Cell type-specific recruitment of *Drosophila* Lin-7 to distinct MAGUK-based protein complexes defines novel roles for Sdt and Dlg-S97

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Summary

Stardust (Sdt) and Discs-Large (Dlg) are membrane-associated guanylate kinases (MAGUKs) involved in the organization of supramolecular protein complexes at distinct epithelial membrane compartments in *Drosophila*. Loss of either Sdt or Dlg affects epithelial development with severe effects on apico-basal polarity. Moreover, Dlg is required for the structural and functional integrity of synaptic junctions. Recent biochemical and cell culture studies have revealed that various mammalian MAGUKs can interact with mLin-7/Veli/MALS, a small PDZ-domain protein. To substantiate these findings for their in vivo significance with regard to Sdt- and Dlg-based protein complexes, we analyzed the subcellular distribution of *Drosophila* Lin-7 (DLin-7) and performed genetic and biochemical assays to characterize its interaction with either of the two MAGUKs. In epithelia, Sdt mediates the recruitment of DLin-7 to the subapical region, while at larval neuromuscular junctions, a particular isoform of Dlg, Dlg-S97, is required for postsynaptic localization of DLin-7. Ectopic expression of Dlg-S97 in epithelia, however, was not sufficient to induce a redistribution of DLin-7. These results imply that the recruitment of DLin-7 to MAGUK-based protein complexes is defined by cell-type specific mechanisms and that DLin-7 acts downstream of Sdt in epithelia and downstream of Dlg at synapses.

Key words: Epithelial polarity, Synapse, L27 domain, Neuromuscular junction, SAP97

Introduction

Many cell types exhibit an asymmetric subdivison of the plasma membrane and the underlying cytocortex into discrete domains with specialized structural and functional properties. This is mediated by the differential localization of distinct, membrane-anchored protein complexes including those forming cellular junctions. The importance of such partitioning is particularly evident in epithelial cells, in which apico-basal polarity largely depends on the well-ordered juxtaposition of molecularly distinct junctions, e.g. adherens junctions, tight junctions or septate junctions (Tepass et al., 2001; Knust, 2002; Knust and Bossinger, 2002). Similarly, diverse multi-protein complexes are established side-by-side along the pre- and postsynaptic membranes of neurons or their non-neuronal targets (Baude et al., 1993; Uchida et al., 1996; Gonzales-Gaitan and Jaeckle, 1997; Sone et al., 2000). The precise spatial arrangement of such complexes relies on the efficient sorting and selective retention of their components. Studies addressing these requirements have revealed that complex-specific combinations of submembraneous proteins provide a scaffold of adapter modules, primarily PDZ-type domains, to which transmembrane proteins and cytoplasmic components can bind concomitantly (Sheng and Sala, 2001; Harris and Lim, 2001). This concept is particularly well-established for membrane-associated guanylate kinases (MAGUKs), a superfamily of evolutionary conserved scaffolding proteins.

In vertebrates, MAGUKs of various subfamilies have been identified as central components of membrane specializations as diverse as epithelial tight junctions, presynaptic active zones, postsynaptic densities and juxtaparanodes of myelinated axons (Mariscal et al., 2000; Garner et al., 2000a; Garner et al., 2000b; Roh et al., 2002; Rasband et al., 2002). The identification of numerous binding partners in conjunction with subsequent biochemical and functional assays in cell culture systems strongly suggest that MAGUKs are required for the targeting and localized clustering of other proteins. Surprisingly, phenotypical analyses on knockout mice failed to confirm this idea (Migaud et al., 1998; McGee et al., 2001; Rasband et al., 2002), possibly as a result of compensatory effects exerted by paralogous isoforms of the mutated MAGUKs. The proposed clustering activity of MAGUKs is, however, strongly supported by genetic studies in invertebrates. For example, the *Drosophila* MAGUK Discs-Large (Dlg) is essential for proper synaptic localization of

Shaker-type potassium channels, the cell adhesion molecule Fasciclin II and the scaffolding protein Scribble (Scrib) at neuromuscular junctions (NMJs) (Tejedor et al., 1997; Thomas et al., 1997; Zito et al., 1997; Mathew et al., 2002). In epithelia, Dlg and Stardust (Sdt), which belong to different MAGUK subfamilies, are involved in the specification of separate epithelial membrane compartments and hence both are required to maintain epithelial polarity (Woods et al., 1996; Bilder et al., 2000; Bachmann et al., 2001; Hong et al., 2001; Bilder et al., 2003; Tanentzapf and Tepass, 2003). Sdt is a prominent component of the subapical region (SAR) (Bachmann et al., 2001), while Dlg localizes basally of adherens junctions, where it is required for the establishment and maintenance of septate junctions (SJ) (Woods et al., 1996; Bilder et al., 2000).

Similar to Dlg, its mammalian homologue synapse-associated protein 97 (SAP97/hDlg), associates with the basolateral rather than apical membrane compartment (Wu et al., 1998). Recent studies have shown that epithelial SAP97 interacts with another MAGUK, mLin-2/CASK, through a homotypic interaction between an L27-type domain in the N-terminal region of SAP97 and the L27N domain in mLin-2 (Lee et al., 2002; Chetkovich et al., 2002). A second L27 domain (L27C) of mLin-2 can bind mLin-7/Veli/MALS, a small PDZ-domain-containing protein, in both epithelial cells and neurons (Straight et al., 2000; Butz et al., 1998). This suggests that SAP97 and mLin-7 are physically coupled at the basolateral membrane in mammalian epithelia and possibly at synaptic junctions too. Apart from the ability to bind mLin-2, the L27 domains of SAP97 and mLin-7 have been implicated in homotypic in vitro interactions with other MAGUKs including Pals1, the putative mammalian homologue of Sdt (Kamberov et al., 2000; Marfatia et al., 2000; Tseng et al., 2001; Karnak et al., 2002). It is not yet known whether these interactions take place within one and the same cell, in a distinct sequential order or in a competitive manner, and to which extent they contribute to the proper targeting of mLin-7. Pals1, for instance, localizes apically within epithelial cells, whereas mLin-7 predominantly colocalizes with mLin-2 and SAP97 at basolateral membranes (Perego et al., 1999; Straight et al., 2000; Straight et al., 2001). Therefore, additional, probably cell-type specific modes of regulation might be involved to favor one interaction over another.

This study was initiated to evaluate a possible association of the fly homologue of mLin-7, DLin-7, with Dlg- or Sdt-based protein complexes in epithelial cells and at synaptic junctions. We found that, in epithelia, DLin-7 is associated with the apical membrane compartment, where it may directly interact with Sdt. Moreover, this interaction promotes recruitment of DLin-7. Although DLin-7 and Dlg segregate into different epithelial protein complexes, both proteins are part of a common postsynaptic protein complex at NMJs. Strikingly, this finding parallels our recent observation that Dlg isoforms containing the SAP97-like N-terminus (Dlg-S97) are expressed at NMJs but not in epithelia (Mendoza et al., 2003). In fact, our genetic analyses demonstrate that the postsynaptic localization of DLin-7 specifically depends on the presence of these Dlg-S97 isoforms. While the role of DLin-7 remains elusive, its recruitment by Dlg-S97 and Sdt reveals novel in vivo functions for both MAGUKs.

Materials and Methods

Fly stocks

The following *dlg*-mutant alleles were used: $dlg^{XI\text{-}2}$, dlg^{m52} and dlg^{m30} (Woods et al., 1996; Mendoza et al., 2003); *w1118* flies served as quasi wild type controls. For targeted expression of *UAS*-transgenes (see below) Gal4 activator strains $ptc\text{-}Gal4^{559.1}$ (Speicher et al., 1994), *Gal4-C57* (Thomas et al., 2000) and *Gal4-C164* (Pennetta et al., 2002) were employed. Effector strains carrying $UAS\text{-}sdt^{MAGUK}$ (line 14) or *UAS-eGFP-dlgA* (line ES50) have been described previously (Bachmann et al., 2001; Koh et al., 1999). Crosses were performed at 25°C.

Transgenic constructs and germline transformation

The complete coding region for Dlg-S97 was assembled by ligation of a 5′-fragment, obtained by PCR on the chimeric EST-clone GH18117, with a 3′-fragment of a *dlgA* cDNA via a common *Sma*I-site. This cDNA corresponds to an RT-PCR product described previously (Mendoza et al., 2003) and lacks the alternatively spliced exon encoding the extended region in-between PDZ2 and PDZ3. Upon in-frame ligation into pEGFP-C1 (BD Clontech) the EGFP-Dlg-S97 fusion gene was cloned into the transformation vector pUAST (Brand and Perrimon, 1993). Dlg-S97N-EGFP was generated by inserting a PCR fragment comprising the entire coding region of the EST clone LP07807 (Lee et al., 2002; Mendoza et al., 2003) into pEGFP-N3 and the fusion gene was then cloned into pUAST.

A *DLin-7* cDNA was obtained by reverse transcription on embryonic polyA$^+$ RNA (primer: lin7-3.1, GTG TTA GAT CTT ATT ATT CGT AAA GGG) followed by PCR (primers: lin7-5.1, CAC GGA ATG GAA TTC TGT GCC CC; lin7-3.2, CGT GCA GAT CTG CAT GGT CTA ATG) and subsequent cloning into pBsc SK$^+$. An N-terminally Flag-tagged version of DLin-7 was generated by introducing a PCR-amplified *DLin-7* cDNA (primers: lin7-Flag-5, TTG AAC AGC GCG GCC GCC GAT AAC; lin7-Flag-3, GAA CTA GTC GAC CTG TAT GGT C) into pUAST-Flag (N. Fischer and A. Wodarz, Institut für Genetik, Universität Düsseldorf, Germany). The construct for *DLin-7*-specific DNA-mediated RNA interference was generated by introducing a PCR-amplified *DLin-7* fragment covering the L27 and PDZ domains (Pro^7 to Pro^{175}; primers: lin7-RNAi-5, CGC GGA TCC ACT GAC TTT GTC; lin7-RNAi-3, CCT CGG GTA CCT TGG GTG TG) into pHIBS followed by cloning into pUAST as described (Nagel et al., 2002). Only sequence-controlled PCR fragments were used for cloning procedures. Transgenic flies were generated by P-element mediated germ line transformation (Spradling, 1986).

