al., 1988), 1:1000 anti-Myc (mouse, Millipore, Billerica, MA), 1:25 CF.6G11 (βPS integrin, mouse, Hybridoma Bank, Iowa City, IA); 1:1000 anti-DMef2 (rabbit, kindly provided by Hanh T. Nguyen, University of Erlangen, Erlangen, Germany); and 1:5000 anti-β-galactosidase (mouse, Promega, Heidelberg). Embryos were then incubated for 1 hour in blocking solution [2% goat serum (Vector Laboratories, Burlingame, CA) in PBT] and afterwards with biotinylated secondary antibody (anti-rabbit 1:500; anti-mouse 1:500; anti-guinea pig 1:500; Vector Laboratories) respectively with Cy2- and Cy3-conjugated secondary antibodies (anti-rabbit, 1:200; anti-mouse, 1:200; anti-guinea pig, 1:200; Dianova, Hamburg) for 2 hours at room temperature (RT). After amplifying the reaction (not for fluorescent immunohistochemistry) using Vectastain Elite AMC Standard Kit (Vector Laboratories), staining was carried out with 10 µl diaminobenzidine, 5 µl 20% H_2O_2 and 1 µl 10% $NiCl_2$ in 600 µl PBT. Numerous embryos were mounted in Epon and examined with a Zeiss Axiophot (Zeiss, Germany). For fluorescence immunohistochemistry, embryos were incubated with Cy2- and Cy3-conjugated secondary antibodies, washed in PBT several times and mounted in Fluoromount G™ (Southern Biotech, Birmingham, AL) and examined under Zeiss Axioplan 2 imaging with ApoTom (Zeiss, Germany).

For double labelling with Phalloidin-TRITC, the embryos were treated as before until devitellinisation. Then, we devitellinised the embryos in PBT with a sharp glass needle. Embryos were incubated with the first antibody and Phalloidin–TRITC [anti-DSwip-1, 1:2000; anti-β-galactosidase, 1:2500; Phalloidin–TRITC, 1:60 (1 µg/µl; Sigma, Steinheim)] overnight at 4℃. The primary antibodies were detected using the corresponding Cy2- and Cy3-conjugated secondary antibodies (1:200, Dianova, Hamburg). Embryos were mounted in Fluoromount G.

In-situ hybridisation

Whole-mount in situ hybridisation was performed as described previously (Tautz and Pfeifle, 1989). DIG-labelled RNA antisense probes were synthesised by in vitro transcription using a RNA-DIG labelling kit (Roche Diagnostics, Mannheim). We used the SD04693 cDNA clone (CG10641 in pOT2; obtained from Drosophila Genomics Resource Center, Bloomington, IN); as a template for transcribing the RNA probe using SP6 polymerase (Roche Diagnostics, Mannheim).

Antibody generation

Antibody against *Drosophila* Swip-1 was generated by the Pineda Antibody Service (Berlin, Germany) against the peptide N-CEERAQPRQQFQQRAAIF-C in rabbits. The serum was affinity purified.

Constructs for cell culture experiments and generation of transgenic flies

Deletion constructs (DSwip-1ΔCC10xMyc, DSwip-1ΔEFPX10xMyc and DSwip-1ΔPX10xMyc) and full-length coding region of *Drosophila* Swip-1 (DSwip-1-fl10xMyc) were amplified by PCR using the following primers:

(1) DSwip-1ΔCC10xMyc constructs: ΔCCfwd: 5′CACCATGTCCGTTTCCTC-GAACGCCTCATCC-3′ and ΔCCrev: 5′GAAGAACTTCTTGGCTCCGCT-3′; (2) DSwip-1DEFPX10xMyc constructs: ΔEFPXfwd: 5′-CACCATGAGCGGAG-CCAAGAACTTCTTC-3′ and ΔEFPXrev: 5′ CTCGAAGATCGCTGCCCGCTG-3′; (3) DSwip-1DPX10xMyc constructs: ΔPXfwd: 5′CACCATGAGCACCAC-CAACACGGACA and DEFPXrev; (4) DSwip-1-full-length constructs: ΔCCfwd and ΔEFPXrev.

PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen, Karlsruhe). With recombination catalysed by the LR Clonase II plus enzyme mix (Invitrogen, Karlsruhe), the constructs were cloned into modified Gateway vectors (pUAST-attB-rfa-10xMyc kindly provided by Sven Bogdan, Universität Münster, Münster, Germany). These vectors were injected into embryos of fly strain BL24749 (Bloomington *Drosophila* Stock Center), where the integration of the construct takes place on the third chromosome. Table 1 summarises constructs and corresponding transgenic *Drosophila* lines.

