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Discs-Large and Strabismus are functionally linked to plasma membrane formation

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Abstract

During early embryogenesis in *Drosophila melanogaster*, extensive vesicle transport occurs to build cell boundaries for 6,000 nuclei. Here we show that this important process depends on a functional complex formed between the tumour suppressor and adaptor protein Discs-Large (Dlg)¹ and the integral membrane protein Strabismus (Stbm)/Van Gogh (Vang)^{2,3}. In support of this idea, embryos with mutations in either *dlg* or *stbm* displayed severe defects in plasma membrane formation. Conversely, overexpression of Dlg and Stbm synergistically induced excessive plasma membrane formation. In addition, ectopic co-expression of Stbm (which associated with post-Golgi vesicles) and the mammalian Dlg homologue SAP97/hDlg^{4,5} promoted translocation of SAP97 from the cytoplasm to both post-Golgi vesicles and the plasma membrane. This effect was dependent on the interaction between Stbm and SAP97. These findings suggest that the Dlg–Stbm complex recruits membrane-associated proteins and lipids from internal membranes to sites of new plasma membrane formation.

Formation of new plasma membranes is dependent on the delivery of post-Golgi vesicles to a pre-existing plasma membrane. An excellent model system to study this process is the cellularization that occurs during early *Drosophila* embryogenesis. During cellularization, the embryonic plasma membrane must rapidly grow inwards to segregate thousands of nuclei, generated by synchronized cycles of nuclear division without cytokinesis, into individual cells⁶. This process occurs through addition of post-Golgi vesicles to defined regions of lengthening plasma membranes and may be required for establishing proper cell polarity⁷. The Dlg tumour suppressor is essential both for establishing proper cell polarity and for assembling multiprotein complexes at specialized cell–cell junctions⁸. Accumulating evidence also indicates that Dlg associates with its targets at intracellular membrane sites before accumulation at the plasma membrane^{9–12}. The integral membrane protein Stbm, however, is a regulator of planar tissue polarity in the fly^{2,3}, and mouse *Loop-tail (Lp)* and zebrafish *trilobite* mutants that also have mutations in *stbm* homologues display defects in gastrulation and neurogenesis^{13,14}. Here, we report that Dlg binds to Stbm and that this complex is required for formation of new plasma membranes during cellularization.

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We identified Stbm as a binding partner of Dlg using the first and second Dlg PDZ domains (Dlg-PDZ1–2; Fig. 1a) as bait in the yeast two-hybrid system. Stbm contains four putative transmembrane domains and a consensus PDZ-domain-binding motif (PBM) at its extreme carboxyl terminus (Fig. 1a)². In glutathione *S*-transferase (GST) pull-down assays, the 17 C-terminal residues of Stbm (GST–Stbm^{PBM}) were sufficient to mediate binding to Dlg-PDZ1–2 (see Supplementary Information, Fig. S1). Furthermore, changing - ETSV to - EASV (GST–Stbm^{PBM*}) abolished this interaction (see Supplementary Information, Fig. S1).

We used GST–Stbm^{WT} as an antigen to generate mouse polyclonal serum that specifically recognizes Stbm (Fig. 1b; also see Supplementary Information, Fig. S2). In embryonic extracts, the anti-Stbm, but not pre-immune, serum recognized a protein that migrates with a relative molecular mass (M_r) of 75K (Fig. 1b, left panel), slightly larger than the predicted 66K mass of Stbm. This 75K band was absent in 27% (8/30) of stage-16 embryos individually collected from the *Df(2R)NP4/CyO* adults, concordant with the predicted 25% inheritance frequency for a homozygous *stbm* deficiency (Fig. 1b, right). In addition, 75K Stbm co-immunoprecipitated with Dlg from fly embryonic extracts (Fig. 1c). Multiple alternative splicing isoforms of fly Dlg have recently been identified¹⁵, and in a reciprocal co-immunoprecipitation assay, three isoforms (110, 95 and 60K) co-immunoprecipitated with Stbm (Fig. 1d).

Similarly, fly Stbm co-immunoprecipitated with SAP97 from extracts of transfected COS-7 cells (Fig. 1e). This interaction is probably conserved as a result of high sequence similarity (60–70% identity) between the PDZ domains of fly and mammalian Dlg^{4,5}, as well as between Stbm family proteins^{2,13,14}. In contrast, Stbm with a disrupted PBM domain (Stbm^{PBM*}) or SAP97 missing the first and second PDZ domains (SAP97^{ΔPDZ1–2}) failed to bind to SAP97^{WT} or Stbm^{WT}, respectively (Fig. 1f), indicating that the interaction is mediated by the Stbm-PBM and Dlg-PDZ1–2 domains.