RT-PCR and northern blotting

RT-PCR was performed on staged polyA$^+$-RNA isolated by using the Omniscript RT Kit (Qiagen) with primer lin7-3.1 (see above) followed by PCR with primers lin7-5.2 (CAG CTG CTC GAA TTC TAT TTC AGG) and lin7-3.2 (see above).

A digoxygenin-labelled RNA antisense probe that recognizes the full-length *DLin-7* cDNA was generated using the DIG RNA labeling Kit (Roche). Northern blotting was performed following standard procedures.

Antibodies and immunofluorescence analyses

Antisera against DLin-7 were obtained by repetitive immunization of a rabbit with an affinity-purified GST-DLin-7 fusion protein (covering the L27 and PDZ domains, Leu^8 to Gln^{195}). Antisera against DLin-7 and mLin-7 (Perego et al., 2002) were both used at a 1:500 dilution for immunofluorescence analyses and at 1:1500 for western blots. Rat and rabbit anti-Dlg_{PDZ1+2} antisera were used as described (Koh et al., 1999), and rabbit anti-Dlg_{S97N} was used as described (Mendoza et al., 2003). Rat anti-Sdt_{MPDZ} (S. Berger and

E.K., unpublished) was used at a dilution 1:500. Anti-Flag M2 monoclonal antibody (Sigma-Aldrich) was used diluted 1:200 and a fluorescein-conjugated goat anti-horseradish peroxidase antibody (Cappel) was diluted 1:200. Fluorescence-labeled secondary antibodies purchased from Jackson ImmunoResearch laboratories (Fluorescein-conjugates) or Molecular Probes (Alexa-568 conjugates) were applied at a 1:200 dilution.

Imaginal discs were dissected from 3rd instar larvae and fixed for 25 minutes in 4% formaldehyde. Body wall preparations of 3rd instar larvae were performed as described (Bellen and Budnik, 2000). Following a 30 minute fixation in 4% paraformaldehyde, washes and antibody incubations were carried out in PBS containing 0.2% Triton X-100. Samples designated for direct comparison were processed in parallel under the same conditions and with identical confocal settings. Confocal imaging was performed on a Leica TCS NT or a Leica DM LFSA microscope.

Pull-down assay, immunoprecipitations and western blot analysis

Biotinylated Sdt-GUK protein was generated by in vitro transcription/translation with the TNT Coupled Reticulocyte Lysate System/Non-Radioactive Translation Detection System (Promega) and incubated with equal amounts of Glutathione Sepharose™ 4B (Amersham)-coupled GST-DLin-7 (see above) and GST, respectively, in pull-down buffer (20 mM Tris pH 8, 0.2 M EDTA, 0.1 M NaCl, 1 mM DTT, 0.2% IGEPAL, 1 μM Pefabloc, 5 μM leupeptin, 1 μM pepstatin, 0.3 μM aprotinin) overnight at 4°C. Beads were pelleted, washed four times in pull-down buffer and eluted by boiling in SDS-PAGE sample buffer. Eluted labelled protein was analyzed by SDS-PAGE and detected with Streptavidin-HRP (Roche, diluted 1:1000).

Immunoprecipitations from embryos overexpressing Sdt-MAGUK (*GAL4^{daG32}/UAS-sdt^{MAGUK}*) were performed essentially as described (Klebes and Knust, 2000). Lysates were incubated with 5 μl anti-mLin-7 antibody (Perego et al., 2002) and 2 μl anti-β-Gal antibody (Promega), respectively.

Immunoprecipitation experiments from larvae were performed at 4°C, largely as described previously (Thomas et al., 1997): for each genotype, body walls from 12 dissected larvae were homogenized in 150 μl of lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% Triton, 1 mM $MgCl_2$ and Complete™ protease inhibitor cocktail (Roche Diagnostics; one tablet per 50 ml lysis buffer). Following a 2 hour extraction on a shaker, the homogenate was cleared from cuticle debris by centrifugation for 5 minutes at 3000 ***g***. 110 μl of the supernatant were loaded onto 30 μl of Protein G sepharose (Pharmacia Biotech), that was pre-incubated for 2 hours with 1.5 μl of anti-Flag antibody (~ 6μg), mixed and rotated overnight. 30 μl of residual supernatant were set aside as input control. The Protein G Sepharose-precipitate was washed three times for 15 minutes in TBST (with intervening centrifugation steps, 5 minutes at 700 ***g***) and then supplied with 15 μl of SDS sample buffer. Equivalents of ~1 body wall (input controls) or 3 body walls (precipitates) were separated by SDS-PAGE and blotted onto nitrocellulose transfer membrane. Upon blockage in TBST/5% dry milk the membrane was incubated with anti-Dlg_{PDZ1+2} at 1:20,000 or anti-DLin-7 (1:1500) overnight. Peroxidase-conjugated secondary antibodies in combination with the ECL system (Amersham Pharmacia Biotech) were employed to detect immunoreactive bands.

Yeast two-hybrid assays

Yeast two-hybrid analyses were based on the Gal4-Matchmaker vector system (BD Clontech). PCR fragments were cloned into pGBKT7 to generate bait clones and into pGADT7 or pACT-2 (Sdt) to generate prey clones. Pairs of bait and prey clones were co-transformed into yeast strain AH109 by electroporation and co-transformants were selected on Trp/Leu-depleted media. Interaction-dependent expression of β-galactosidase activity was monitored by a colony-lift filter assay as described (BD Clontech, protocol PT3024-1). All combinations were tested in parallel and in at least two independent experiments.

Results

Identification of a close *Drosophila* homologue of Lin-7/Veli

Searching the *Drosophila* genome database at http://www.BDGP.org revealed that a single gene annotated as CG7662 (Fig. 1A) encodes two conceptual splice isoforms representing fly homologues of Lin-7. RT-PCR analyses performed on mRNA samples from different developmental stages and northern blot analysis of embryonic mRNA, however, indicated that only one major gene product is expressed throughout development (Fig. 1B,C). An embryonic RT-PCR product was subcloned and sequenced. The deduced 195 aa gene product comprises an N-terminal L27-type domain and a C-

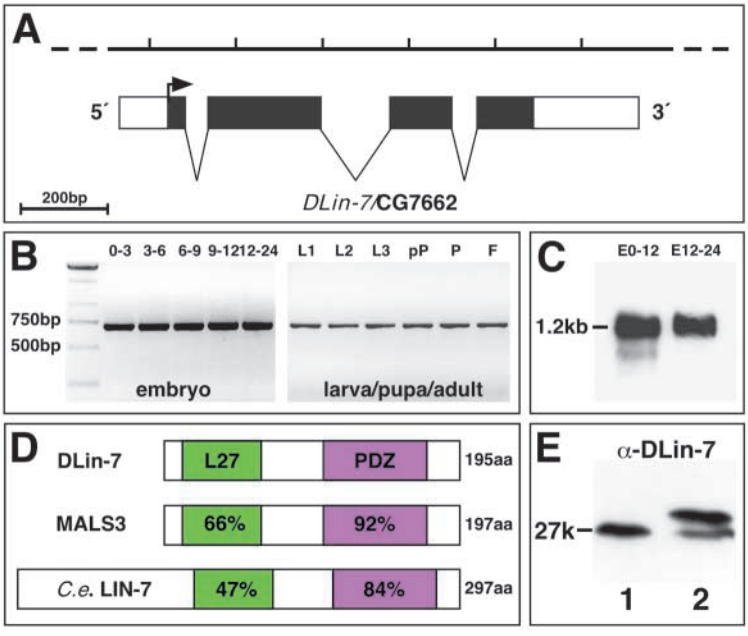
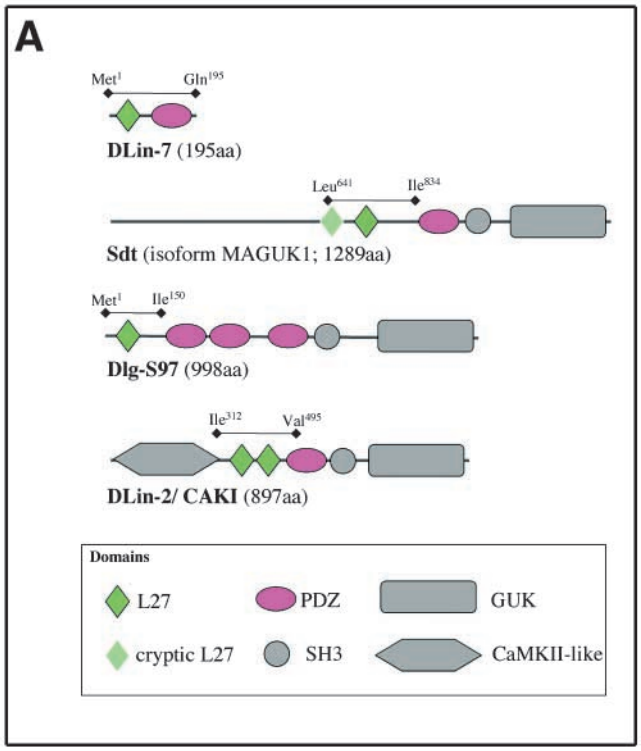


Fig. 1. *DLin-7* encodes a single-PDZ domain protein. (A) Exon-intron structure of the *DLin-7*/CG7662 locus (black boxes, ORF) from the cytological interval 96B19 (genomic scaffold AE003750). (B) RT-PCR performed on poly(A)$^+$ RNA of different developmental stages (staged embryos in hours, L1-L3, larval stages; pP, prepupa; P, pupa; F, female adult). (C) Northern blot of embryonic poly(A)$^+$ RNA hybridized with a probe covering the L27 and PDZ domain of DLin-7. (D) Protein structure of DLin-7 and two putative orthologues from mammals (MALS3) and *C. elegans* (LIN-7). The green and lilac colours indicate the L27 and the PDZ domains, respectively. The percentages of amino acid identities of the two domains with respect to the corresponding domains of DLin-7 are shown. (E) Western blot analysis of wild-type embryos (1) and embryos overexpressing a Flag-tagged DLin-7 transgene (2) probed with anti-DLin-7 antibodies. Note the additional, slightly larger band in lane 2 that corresponds to the Flag-DLin-7 protein.

terminal PDZ domain. DLin-7 exhibits 70% sequence identity with the corresponding region of LIN-7 in *C. elegans* and is even more closely related to the three paralogous isoforms of mammals (collectively referred to as mLin-7; Fig. 1D). Western blot analyses using an antibody raised against DLin-7 yielded a prominent immunoreactive band at ~27 kDa. As expected, a slightly increased molecular weight was apparent for a transgenically expressed, Flag epitope-tagged version of DLin-7 (Fig. 1E).