SL2 cell culture, transfection and antibody staining

SL2 cells were cultured in Schneider's *Drosophila* Medium (Invitrogen, Karlsruhe, Germany) with 1% penicillin-streptomycin (Invitrogen) and 10% fetal calf serum (Sigma) at 25℃. For each transfection, we split cells on multititer plates (3×10^5 cells/ml per well) and after 24 hours, transfected with 0.5 μg DNA, 100 μl FCS-free medium, 3 μl Transfectin (Bio-Rad, Munich), *act-GAL4* and 400 μl medium for 2 days.

For antibody staining, we incubated glass dishes with concanavalin A (Sigma) for 40 minutes at room temperature (RT). After incubation with the transfected cells for 2 hours, we fixed them with 4% F-PBS for 15 minutes at RT. Cells were then washed twice with PBS, before permeabilising with 0.5% Triton X-100 in PBS and washing again. After this, cells were blocked with 3% BSA-PBS for 30 minutes and washed three times in PBS and H_2O. Cells were next incubated with the primary antibodies [(anti-DSwip-1, 1:1000; anti-Myc, 1:1000; anti-syntaxin-16, 1:50 (mouse, Abcam, Cambridge, MA), anti-Rab5, 1:200 (rabbit, Abcam)] for 2 hours at RT and washed three times in PBS and H_2O. The primary antibodies were detected using the corresponding Cy2- and Cy3-conjugated secondary antibodies (1:100, Dianova, Hamburg, Germany) and by incubating the cells for 1 hour at RT. After washing again, cells were incubated with DAPI (1 μg/μl; Sigma) to visualise nuclei, washed and finally mounted in Fluoromount G.

Preparation of protein extracts and western blot analysis

Cells were transfected as described above (3×10^5 cells/ml in 24-well plates). Two days after transfection, protein extracts were prepared. Cells were harvested and after centrifugation (3 minutes, 1000 ***g***) dissolved in lysis buffer [1% Triton X-100, 1 mM PMSF, 1 mM DTT, protease inhibitor (Roche Diagnostics) in PBS]. After centrifugation, the supernatant was taken for SDS-PAGE.

For generation of protein extracts of *Drosophila* embryos, larvae and pupae, we homogenised each tissue and added two volumes of lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 5% glycerol, 0.3% NP_4O, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM DTT, proteinase inhibitor in PBS). After centrifugation (20 minutes, 15,000 ***g***), supernatant was taken for SDS-PAGE. Larvae were homogenised and additionally precipitated with TCA. After centrifugation (20 minutes, 15,000 ***g***), the pellet was washed in 20% TCA, followed by 5% TCA, 100% ethanol and 1 M Tris-HCl (pH 8). After centrifugation (20 minutes, 15,000 ***g***), supernatant was taken for SDS-PAGE.

SDS-PAGE (15% acrylamide) was carried out as described by (Laemmli, 1970). Proteins were blotted on a nitrocellulose membrane (1 hour, 0.8 mA/cm^2). After washing the membranes in TBST (10 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween20) for 10 minutes, blocking in 5% milk powder in TBST for 1 hour and washing again in TBST, extracts were incubated with primary antibody overnight at 4℃ (anti-Myc, 1:2000; anti-DSwip-1, 1:1000; anti-actin (rabbit, Cell Signaling Technology, Boston, MA), 1:1000). Membranes were then washed four times in TBST and incubated with peroxidase-coupled corresponding secondary antibody (Dianova, Hamburg, 1:1000) and developed with the help of an ECL Western Blotting Analysis System (GE Healthcare, Munich).

Acknowledgements

We thank Christiana Stute and Markus Kallin for the initial analysis of *Drosophila* Swip-1 and Nadja Ring for generating some constructs. We also thank Sven Bogdan (Universität Münster) for providing modified Gateway vector. We would also like to thank Katja Gessner for excellent secretarial assistance, Susanne Önel for critical reading, Anja Rudolf and Jessica Kuckwa for helping with Phalloidin and anti-DMef2 staining, Ruth Willmott for excellent editing as well as Bettina Bonn for advice with cell culture transfection and Monika Hassel for sharing cell culture facilities. The Hybridoma antibody developed by Danny Brower was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA52242. We received gratefully fly strains from Vienna *Drosophila* RNAi Center, Austria. This work was supported by the Deutsche Forschungsgemeinschaft Re 628/14-3 and Re 628/15-2 to R.R.-P. and the EU Network of Excellence MYORES.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.083907/-/DC1

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