Consistent with reports that Stbm functions during early embryogenesis², we detected expression of Stbm throughout fly embryogenesis, including the 0–3-h stage (data not shown), indicating that Stbm is maternally contributed to fly eggs. We also found that 100% of *stbm*¹⁵/*stbm*¹⁵ or *stbm*⁶/*stbm*⁶ embryos, 94% (268/284) of *stbm*⁷⁻⁶/*stbm*⁷⁻⁶ embryos, 81% (281/318) of *stbm*⁷⁻⁶/*stbm*¹⁵³ embryos and 20% (92/471) of *stbm*¹⁵³/*stbm*¹⁵³ embryos obtained from *stbm* homozygous parents failed to hatch, and their phenotype was similar to unfertilized eggs (data not shown). The fact that only 1% of wild-type *Canton-S* (*CS*) embryos (5/390) displayed this defect indicates that parental Stbm is important during gametogenesis, fertilization or possibly both.

To evaluate *stbm* mutant embryos for hypomorphic phenotypes during early-stage embryogenesis, we crossed homozygous *stbm* adults and looked for defects in resulting *stbm*⁷⁻⁶/*stbm*⁷⁻⁶ or *stbm*⁷⁻⁶/*stbm*¹⁵³ embryos that were capable of reaching the 2–5-h stage of development (~10% and ~23% of embryos, respectively). In such *stbm* mutant embryos, we found that Dlg and Discs Lost (Dlt), which are maternally provided to eggs and show dynamic expression patterns during early embryogenesis¹⁶, failed to localize properly to the plasma membrane during the early cellularization stage (Fig. 2b). In contrast, control wild-type *CS* embryos exhibited regular honeycomb-like staining patterns for Dlg and Dlt at the plasma membrane (Fig. 2a). At later stages of cellularization, mutant embryos displayed increasingly severe abnormalities, particularly speckled Dlt and diffused Dlg staining (Fig. 2e–g). In control *CS* embryos, however, Dlg was distributed continuously along the plasma membrane at regions of cell–cell contact, and Dlt was enriched at invaginating cleavage furrows (Fig. 2c, d). Therefore, Stbm is required for proper localization of Dlg and Dlt during cellularization.

The absence of Dlg and Dlt in newly forming membranes of *stbm* mutant embryos (Fig. 2b) has two different possible interpretations: first, Dlg and Dlt fail to localize to plasma membranes, second, plasma membranes do not form. To distinguish between these two possibilities, we visualized plasma membranes in embryos by staining with fluorescein-conjugated concanavalin A (conA)¹⁷, which binds membrane glycoproteins and glycolipids. In wild-type *CS* embryos, Dlg and Dlt staining uniformly coincided with conA staining at the plasma membrane (100%, $n = 20$; Fig. 2h). In contrast, 60% of *stbm* embryos ($n = 20$) displayed regions lacking both Dlg and Dlt staining and, significantly, these regions invariably also lacked conA staining (Fig. 2i). This finding indicates that the formation of new plasma membranes is impaired in *stbm* mutant embryos.

To ascertain whether zygotic *Stbm* is involved in proper formation of plasma membranes, we analysed embryos obtained from *stbm*¹⁵/*CyO-GFP* parents. *stbm*¹⁵ (or *stbm*⁶) homozygous embryos, which contain maternal *Stbm*, but not zygotic wild-type *Stbm*, lacked detectable Dlg and Dlt staining in some cells, whereas this defect was absent in control embryos containing both wild-type maternal and zygotic *Stbm* (see Supplementary Information, Fig. S3). Therefore, we conclude that zygotic *Stbm* is required for membrane formation. A similarly important role for maternal *Stbm* in membrane formation was demonstrated in experiments showing that during cellularization, embryos derived from homozygous *stbm*⁷⁻⁶ females and wild-type *CS* males (and thereby containing zygotic but not maternal wild-type *Stbm*) also exhibited cells lacking detectable Dlg staining (data not shown).