We next tested whether the sequence homology between DLin-7 and its orthologues is significant with regard to protein-protein interactions. Since the PDZ domains of DLin-7 and mLin-7 are almost identical (Fig. 1D) we may assume that they exhibit identical binding specificities. A couple of binding partners of the PDZ domain have been identified in other species (Jo et al., 1999; Perego et al., 1999; Perego et al., 2000; Straight et al., 2001); however, their putative fly homologues carry no C-terminal PDZ-binding motifs. We therefore focussed on potential interactions corresponding to those mediated by the L27 domain of mLin-7 (Fig. 2A). Using a yeast two-hybrid assay we demonstrated that DLin-7 binds to L27 domains in the fly homologues of mLin-2 (DLin-2/CamGUK/CAKI) and Pals1 (Sdt) (Fig. 2B). Whereas in a previous study a direct interaction between mLin-7 and SAP97 was not detectable (Lee et al., 2002), we noticed a weak interaction between DLin-7 and the N-terminal region of Dlg-S97. Similarly to mLin-7, DLin-7 did not display homophilic interactions (Fig. 2B). We thus conclude that, for DLin-7 and mLin-7, the in vitro binding properties of their L27 domains with members of distinct MAGUK subfamilies are very similar.



B

prey / bait	DLin-7	Sdt	Dlg-S97	DLin-2 / CAKI
DLin-7	-	+++	+	++++
pGBKT7 (vector)	-	-	-	-

Fig. 2. In vitro interaction of DLin-7 with members of different MAGUK subfamilies. (A) Proteins that were tested for interaction in a yeast two-hybrid assay. Subregions expressed as baits or preys are indicated as bars together with the positions of the first and last amino acid residues. The symbols used for the various protein domains are summarized in the box below. See Discussion for an interpretation of the cryptic L27 domain of Sdt. (B) Results of the yeast two-hybrid assay. The strength of each interaction was classified by the time period between the onset of the β-Gal filter assay and the detection of a clearly visible color reaction; ++++, within 30 minutes or less; +++, within 1 hour; ++, within 3 hours; +, within 5 hours; –, more than 5 hours. The empty bait vector pGBKT7 served as a negative control. The weak interaction between DLin-7 and Dlg-S97 was also observed in the reverse bait-prey combination.

DLin-7 is associated with the Crb/Sdt complex in epithelial cells

To assess the subcellular localization of DLin-7 we employed crossreacting antibodies raised against mLin-7 (Perego et al., 2002) as well as antisera raised against DLin-7. Both antisera yielded virtually identical results in western blot and immunofluorescence analyses. The specificity of the antisera was further evident from increased or decreased immunoreactivities upon transgenic overexpression or reduction of DLin-7, respectively (Fig. 1E, Fig. 3A,B).

In the embryonic epidermis and in imaginal disc epithelia, DLin-7-specific immunofluorescence was found highly enriched at the apical membrane. Accordingly, confocal microscopy on double-stained imaginal discs revealed that the immunoreactivities for DLin-7 and Dlg, which localizes to septate junctions, were totally disjunct (Fig. 4A-C), whereas a striking colocalization of DLin-7 with Sdt was evident in these epithelia (Fig. 4D-F). Considering the in vitro interaction between DLin-7 and Sdt we assumed that DLin-7 is a component of the previously described subapical Crb/Sdt complex (Klebes et al., 2000; Hong et al., 2001; Bachmann et al., 2001).

To further substantiate this assumption, we used a transgenic approach to alter the amount of Sdt in restricted areas of imaginal discs and monitored the effects on DLin-7 localization. Targeted overexpression of a *UAS-sdt*^{MAGUK} transgene using a *ptc-Gal4* activator strain (Bachmann et al., 2001; Speicher et al., 1994) resulted in increased levels of both Sdt and DLin-7 at the apical cortex of epithelial cells within the respective area (Fig. 4G-I). In contrast, transgenically expressed Flag-DLin-7 had no obvious effect on the localization of Sdt (data not shown).

Further support for a close association of Sdt and DLin-7 was obtained from biochemical experiments. In a pull-down

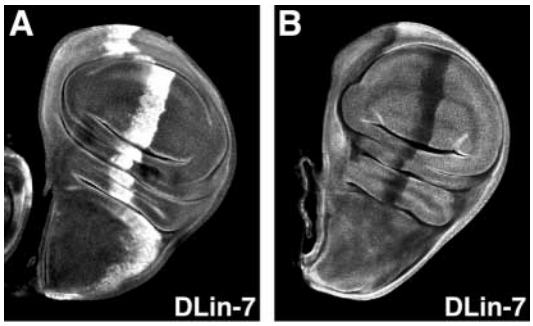


Fig. 3. Modulation of DLin-7 protein levels in wing imaginal discs by means of the GAL4/UAS-system. (A) *ptc-Gal4*-driven overexpression of Flag-DLin-7 strikingly increases Flag-DLin-7 protein levels. (B) Upon overexpression of *DLin-7*-ds-RNA with *ptc-Gal4* the amount of DLin-7 protein is significantly reduced.

assay an in vitro translated Sdt isoform missing the PDZ and SH3 domains [Sdt-GUK (Bachmann et al., 2001)] was bound by a GST-DLin-7 fusion protein but not by GST alone (Fig. 4J). Using anti-mLin-7 antibodies we co-immunoprecipitated Sdt from lysates of Sdt-overexpressing embryos (Fig. 4K). Together these findings strongly suggest that Sdt recruits DLin-7 to the SAR, most probably through a homotypic interaction between the L27 domains of the two proteins.

Postsynaptic colocalization of Dlg and DLin-7 at larval NMJs

In mammals, mLin-7 has originally been identified as a synaptic protein component with isoform-specific neuronal expression patterns (Butz et al., 1998; Jo et al., 1999; Misawa et al., 2001). We thus asked whether DLin-7 is also associated with synapses in flies. In fact, in situ hybridization revealed an expression of *DLin-7* transcripts in the embryonic CNS (data not shown) and immunostaining revealed an enrichment of DLin-7 in the neuropil regions of the larval CNS (Fig. 5A). To further resolve a potential association of DLin-7 with synaptic protein complexes, we focussed on larval NMJs. Various types of motornerve terminals (boutons) can be classified based on differences in morphology and neurotransmitter specificity (Gramates and Budnik, 1999). DLin-7-specific immunoreactivity was easily detectable at glutamatergic NMJs, i.e. most prominently at type Ib boutons and to a lesser extent at type Is boutons. At non-glutamatergic boutons DLin-7 immunoreactivity was much weaker (type II boutons) or even undetectable (type III boutons) (Fig. 5B-D). Confocal analysis of synaptic boutons double-stained with the neuronal marker anti-HRP and anti-DLin-7 antibodies further revealed that DLin-7 is strongly enriched postsynaptically (Fig. 5E-G). A striking colocalization was evident for DLin-7 and Dlg (Fig. 5H-J). Notably, the distribution of both proteins is even congruent with respect to non-immunoreactive sites along the bouton rims (arrowheads in Fig. 5H-J), which might represent glutamate receptor fields or initiation sites of bouton budding (Sone et al., 2000; Mathew et al., 2002). Dlg-specific immunofluorescence at NMJs of 3rd instar larvae is predominantly attributable to a postsynaptic enrichment of the protein in the subsynaptic reticulum

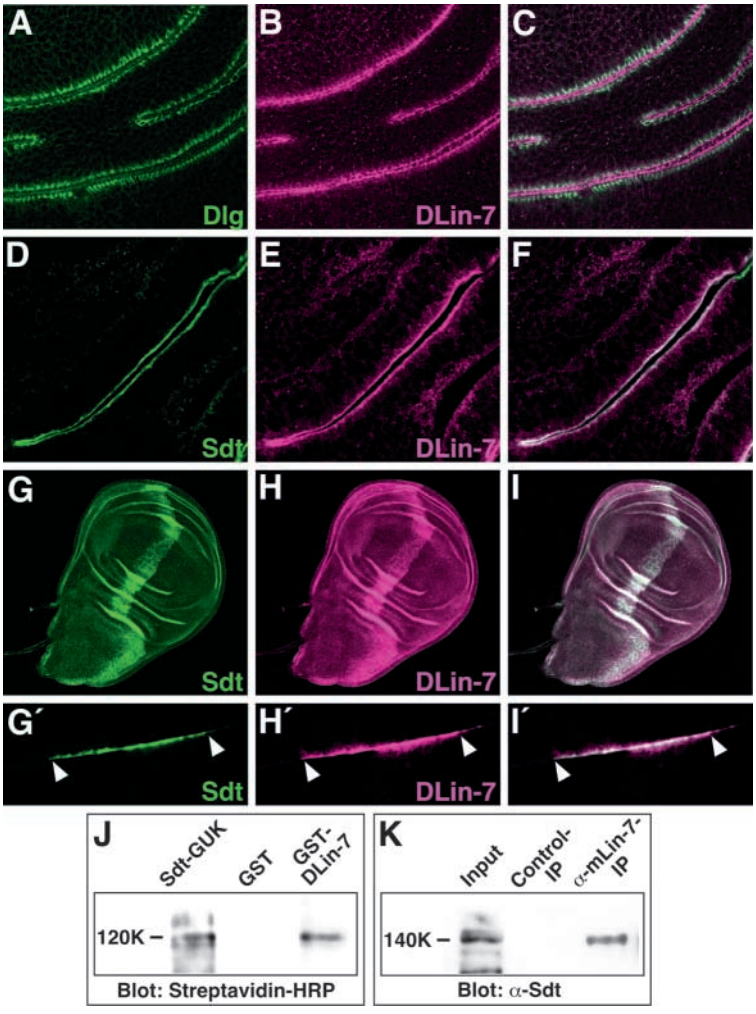


Fig. 4. Subcellular localization of DLin-7 in wing imaginal discs and its recruitment by Sdt. (A-F) In wing imaginal discs DLin-7 is localized apical to Dlg, which is restricted to the septate junction in epithelia (A-C), and colocalizes with Sdt in the SAR (D-F). (G-I) *ptc-Gal4*-driven overexpression of Sdt increases apical levels of DLin-7. (G´-I´) Note that both overexpressed Sdt and, as a consequence, DLin-7 are targeted to the SAR (white arrowheads mark the borders of the overexpression domain). (J) DLin-7 and Sdt-GUK interact in vitro. Sepharose beads carrying a GST-DLin-7 fusion protein or GST alone were incubated with biotinylated Sdt-GUK. Bound protein was eluted and analysed by SDS-PAGE. Binding was observed between GST-DLin-7 and Sdt-GUK (right lane), but not between GST and Sdt-GUK (middle lane). The left lane shows labelled Sdt-GUK protein equivalent to 10% of the material analysed in the experimental lanes. (K) Anti-mLin-7 antibody (Perego et al., 2002) immunoprecipitates Sdt from embryos overexpressing Sdt-MAGUK protein (*GAL4*^{daG32}*/UAS-sdt*^{MAGUK}, left lane), whereas a control IgG (anti-β-Gal) does not (middle lane). The western blot was then probed with anti-Sdt antibody. The left lane shows the corresponding input control with approximately 50 μg of total protein from embryonic extracts.

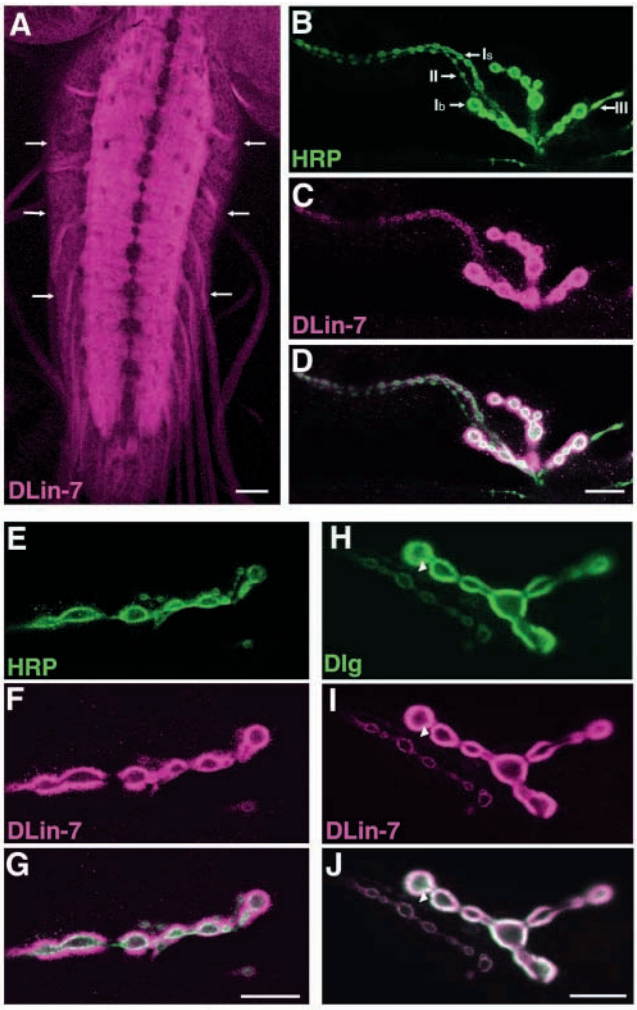


Fig. 5. DLin-7 is expressed in the CNS and at NMJs. (A) Ventral nerve cord of a 3rd instar larva. DLin-7 is enriched in the neuropil area. Arrows mark the periphery of the cortical area. (B-D). Muscle 12 at abdominal segment A3 is innervated by four different motornerve terminals (Ib, Is, II, III) as revealed by anti-HRP immunoreactivity (B), but only type I boutons exhibit clear DLin-7 specific immunoreactivity (C,D). (E-G) Confocal section of a branch of the NMJ at muscle 6. Note that the presynaptic marker HRP (E) is largely surrounded by DLin-7 specific immunoreactivity (F,G). (H-J) Confocal section of a NMJ at muscle 12, double-labeled with anti-Dlg (I) and anti-DLin-7 (J) antibodies. Arrowhead marks a site of reduced immunoreactivity. Bar, 20 μm (A); 10 μm (B-J).

[SSR (Lahey et al., 1994)]. We therefore conclude, that DLin-7 and Dlg are colocalized in the SSR of type I boutons at larval NMJs.

Dlg-dependent localization of DLin-7 at NMJs

Previous studies imply that Dlg ensures proper synaptic localization of several other proteins at type I boutons either by direct interaction or through recruitment of additional scaffolding molecules (Tejedor et al., 1997; Thomas et al., 1997; Zito et al., 1997; Mathew et al., 2002). To test whether Dlg is as well required for postsynaptic localization of DLin-7, we examined body wall muscle preparations from *dlg*-mutant larvae for DLin-7-specific immunofluorescence. Compared with wildtype, a severe reduction of DLin-7 was obvious at boutons from $dlg^{XI\text{-}2}$ and dlg^{m52} mutant larvae (Fig. 6A-F), which express only low amounts of truncated Dlg at NMJs (Mendoza et al., 2003). Postsynaptic DLin-7 appeared unaffected in dlg^{m30} mutant larvae, which express a mutant isoform with a single amino acid exchange in the SH3 domain (Fig. 6G-H). To rule out the possibility that reduced expression levels of DLin-7 are the major cause for its poor localization at *dlg*-mutant NMJs, we employed the muscle-specific Gal4-activator strain *Gal4-C57* (Koh et al., 1999) to express Flag-tagged DLin-7 in dlg^{+} and $dlg^{XI\text{-}2}$ mutant larvae. A considerable and *dlg*-independent overexpression of the transgene-encoded protein in comparison to endogeneous DLin-7 was evident from western blot analysis (Fig. 7A). Consequently, increased levels of DLin-7-specific immunoreactivity could be monitored throughout the muscles irrespective of the *dlg*-genotype (not shown). DLin-7-related anti-Flag immunoreactivity, however, was only enriched at NMJs in the presence of wild-type Dlg, whereas it was barely detectable at NMJs of $dlg^{XI\text{-}2}$ mutant larvae (Fig. 7B-G). We thus conclude that Dlg is essential for postsynaptic recruitment of DLin-7.

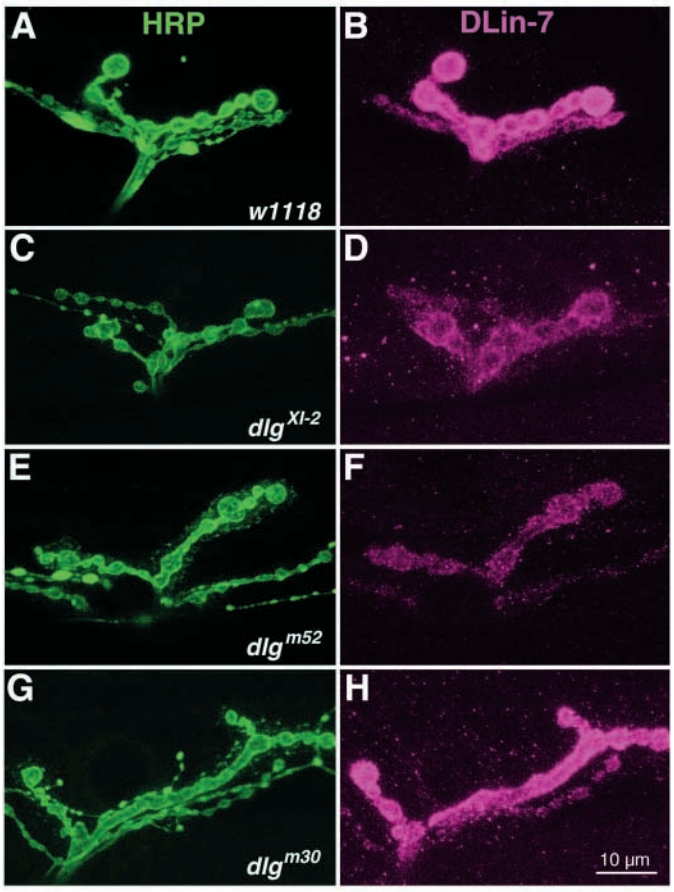


Fig. 6. Mutations in *dlg* affect the localization of DLin-7 at larval NMJs. NMJs at muscle 12 of *w1118* control larvae (A,B) and *dlg*-mutant larvae (C-H) were double-labeled with anti-HRP antibodies (A,C,E,G) and antibodies against DLin-7 (B,D,F,H). Each image represents a stack of 15 to 17 optical sections taken at 0.5 μm steps.