Next, we determined the sub-cellular location of *Stbm*. In epithelial cells of larval imaginal discs, *Stbm* colocalized with Dlg at membrane cell-cell junctions (Fig. 3a) and was also detected in association with cytoplasmic vesicular structures (Fig. 3a'). Similar vesicular structures containing either Dlg, *Stbm* or both were also abundant within the cytoplasm of salivary gland duct cells (Fig. 3b), suggesting that *Stbm* may associate with endosomes or Golgi vesicles. In this regard, mammalian Dlg and Hrs (an endosomal protein that regulates protein sorting in yeast, fly and mammalian cells) have been reported to form an endosome-associated complex¹⁸. We consistently found that Dlg partially colocalizes with vesicular Hrs (Fig. 3c'', 3c'''). Although *Stbm* also associated with vesicle-like structures in embryos during cellularization (Fig. 3c'), its staining pattern did not coincide with vesicular Hrs (Figs. 3c', 3c'''), suggesting that unlike Dlg, *Stbm* does not localize to endosomes. Dlg and *Stbm* instead colocalized on distinct vesicle-like structures in the cytoplasm and near the plasma membrane within apical regions of epithelial cells (Fig. 3d). This finding prompted us to explore whether *Stbm* associates with the *Drosophila* Golgi apparatus. Confocal microscopic analyses revealed that *Stbm* colocalizes with the Golgi-specific marker GM130 (refs 19, 20) in vesicular structures of *CS* embryos both before and during cellularization (Fig. 3e, f), albeit most prominently during cellularization (Fig. 3g). Thus, *Stbm* dynamically localizes on Golgi vesicles and at the plasma membrane in various cell types.

Whereas the mammalian Golgi apparatus has characteristic stacked structures, or cisternae, which fragment transiently during mitosis, the fly Golgi apparatus remains largely dispersed throughout the cell cycle^{21,22}. Using *Stbm* antiserum that also reacts with mammalian *Stbm* in immunoblot assays (Fig. 3h), we showed that similarly to fly *Stbm*, mammalian *Stbm* localized predominantly on vesicles in TE85 and COS-7 cells. In TE85 cells that had just undergone mitosis (and therefore lacked defined Golgi stack structures) the majority of *Stbm* colocalized with GM130-containing vesicles (Fig. 3i). The size and pattern of vesicular structures containing *Stbm*, GM130, or both proteins, were similar in fly and mammalian cells (compare Fig. 3g with 3i). In TE85 cells displaying mature Golgi cisternae, however, *Stbm* was largely excluded from this structure, which was marked by endogenous GM130, a *cis*-Golgi marker¹⁹, or exogenous yellow fluorescent protein (YFP)–

p58, a *trans*-Golgi marker²³ (Fig. 3j, k). These observations demonstrate that Stbm associates with post-Golgi vesicles, but not with the mature Golgi apparatus, in mammalian cells.

We next investigated whether the interaction between Dlg and Stbm may localize one or both proteins to post-Golgi vesicles or to the plasma membrane. In TE85 cells, which express low endogenous levels of Stbm and SAP97, exogenously expressed fly Stbm localized to cytoplasmic vesicles (Fig. 4a), similarly to endogenous mammalian Stbm (Fig. 3i–k). Exogenously expressed YFP–SAP97 remained diffuse in the cytoplasm, with small amounts also detected at plasma membrane (Fig. 4b). In cells ectopically co-expressing Stbm and YFP–SAP97, however, YFP–SAP97 instead associated with Stbm-containing vesicles located proximal either to the nucleus or to the plasma membrane (27/30 cells; Fig. 4c–f). This suggests that an interaction between Stbm and SAP97 is required for mobilization of SAP97, because YFP–SAP97 and SAP97 were efficiently recruited to vesicles by wild-type Stbm (Fig. 4c, e, g), but not by Stbm^{PBM*} (27/32 cells; Fig. 4h). Similarly, we failed to detect vesicle mobilization of SAP97 in cells ectopically co-expressing either YFP–SAP97 and Stbm^{PBM*} (data not shown), or SAP97^{ΔPDZ1–2} and wild-type Stbm (Fig. 4i). In addition, wild-type Stbm or Stbm^{PBM*} expressed alone localized to vesicle-like structures, suggesting that Dlg does not function reciprocally to recruit Stbm to vesicles (Fig. 4a; data not shown). It should also be noted that cells expressing both SAP97 and Stbm exhibited extensive membrane ruffling and filopodial formation (Fig. 4e, g), suggesting that this complex may function to regulate plasma membrane growth and maintenance.

The proposed role of Dlg and Stbm in plasma membrane formation was further investigated in flies by testing the effects of overexpressing SAP97 and Stbm on the distribution of Neurexin (Nrx), a transmembrane protein concentrated at septate junctions of fly cells²⁴. Wing epithelial cells overexpressing SAP97 alone exhibited abnormal aggregations of SAP97 in the cytoplasm (Fig. 4j), and there was no noticeable change in the Nrx pattern at the basolateral membrane (Fig. 4l). However, wing epithelial cells expressing both SAP97 and Stbm showed abnormally extended basolateral membrane regions, where both SAP97 and Nrx were localized (Fig. 4k, m). A similar effect was observed in peripodial cells of eye-antenna discs overexpressing fly Dlg and Stbm (see Supplementary Information, Fig. S4). These findings suggest that elevated levels of Dlg–Stbm complexes in cells stimulate formation of plasma membranes, perhaps by increasing vesicle transport to these sites.