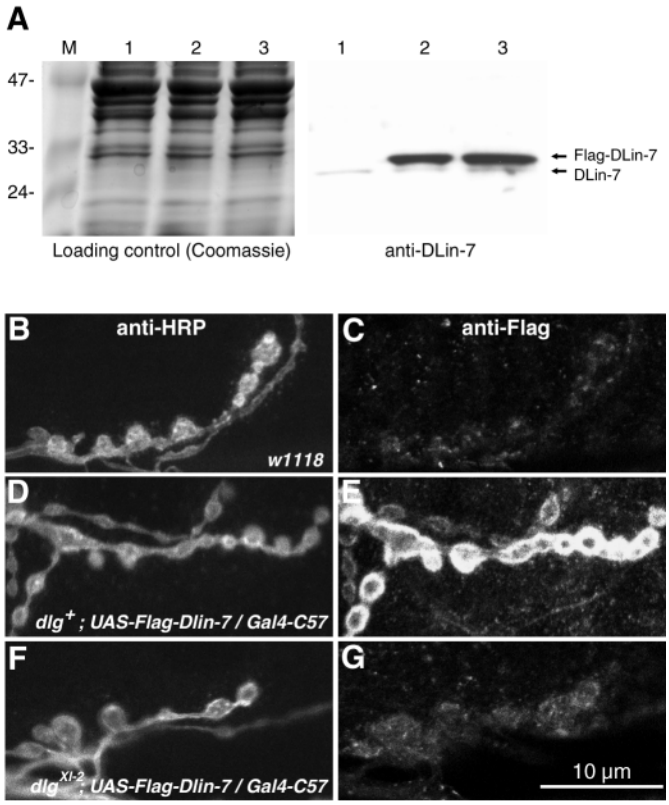


Fig. 7. Dlg-dependent localization of transgenically expressed Flag-DLin-7 at NMJs. (A) Western blot analysis on body wall muscle extracts using anti-DLin-7. A Coomassie staining was used as a loading control to ensure that equal amounts of body wall extracts were analyzed. M, molecular weight marker; lane 1, *w1118*; lane 2, *dlg*$^{+}$*;;UAS-Flag-DLin-7/Gal4-C57*; lane 3, *dlg*$^{XI-2}$/Y;;*UAS-Flag-DLin-7/Gal4-C57*. (B-G) Confocal sections of synaptic boutons at muscle 12 double-labeled with anti-HRP (B,D,F) and anti-Flag (C,E,G). (B,C) *w1118*; (D,E) *dlg*$^{+}$*;;UAS-Flag-DLin-7/Gal4-C57*; (F,G) *dlg*$^{XI-2}$/Y;;*UAS-Flag-DLin-7/Gal4-C57*.

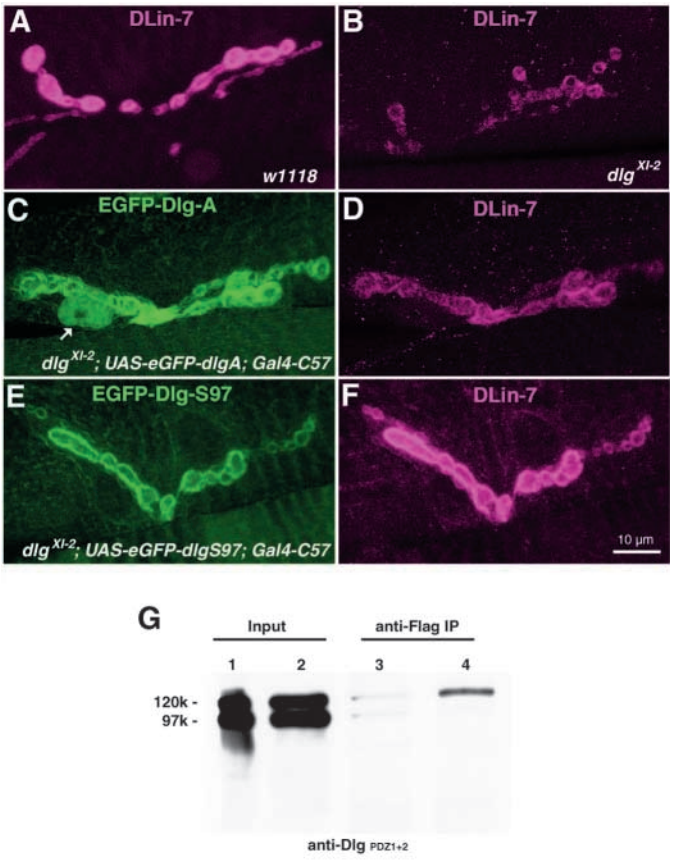


Fig. 8. Isoform-specific interaction between Dlg-S97 and DLin-7. (A-F) Rescue of DLin-7 at *dlg*-mutant NMJs on muscle 12 upon postsynaptic expression of EGFP-Dlg-A versus EGFP-Dlg-S97. DLin-7-specific immunofluorescences of *w1118* (A) and *dlg*$^{XI-2}$ (B) are shown for reference. (C) *Gal4-C57*-driven EGFP-Dlg-A was targeted to NMJs and to nuclei (arrow), whereas co-staining reveals no obvious enrichment for DLin-7 at either compartment (D). Postsynaptic targeting of EGFP-Dlg-S97 (E) was accompanied by effective restoration of DLin-7 (F). Each image represents a stack of 17 optical sections taken at 0.5 µm steps. (G) Co-immunoprecipitation assay performed on body wall muscle extracts from *w1118*- (lanes 1, 3) and *UAS-Flag-DLin-7/Gal4-C57* (lanes 2, 4) 3rd instar larvae using anti-Flag antibody. Input control samples (lanes 1, 2) and immunoprecipitated protein samples (lanes 3, 4) were analyzed by western blotting using anti-Dlg$_{PDZ1+2}$. Note the enrichment of the 120 kDa isoform in lane 4.

The SAP97-type N-terminus of Dlg is required for synaptic recruitment of DLin-7 to NMJs

The close association of DLin-7 with Dlg at NMJs contrasts with the separate distribution of both proteins in epithelial cells. This finding coincides with the differential expression of the Dlg-S97 isoform, which is found at NMJs but not in epithelia (Mendoza et al., 2003). In contrast to Dlg-A, which is expressed in epithelia, Dlg-S97 contains an N-terminal segment with striking homology to a corresponding region in SAP97/hDlg. We therefore asked, whether the SAP97-type N-terminus is involved in synaptic recruitment of DLin-7. This hypothesis was tested by rescue experiments, in which we monitored the reconstitution of DLin-7 at *dlg*$^{XI-2}$ mutant NMJs upon muscle-specific expression of various *dlg*-transgenes (Fig. 8). Expression of a previously described *UAS-eGFP-dlgA*-construct in *dlg*$^{XI-2}$ mutants (Koh et al., 1999) resulted in poor if any reconstitution of postsynaptic DLin-7, although a considerable amount of EGFP-Dlg-A was targeted to NMJs (Fig. 8A-D). In striking contrast, expression of a newly generated *UAS-eGFP-Dlg-S97* transgene clearly restored DLin-7 localization at NMJs (Fig. 8E-F). Similarly, expression of EGFP-SAP97 was found to rescue synaptic localization of DLin-7 (data not shown).

We next performed co-immunoprecipitation experiments to evaluate whether DLin-7 and Dlg-S97 are physically linked in vivo. To this end, body wall muscle extracts from Flag-DLin-7 expressing larvae were incubated with monoclonal anti-Flag antibodies coupled onto Protein-G-Sepharose. Equally treated extracts from *w1118* body walls served as a control. Typically, western blot analysis on body wall muscle extracts using anti-Dlg$_{PDZ1-2}$ antibody (Koh et al., 1999) yields two prominent Dlg-specifc immunoreactive bands of ~120 kDa and ~97 kDa (Fig. 8G, lanes 1 and 2). The ~120 kDa Dlg isoform was selectively precipitated together with Flag-DLin-7, whereas the amount of the 97 kD isoform present in the precipitate did not

exceed control levels (Fig. 8G, lanes 3 and 4). We have recently demonstrated, that the 120 kDa band largely consists of Dlg-S97 and that the 97 kD isoform lacks the SAP97-type N-terminus (Mendoza et al., 2003). Our results thus indicate, that DLin-7 and Dlg-S97 are part of the same protein complexes in body wall muscles and that their association requires the N-terminal domain of Dlg-S97.

Ectopic expression of Dlg-S97 in epithelia does not affect the apical localization of DLin-7

To test whether the presence of Dlg-S97 at a distinct membrane compartment is sufficient to recruit DLin-7 we used the *ptc-Gal4* activator strain to induce ectopic expression of the *UAS-eGFP-dlgS97* transgene in imaginal disc epithelia. EGFP-Dlg-S97 was found along the plasma membrane, clearly enriched at lateral regions that most likely correspond to the regions of the septate junctions (Fig. 9A). We did not observe, however, any redistribution of endogeneous DLin-7 from the SAR to sites enriched for EGFP-Dlg-S97 (Fig. 9B,C). This might simply be explained by a strong competitive influence of Sdt. To test this idea, we used *ptc-Gal4* to express Flag-DLin-7 either alone or in combination with EGFP-Dlg-S97 (Fig. 9D-F). In both cases, surplus DLin-7-specific immunofluorescence largely accumulated in the cytosol rather than at the SAR (Fig. 9E), which again suggests that the amount of Sdt is limiting to the subapical localization of DLin-7 (Fig. 4G-I). Despite the presence of excess DLin-7, we still did not monitor a co-enrichment with EGFP-Dlg-S97 (Fig. 9F). This suggests that additional, cell-type-specific requirements underlie the Dlg-S97-dependent recruitment of DLin-7 as observed at the NMJ. To elaborate on this latter idea, we took advantage of a *UAS-dlg-S97N-eGFP* transgene, which encodes the short, previously described Dlg-S97N isoform [also denoted as CPD (Lee et al., 2002; Mendoza et al., 2003)] with EGFP fused to its C-terminus. This fusion protein contains the SAP97-type N-terminus but lacks all the canonical MAGUK domains. Targeted expression of this transgene in both epithelia or body wall muscles resulted in nuclear enrichment of Dlg-S97N-EGFP in either tissue (Fig. 9G,J). As for EGFP-Dlg-S97, no redistribution of DLin-7 was detectable in epithelia (Fig. 9H,I). In striking contrast, expression of Dlg-S97N-EGFP in muscles caused an obvious nuclear enrichment of DLin-7 compared with controls (Fig. 9K,L). Notably, EGFP-Dlg-A, which aside from its enrichment at NMJs, also exhibits some nuclear localization, does not induce a detectable enrichment of DLin-7 in muscle nuclei (Fig. 8C,D).

Assuming that a fairly weak interaction between Dlg-S97 and DLin-7 accounts for the recruitment of DLin-7 to NMJs, we would expect that ectopic expression of Sdt in muscles could easily affect the synaptic localization of DLin-7. This possibility was tested by *Gal4-C57*-induced muscle expression of the *UAS-sdt*^{MAGUK} transgene. Overexpression was verified by western blotting (not shown). Sdt-specific immunofluorescence did not enrich at NMJs or at any distinct sites within muscles but remained diffusely distributed. Most notably, no effect on the synaptic localization of DLin-7 was evident (data not shown), implying a robust linkage to Dlg-S97. We therefore propose that a differentially expressed factor is required to link both proteins and thus to mediate Dlg-S97-dependent recruitment of DLin-7 to NMJs.

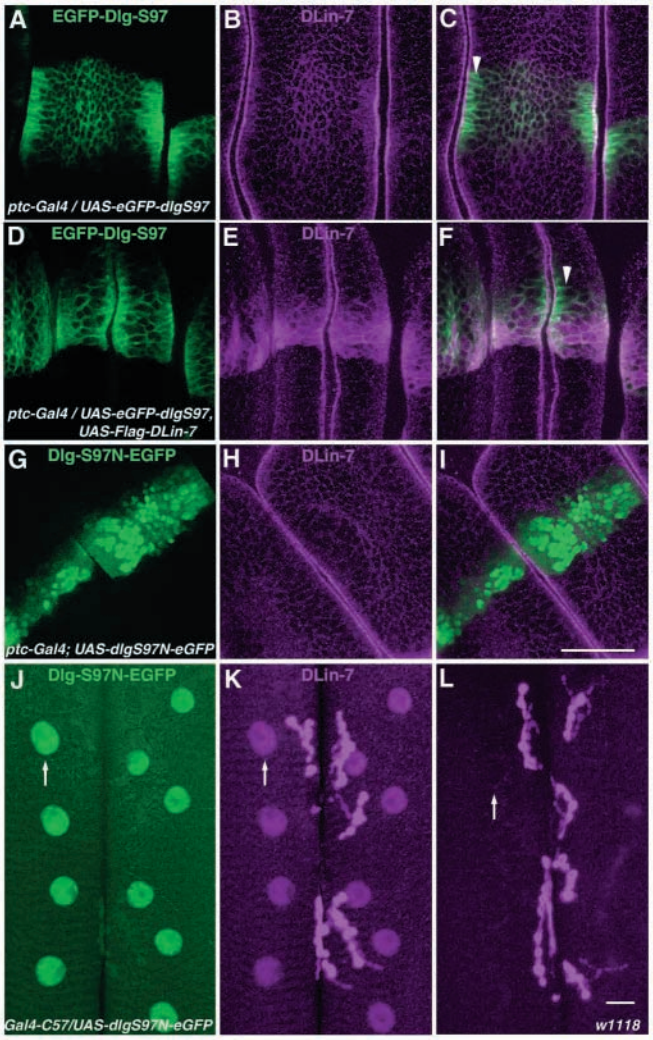


Fig. 9. Ectopic expression of EGFP-Dlg-S97 and Dlg-S97N-EGFP. (A-I) Confocal sections of wing imaginal disc epithelia expressing EGFP-Dlg-S97 (A-C), EGFP-Dlg-S97 and Flag-DLin-7 (D-F) or Dlg-S97N-EGFP (G-I) under the control of *ptc-Gal4*, co-stained with anti-DLin-7 antibodies (B,E,H). Areas flanking the *ptc*-expression domain served as an internal control to assess possible effects on the subcellular distribution of DLin-7. Merged images are shown in C, F and I. No striking redistribution of DLin-7 was caused by either EGFP-Dlg-S97, which was enriched basolaterally (depicted by arrowheads in C,F), or Dlg-S97N-EGFP, which exhibited strong nuclear localization (G). (J-L) Mislocalization of DLin-7 in nuclei of muscles 6 and 7 upon *Gal4-C57* driven expression of Dlg-S97N-EGFP. Each image represents a stack of 20 optical sections taken at 0.5 μm steps. Nuclear enrichment of Dlg-S97N-EGFP (G) was paralleled by partial targeting of DLin-7 to nuclei (H), which was not observed in *w1118* (I). Arrows mark selected nuclei. Bars, 10 μm (in I for panels A-I; in L, for panels J-L).

Discussion

We have shown that the single fly homologue of Lin-7 is a component of different MAGUK-based protein complexes in epithelia and at synaptic junctions. This finding is in line with the previously reported association of LIN-7 and mLin-7 with various membrane specializations in worms or mammals, respectively (Simske et al., 1996; Butz et al., 1998; Jo et al.,

1999; Perego et al., 2000; Straight et al., 2000). Nonetheless, our results deviate from these earlier reports in several regards and thereby imply novel roles for Sdt and Dlg-S97. Most notably, the requirement for either MAGUK to recruit DLin-7 to distinct membrane domains was not simply predictable from studies on their homologues in other species.

Pals1, a putative mammalian homologue of Sdt, binds mLin-7 in vitro (Kamberov et al., 2000). The physiological significance of this finding remains unclear since Pals1 localizes to tight junctions of epithelial cells (Roh et al., 2002), whereas a basolateral localization for mLin-7 was emphasized in several other reports (Perego et al., 1999; Straight et al., 2000; Straight et al., 2001). In Madin-Darby canine kidney cells, however, mLin-7 has also been detected at tight junctions (Irie et al., 1999; Perego et al., 2000). Our results now indicate that the interaction between the fly orthologues of Pals1 and mLin-7 is employed in epithelia for the recruitment of DLin-7 to the Crb-Sdt complex within the SAR.

The virtual absence of DLin-7 from basolateral plasma membrane compartments in *Drosophila* imaginal disc epithelia is in striking contrast to the situation in both mammals and nematodes. Differences in the expression, subcellular localization and binding capacity of potential interaction partners may account for this discrepancy. Two types of evolutionary conserved proteins have been implicated in the basolateral membrane recruitment of LIN-7 and mLin-7: the MAGUKs LIN-2/mLin-2 (CASK) and β-catenin. The latter was found to recruit mLin-7 to cadherin-based epithelial junctions via its C-terminal PDZ-binding motif (Perego et al., 2000). Although this motif (*t*DTDL) is conserved in *C. elegans* β-catenin, it is aberrant in the fly orthologue, Armadillo (*t*DTDC). In fact, a direct interaction between DLin-7 and Armadillo was not detectable in a yeast two-hybrid assay (A.B., unpublished). Hence it appears unlikely that DLin-7 and Armadillo exhibit a mode of interaction similar to that of their counterparts in mammals. In contrast, DLin-2 (Caki/CamGUK) and DLin-7 displayed strong interaction in the yeast two-hybrid assay. Therefore we would expect DLin-2 to compete with Sdt for binding to the L27 domain of DLin-7 when expressed in epithelia. An epithelial expression of DLin-2, however, has not yet been documented and, instead, both immunostainings and mRNA analyses revealed that DLin-2 is predominantly expressed in the CNS (Martin and Ollo, 1996).

Sdt is not expressed at detectable levels at larval NMJs (A.B. and U.T., unpublished) and thus cannot contribute to the postsynaptic enrichment of DLin-7 at these junctions. Instead we could demonstrate that Dlg-S97 is required for the recruitment of DLin-7 to scaffolding complexes within the SSR around type I boutons. Severe mutations in *dlg* cause a decrease in the length of the SSR to about 40% [relative to bouton size (Lahey et al., 1994; Koh et al., 1999)]. In immunofluorescence analyses, however, the reduction of both endogeneous or Flag-tagged DLin-7 at $dlg^{XI\text{-}2}$ mutant NMJs appeared clearly more dramatic, suggesting that impaired recruitment of DLin-7 is not simply due to reduced SSR complexity. This reasoning is supported by our co-immunoprecipitation experiments that revealed a physical linkage between DLin-7 and Dlg-S97. This linkage is most likely indirect, as implied by the failure of EGFP-Dlg-S97 to recruit cytosolic DLin-7 in epithelia. Although we could monitor an interaction between DLin-7 and the N-terminal domain of Dlg-S97 in yeast, we also noted that this interaction is much weaker compared with those displayed by DLin-7 in combination with DLin-2 or Sdt. In accordance with recent biochemical studies and cell culture assays, which imply the coupling of SAP97 and mLin-7 via MAGUKs such as mLin-2 or MPP3 (Lee et al., 2002; Karnak et al., 2002), we therefore propose that Dlg-S97 and DLin-7 are linked via an intermediate protein factor. In fact, both the N-terminal domain of Dlg-S97 and DLin-7 can bind to L27 domains of DLin-2 in vitro (Lee et al., 2002) (M.T. and U.T., unpublished). The presence of DLin-2 at larval NMJs, however, remains questionable. Unfortunately we were not successful in employing an antibody against DLin-2 (Ollo and Martin, 1996) to address this issue in further detail. Third instar larvae that are homo- or hemizygous for the *DLin-2* mutant allele $caki^{x\text{-}307}$ exhibit normal levels of both DLin7- and Dlg-S97-specific immunofluorescence (U.T., unpublished). This allele has been characterized as a deletion that removes large portions of the gene including the region encoding the PDZ-, SH3- and GUK domains of DLin-2 (Ollo and Martin, 1996). Nonetheless some residual function might be displayed by a truncated DLin-$2^{x\text{-}307}$ mutant isoform. Thus, our observations strongly argue against, but do not completely rule out, an involvement of DLin-2 in the recruitment of DLin-7 to NMJs. It should be noted, however, that Dlg-S97, DLin-2 and DLin-7 could co-assemble into synaptic protein complexes in the CNS where they are found equally enriched within the neuropil regions (Mendoza et al., 2003; Ollo and Martin, 1996) (this study).