These observations suggest that similarly to Stbm, Dlg should be necessary for formation of plasma membranes. To test this hypothesis, we investigated whether temperature-sensitive *dlg*^{HF321} embryos cultured at the restrictive temperature showed defects in localization of Dlt. *dlg*^{HF321} embryos cultured at the restrictive temperature are known to exhibit developmental defects, such as dorsal closure and head involution²⁵, but a possible role for *dlg* in embryonic cellularization has not yet been addressed. We found that Dlg in *dlg*^{HF321} embryos incubated at the restrictive temperature is present primarily in cytoplasmic vesicle-like structures, whereas Dlt localization is either diffuse or aggregated (Fig. 5a, b). These results are in marked contrast to those obtained in *CS* embryos, where Dlg and Dlt exhibited prominent localization at the plasma membrane (Fig. 2a). In *dlg*^{HF321} embryos, Stbm retained a normal vesicular staining pattern (Fig. 5c).

If Dlg were essential for formation of plasma membranes, then this process should also be impaired in rapidly proliferating epithelial cells of *dlg* mutant imaginal discs. In small *dlg*^{m52} mutant clones where Dlg was not detected, Nrx levels were greatly reduced, resulting in disruption of septate junctions, whereas Nrx was not detected in larger mutant clones (Fig. 5d). As determined by Dlt staining, plasma membranes were also malformed and

disrupted into vesicles in *dlgm*⁵² clones (Fig. 5e). Similar defects were evident in *dlgm*³⁰ and *dlg*^{X1-2} mutant clones (data not shown). As expected, the staining pattern of Stbm remained vesicular in the *dlg* mutant clones, although the number of Stbm-containing vesicles located apically was somewhat reduced (data not shown). These results indicate that similarly to Stbm, Dlg is essential for plasma membrane formation in both embryos and larvae.

Proteins involved in establishing planar polarity, such as Stbm, Dishevelled (Dsh), Frizzled, Flamingo and Prickle (Pk), show dynamic changes in their localization between the plasma membrane and cytoplasmic vesicular structures^{26–28}. The localization of these proteins most probably requires transport machinery capable of rapid activation at precise moments during development. Stbm was recently shown to bind directly to Dsh and Pk, and to induce their translocation to the plasma membrane²⁸. Therefore, the processes of planar polarity and plasma membrane formation may depend on the ability of Stbm to mediate vesicle transport from the Golgi apparatus to the plasma membrane. Whether these tissue polarity proteins are simply mobilized by Stbm or actively participate in vesicle transport remains to be determined.

Tumour suppressors, such as Dlg, Lethal-giant larvae (Lgl) and Scribble (Scrib), as well as other PDZ domain- or PBM-containing proteins such as Bazooka, Crumbs (Crb), Stardust and Dlt, have important roles in assembling cell–cell junctions and in establishing and maintaining cell polarity^{8,29,30}. Interestingly, the yeast and mammalian Lgl homologues function in fusion of post-Golgi vesicles to the plasma membrane by interacting with Syntaxin^{31,32}. Syntaxin, which controls membrane traffic in both neuronal and non-neuronal cells, has also been shown to be essential for embryonic cellularization¹⁷. In addition, *stbm* and *scrib* exhibit a genetic interaction in wild-type mice, and *Lp* and *circletail* mutant mice with mutations in *stbm* and *scrib*, respectively, develop neural tube defects³³. Taken together, our results and those of others suggest that Dlg, Lgl, Scrib and Stbm functionally interact with components of an undetermined vesicular transport apparatus to promote formation of plasma membranes with proper apical–basal polarity. Interestingly, a recent report has shown that SAP102, a brain-enriched mammalian homologue of fly Dlg, regulates the trafficking of NMDA receptors through an interaction with Sec8, a component of the exocyst complex³⁴. Components of the exocyst complex often concentrate at points of rapid membrane addition, and it is believed that this complex functions to direct intracellular membrane vesicles to their sites of fusion with the plasma membrane³⁵. Determining whether the exocyst complex contributes to the vesicle transport pathway that we suggest is controlled by the Dlg–Stbm complex requires further investigation. In conclusion, dissecting molecular mechanisms for Stbm and Dlg may be key to understanding how vesicle transport is coupled to the formation of plasma membranes and cell–cell junctions, as well as to the establishment of cell polarity.