In light of recent work, which has revealed complex intramolecular interactions displayed by SAP97 (Wu et al., 2000; Paarmann et al., 2002), one should also consider the possibility that the SAP97-type N-terminus is only accessible upon binding of tissue- or compartment-specific factors to other domains within Dlg-S97. This mode of regulation, however, would not apply to Dlg-S97N-EGFP and thus cannot explain its inability to induce nuclear targeting of DLin-7 in epithelia (Fig. 9H-J).

It has been proposed that the targeting of SAP97 to epithelial membranes depends on mLin-2. This hypothesis was based on the finding that the expression of truncated mLin-2 exerts a dominant-negative effect on the localization of SAP97 in cultured epithelial cells (Lee et al., 2002). We would like to stress that this hierarchical mode does not apply to the respective fly homologues, since Dlg-A, which lacks the SAP97-type N-terminus, is efficiently targeted to epithelial septate junctions and to NMJs (Hough et al., 1997; Thomas et al., 2000). Moreover, the recruitment of Dlg-S97 by a DLin-7 binding MAGUK could hardly explain the Dlg-S97-dependent recruitment of DLin-7.

Our analyses strongly suggest that the interactions between DLin-7 and Sdt or Dlg-S97 take place within the respective submembraneous target regions. In addition, these interactions could play a role during the trafficking of DLin-7. In mammalian neurons mLin-7 was found in a complex with mLin-2, mLin-10 and the NMDA-type glutamate receptor subunit NR2B on dendritic vesicles, which are transported along microtubules (Setou et al., 2000). Likewise, the subcellular targeting of Dlg-like MAGUKs involves the association with vesicles and/ or intracellular membrane

compartments and depends on microtubular transport (El-Husseini et al., 2000; Thomas et al., 2000).

In vertebrates, mLin-7 isoforms have been detected in axonal and dendritic compartments (Butz et al., 1998; Jo et al., 1999). The postsynaptic colocalization of DLin-7 and Dlg-S97 is reminiscent of the association of mLin-7 with PSD-95/SAP90, a prominent Dlg-like MAGUK present in postsynaptic densities of vertebrate neurons (Jo et al., 1999). Interestingly, a recently discovered isoform of PSD-95 (PSD-95β) exhibits a SAP97-type N-terminus with conserved binding properties (Chetkovich et al., 2002). In light of our findings we speculate that PSD-95β, as opposed to conventional PSD-95, is involved in the postsynaptic recruitment of mLin-7. SAP97 could also serve this role, although a physical association of SAP97 and mLin-7 at synaptic junctions has not yet been reported. A possible association of DLin-7 with the presynaptic membrane of synaptic boutons can hardly be resolved by confocal microscopy in the presence of strong postsynaptic immunoreactivity. Targeted expression of Flag-DLin-7 in motorneurons did not yield considerable immunofluorescence signals at NMJs, suggesting that DLin-7 is barely targeted to presynaptic nerve terminals (data not shown). It should be noted, however, that the relative pre- versus postsynaptic abundance of a protein does not necessarily reflect its functional impact on either side of the synaptic cleft. For instance, while Dlg, Scrib and D-VAP-33A are clearly enriched postsynaptically at larval NMJs, genetic rescue and gain-of-function experiments have highlighted the importance of the minor presynaptic component in all three cases (Thomas et al., 1997; Roche et al., 2002; Pennetta et al., 2002).

The roles of DLin-7 within the SAR and at synapses remain elusive. Overexpression of Flag-DLin-7 did not result in easily detectable phenotypes within epithelia or at NMJs. Our analyses allow us to predict that DLin-7 acts downstream of Sdt or Dlg-S97. Accordingly, loss-of-function alleles of *DLin-7* are expected to mimic previously described or yet concealed phenotypical aspects of *sdt* and *dlg* mutants. The partial reduction of DLin-7 as achieved by transgenic expression of dsRNA had no obvious effect on the shape of boutons or on epithelial polarity. Current studies in our labs are therefore aimed at both the generation of complete loss-of-function alleles and monitoring more subtle phenotypes. In accordance with previous studies in other species, we can expect DLin-7 to bind at least one ligand via its single PDZ domain. Thereby it may help to retain this ligand within the respective compartment and/or to regulate its endosomal sorting (Kaech et al., 1998; Perego et al., 1999; Straight et al., 2001; Chetkovich et al., 2002).

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References

Bachmann, A., Schneider, M., Theilenberg, E., Grawe, F. and Knust, E. (2001). Drosophila Stardust is a partner of Crumbs in the control of epithelial cell polarity. *Nature* **414**, 638-643.

Baude, A., Nusser, Z., Roberts, J. D., Mulvihill, E., McIlhinney, R. A. and Somogyi, P. (1993). The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron* **11**, 771-787.

Bellen, H. J. and Budnik, V. (2000). The neuromuscular junction, In *Drosophila, a Laboratory Manual* (ed. M. Ashburner, S. Hawley and B. Sullivan), pp. 175-200. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Bilder, D., Li, M. and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science* **289**, 113-116.

Bilder, D., Schober, M. and Perrimon, N. (2003). Integrated activity of PDZ protein complexes regulates epithelial polarity. *Nat. Cell Biol.* **5**, 53-58.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Butz, S., Okamoto, M. and Südhof, T. C. (1998). A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain. (1998). *Cell* **94**, 773-782.

Chetkovich, D. M., Bunn, R. C., Kuo, S.-H., Kawasaki, Y., Kohwi, M. and Bredt, D. S. (2002). Postsynaptic targeting of alternative Postsynaptic Density-95 isoforms by distinct mechanisms. *J. Neurosci*. **22**, 6415-6425.

El-Husseini, A. E., Craven, S. E., Chetkovich, D. M., Firestein, B. L., Schnell, E., Aoki, C. and Bredt, D. S. (2000). Dual palmitoylation of PSD-95 mediates its vesiculotubular sorting, postsynaptic targeting, and ion channel clustering. *J. Cell Biol.* **148**, 159-172.

Garner, C. C., Kindler, S. and Gundelfinger, E. D. (2000a). Molecular determinants of presynaptic active zones. *Curr. Opin. Neurobiol*. **10**, 321-327.

Garner, C. C., Nash, J. and Huganir, R. L. (2000b). PDZ domains in synapse assembly and signalling. *Trends Cell Biol*. **10**, 274-280.

Gonzalez-Gaitan, M. and Jaeckle, H. (1997). Role of Drosophila alpha-adaptin in presynaptic vesicle recycling. *Cell* **88**, 767-776.

Gonzalez-Mariscal, L., Betanzos, A. and Avila-Flores, A. (2000). MAGUK proteins: structure and role in the tight junction. *Semin. Cell Dev. Biol*. **11**, 315-324.

Gramates, L. S. and Budnik, V. (1999). Assembly and maturation of the Drosophila larval neuromuscular junction, In *Neuromuscular Junctions in Drosophila* (ed. L. S. Gramates and V. Budnik), pp. 93-117. San Diego: Academic Press.

Harris, B. Z. and Lin, W. A. (2001). Mechanism and role of PDZ domains in signaling complex assembly. *J. Cell Sci.* **114**, 3219-3231.

Hong, Y., Stronach, B., Perrimon, N., Jan, L. Y. and Jan, Y. N. (2001). Drosophila Stardust interacts with Crumbs to control polarity of epithelia but not neuroblasts. *Nature* **414**, 634-638.

Hough, C. D., Woods, D. F., Park, S. and Bryant, P. J. (1997). Organizing a functional junctional complex requires specific domains of the Drosophila MAGUK Discs large. *Genes Dev.* **11**, 3242-3253.

Irie, M., Hata, Y., Deguchi, M., Ide, N., Hirao, K., Yao, I., Nishioka, H. and Takai, Y. (1999). Isolation and characterization of mammalian homologues of *Caenorhabditis elegans lin-7*: localization at cell-cell junctions. *Oncogene* **18**, 2811-2817.

Jo, K., Derin, R., Li, M. and Bredt, D. S. (1999). Characterization of MALS/Velis-1, -2, and -3: a family of mammalian Lin-7 homologs enriched at brain synapses in association with the Postsynaptic Density-95/ NMDA receptor postsynaptic complex. *J. Neurosci*. **19**, 4189-4199.

Kaech, S. M., Whitfield, C. W. and Kim, S. K. (1998). The LIN-2/LIN-7/LIN-10 complex mediates basolateral membrane localization of the C. elegans EGF receptor LET-23 in vulval epithelial cells. *Cell* **94**, 761-771.

Kamberov, E., Makarova, O., Roh, M., Liu, A., Karnak, D., Straight, S. and Margolis, B. (2000). Molecular cloning and characterization of Pals, proteins associated with mLin-7. *J. Biol. Chem.* **275**, 11425-11431.

Karnak, D., Lee, S. and Margolis, B. (2002). Identification of multiple binding partners for the amino-terminal domain of synapse-associated protein 97. *J. Biol. Chem.* **277**, 46730-46735.

Klebes, A. and Knust, E. (2000). A conserved motif in Crumbs is required for E-cadherin localisation and zonula adherens formation in Drosophila. *Curr. Biol.* **10**, 76-85.

Knust, E. (2002). Regulation of epithelial cell shape and polarity by cell-cell adhesion. *Mol. Membr. Biol.* **19**, 113-120.

Knust, E. and Bossinger, O. (2002). Composition and formation of intercellular junctions in epithelial cells. *Science* **298**, 1955-1959.

Koh, Y. H., Popova, E., Thomas, U., Griffith, L. C. and Budnik, V. (1999).

Regulation of DLG localization at synapses by CaMKII-dependent phosphorylation. *Cell* **98**, 353-363.

Lahey, T., Gorzcyca, M., Jia, X.-X. and Budnik, V. (1994). The Drosophila tumor suppressor gene dlg is required for normal synaptic bouton structure. *Neuron* **13**, 823-835.

Lee, S., Fan, S., Makarova, O., Straight, S. and Margolis, B. (2002). A novel and conserved protein-protein interaction domain of mammalian Lin-2/CASK binds and recruits SAP97 to the lateral surface of epithelia. *Mol. Cell. Biol.* **22**, 1778-1791.

Marfatia, S. M., Byron, O., Campbell, G., Liu, S. C. and Chishti, A. H. (2000). Human homologue of the Drosophila discs large tumor suppressor protein forms an oligomer in solution. Identification of the self-association site. *J. Biol. Chem.* **275**, 13759-13770.

Martin, J.-R. and Ollo, R. (1996). A new Drosophila Ca^{2+}/ calmodulin-dependent protein kinase (Caki) is localized in the central nervous system and implicated in walking speed. *EMBO J.* **15**, 1865-1876.

Mathew, D., Gramates, L. S., Packard, M., Thomas, U., Bilder, D., Perrimon, N., Gorzcyca, M. and Budnik, V. (2002). Recruitment of Scribble to synaptic scaffolding complex requires GUK-holder, a novel DLG binding protein. *Curr. Biol.* **12**, 1-20.

McGee, A. W., Topinka, J. R., Hashimoto, K., Petralia, R. S., Kakizawa, S., Kauer, F., Aguilera-Moreno, A., Wenthold, R. J., Kano, M. and Bredt, D. S. (2001). PSD-93 knockout mice reveal that neuronal MAGUKs are not required for development or function of parallel fiber synapses in cerebellum. *J. Neurosci.* **21**, 3085-3091.

Mendoza, C., Olguin, P., Lafferte, G., Thomas, U., Ebitsch, S., Gundelfinger, E. D., Kukuljan, M. and Sierralte, J. (2003). Novel isoforms of Dlg are fundamental for neuronal development in Drosophila. *J. Neurosci*. **23**, 2093-2101.

Migaud, M., Charlesworth, P., Dempster, M., Webster, L. C., Watabe, A. M., Makhinson, M., He, Y., Ramsay, M. F., Morris, R. G. M., Morrison, J. H., O'Dell, T. J. and Grant, S. G. N. (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* **396**, 433-439.

Misawa, H., Kawasaki, Y., Mellor, J., Sweeney, N., Jo, K., Nicoll, R. A. and Bredt, D. S. (2001). Contrasting localizations of MALS/LIN-7 PDZ proteins in brain and molecular compensation in knockout mice. *J. Biol. Chem.* **276**, 9264-9272.

Müller, B. M., Kistner, U., Veh, R. W., Cases-Langhoff, C., Becker, B., Gundelfinger, E. D. and Garner, C. C. (1995). Molecular characterization and spatial distribution of SAP97, a novel presynaptic protein homologous to SAP90 and the Drosophila discs-large tumor suppressor protein. *J. Neurosci*. **15**, 2354-2366.

Nagel, A. C., Maier, D. and Preiss, A. (2002). Green fluorescent protein as a convenient and versatile marker for studies on functional genomics in *Drosophila*. *Dev. Genes Evol.* **212**, 93-98.

Paarmann, I., Spangenberg, O., Lavie, A. and Konrad, M. (2002). Formation of complexes between Ca^{2+} calmodulin and the synapse-associated protein SAP97 requires the SH3 domain-guanylate kinase domain-connecting HOOK region. *J. Biol. Chem*. **277**, 40832-40838.

Pennetta, G., Hiesinger, P. R., Fabian-Fine, R., Meinertzhagen, I. A. and Bellen, H. J. (2002). Drosophila VAP-33A directs bouton formation at neuromuscular junctions in a dosage-dependent manner. *Neuron* **35**, 291-306.

Perego, C., Vanoni, C., Villa, A., Longhi, R., Kaech, S. M., Frohli, E., Hajnai, A., Kim, S. K. and Pietrini, G. (1999). PDZ-mediated interactions retain the epithelial GABA transporter on the basolateral surface of polarized epithelial cells. *EMBO J.* **18**, 2384-2393.

Perego, C., Vanoni, C., Massari, S., Longhi, R. and Pietrini, G. (2000). Mammalian LIN-7 PDZ proteins associate with β-catenin at the cell-cell junctions of epithelia and neurons. *EMBO J.* **19**, 3978-3989.

Perego, C., Vanoni, C., Massari, S., Raimondi, A., Pola, S., Cattaneo, M. G., Francolini, M., Vicentini, L. M. and Pietrini, G. (2002). Invasive behaviour of glioblastoma cell lines is associated with altered organisation of the cadherin-catenin adhesion system. *J. Cell Sci.* **115**, 3331-3340.

Rasband, M. N., Park, E. W., Zhen, D., Arbuckle, M. I., Poliak, S., Peles, E., Grant, S. G. N. and Trimmer, J. S. (2002). Clustering of neuronal potassium channels is independent of their interaction with PSD-95. *J. Cell Biol*. **159**, 663-672.

Roche, J. P., Packard, M. C., Moeckel-Cole, S. and Budnik, V. (2002). Regulation of synaptic plasticity and synaptic vesicle dynamics by the PDZ protein Scribble. *J. Neurosci*. **22**, 6471-6479.

Roh, M., H., Makarova, O., Liu, C.-J., Shin, K. Y., Lee, S., Laurinec, S., Goyal, M., Wiggins, R. and Margolis, B. (2002). The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. *J. Cell Biol.* **157**, 161-172.

Setou, M., Nakagawa, T., Seog, D.-H. and Hirokawa, N. (2000). Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport. *Science* **288**, 1796-1802.

Sheng, M. and Sala, C. (2001). PDZ domains and the organization of supramolecular complexes. *Annu. Rev. Neurosci*. **24**, 299-325.

Simske, J. S., Kaech, S. M., Harp, S. A. and Kim, S. K. (1996). LET-23 receptor localization by the cell junction protein LIN-7 during C. elegans vulval induction. *Cell* **85**, 195-204.

Sone, M., Suzuki, E., Hoshino, M., Hou, D., Kuromi, H., Fukata, M., Kuroda, S., Kaibuchi, K., Nabshima, Y.-I. and Hama, C. (2000). Synaptic development is controlled in the periactive zone of Drosophila synapses. *Development* **127**, 4157-4168.

Speicher, S. A., Thomas, U., Hinz, U. and Knust, E. (1994). The *Serrate* locus of *Drosophila* and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. *Development* **120**, 535-544.

Spradling, A. C. (1986). P element-mediated transformation, In *Drosophila – A Practical Approach* (ed. D. B. Roberts), pp. 175-197. Oxford and Washington DC: IRL Press.

Straight, S. W., Karnak, D., Borg, J.-P., Kamberov, E., Dare, H., Margolis, B. and Wade, J. B. (2000). mLin-7 is localized to the basolateral surface of renal epithelia via its NH_2 terminus. *Am. J. Physiol. Renal Physiol.* **278**, F464-F475.

Straight, S. W., Chen, L., Karnak, D. and Margolis, B. (2001). Interaction with mLin-7 alters the targeting of endocytosed transmembrane proteins in mammalian epithelial cells. *Mol. Biol. Cell* **12**, 1329-1340.

Tanentzapf, G. and Tepass, U. (2003). Interactions between the *crumbs, lethal giant larvae* and *bazooka* pathways in epithelial polarization. *Nat. Cell Biol*. **5**, 46-52.

Tejedor, F. J., Bokhari, A., Rogero, O., Gorczyca, M., Zhang, J., Kim, E., Sheng, M. and Budnik, V. (1997). Essential role for *dlg* in synaptic clustering of shaker K channels in vivo. *J. Neurosci*. **17**, 152-159.

Tepass, U., Tanentzapf, G., Ward, G. and Fehon, R. (2001). Epithelial cell polarity and cell junctions in Drosophila. *Annu. Rev. Genet.* **35**, 747-784.

Thomas, U., Kim, E., Kuhlendahl, S., Koh, Y.-H., Gundelfinger, E. D., Sheng, M., Garner, C. C. and Budnik, V. (1997). Synaptic clustering of the cell adhesion molecule fasciclin II by discs-large and its role in the regulation of presynaptic structure. *Neuron* **19**, 787-799.

Thomas, U., Ebitsch, S., Gorzcyca, M., Koh, Y.-H., Hough, C. D., Woods, D., Gundelfinger, E. D. and Budnik, V. (2000). Synaptic targeting and localization of Discs-large is a stepwise process controlled by different domains of the protein. *Curr. Biol.* **10**, 1108-1117.

Tseng, T. C., Marfatia, S. M., Bryant, P. J., Pack, S., Zhuang, Z., O'Brien, J. E., Lin, L., Hanada, T. and Chishti, A. H. (2001). VAM-1: a new member of the MAGUK family binds to human Veli-1 through a conserved domain. *Biochim. Biophys. Acta.* **1518**, 249-259.

Uchida, N., Honjo, Y., Johnson, K. R., Wheelock, M. J. and Takeichi, M. (1996). The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones. *J. Cell Biol*. **135**, 767-779.

Woods, D. F., Hough, C., Peel, D., Callaini, G. and Bryant, P. J. (1996). Dlg protein is required for junction structure, cell polarity, and proliferation control in Drosophila epithelia. *J. Cell Biol.* **134**, 1469-1482.

Wu., H., Reuver, S. M., Kuhlendahl, S., Chung, W. J. and Garner, C. C. (1998). Subcellular targeting and cytoskeletal attachment of SAP97 to the epithelial lateral membrane. *J. Cell Sci.* **111**, 2365-2376.

Wu, H., Reissner, C., Kuhlendahl, S., Coblentz, B., Reuver, S., Kindler, S., Gundelfinger, E. D. and Garner, C. C. (2000). Intramolecular interactions regulate SAP97 binding to GKAP. *EMBO J.* **19**, 5740-5751.

Wu, H., Nash, J. E., Zamorano, P. and Garner, C. C. (2002). Interaction of SAP97 with minus-end-directed actin motor myosin VI. *J. Biol. Chem.* **277**, 30928-30934.

Zito, K., Fetter, R. D., Goodman, C. S. and Isacoff, E. Y. (1997). Synaptic clustering of Fascilin II and Shaker: essential targeting sequences and role of Dlg. *Neuron* **19**, 1007-1016.