METHODS

Fly strains

Stbm mutants, *dlg* mutants and *UAS-dlg*, or *UAS-eGFPSAP97* were kindly provided by T. Wolff, P. Bryant and U. Thomas, respectively. *UAS-stbm* flies were generated using a *stbm* cDNA obtained from T. Wolff². Other fly strains were from the Bloomington Stock Center.

To evaluate *dlg* mutant phenotypes, *dlg*^{HF321} embryos were collected from homozygous mutant adults maintained at 25 °C for 5 h. *dlg* mutant clones in imaginal discs were generated by Flp-mediated mitotic recombination in late-first- to early-second-instar larval

progeny obtained by crossing *dlg FRT18A* females to *w, hs-FLP, P[mini- w^+ *hs-pM*]5A, 10D, P[ry⁺; *hs-neo*; *FRT*]18A* males.

Yeast two-hybrid screen

A cDNA encoding Dlg PDZ1 and PDZ2 (Dlg-PDZ1–2) was generated by PCR using larval poly(A)⁺ RNA as a template and cloned into the *NdeI* site of the pAS1 vector to generate pAS-dlg. A larval cDNA library constructed in the pACT vector was screened with the bait plasmid pAS-dlg, as described³⁶.

Antibodies

The Dlg-PDZ1–2 cDNA was subcloned into the pGEX vector. The fusion protein expressed by this plasmid was affinity purified and used as an antigen to generate Dlg antiserum in rabbits. Stbm polyclonal antiserum raised to an affinity purified GST–Stbm lacking the amino-terminal 41 residues was produced by the Caltech Monoclonal facility, California Institute of Technology, Pasadena, CA (see Supplementary Information, Fig. S1).

Transfections

COS-7 or TE85 cells were maintained in DMEM supplemented with gentamicin and 10% foetal bovine serum. Transfections were performed with Fugene6 according to manufacturers' recommendations (Roche, Indianapolis, IN). Experimental analyses were routinely conducted 48 h after transfection. All SAP97 expression constructs were derived from a GW1-SAP97 plasmid that expresses the full-length I2 isoform. The YFP cDNA present in the GW1-YFP-SAP97 plasmid was derived from the pEYFP-C1 vector (Clontech, Palo Alto, CA). The GW1-SAP97 Δ PDZ1–2 plasmid contains a SAP97 cDNA lacking SAP97 amino-acid residues 193–336 (ref. 5).

Immunoprecipitation and immunoblot assays

For immunoblot analyses, fly embryos were homogenized in homogenization buffer (20 mM Hepes at pH 7.6, 70 mM potassium chloride, 2 mM dithiothreitol, 0.1% NP40, 8% glycerol, 1 mM phenyl methylsulphonyl fluoride, Complete protease inhibitors (Roche), EGTA and 1,10-phenanthroline). Homogenates were precleared by centrifugation for 5 min at 5,000g and equal amounts of protein (as determined by a Bradford assay (Bio-Rad, Hercules, CA)) were separated by SDS–PAGE and transferred to nitrocellulose membrane. Mouse anti-Stbm and rabbit anti-Dlg antisera were used at 1:1,000 and 1:10,000, respectively, for immunoblot analyses. For immunoprecipitation assays, fly embryonic extracts (10 mg of protein in 1 ml of homogenization buffer) were incubated overnight at 4 °C with 20 μ l anti-Stbm or 6 μ l anti-Dlg antibody, and 30 μ g Sepharose 6MB (Amersham). Sepharose beads were then washed extensively with the homogenization buffer. Mammalian cell extracts were generated in NETN buffer (20 mM Tris-HCl at pH 8.0, 100 mM sodium chloride, 1 mM EDTA and 0.5% NP-40) supplemented with protease inhibitors and phosphatase inhibitors. Immunoprecipitation was performed as previously described³⁷.

Immunocytochemistry

Embryos were fixed in 4% methanol-free formaldehyde (Polysciences, Warrington, PA). Samples were then stained with mouse antibodies to Stbm and rabbit antibodies to Dlg and GM130 (both at 1:1,000 dilution). TE85 or COS-7 cells were transfected on glass coverslips, fixed for 30 min in 4% paraformaldehyde (Polysciences) and then permeabilized for 5 min in 0.5% Triton X-100. Cells were blocked with Tris-buffered saline containing 10% goat serum, incubated with pre-immune serum or the indicated antiserum and then appropriate secondary antibodies. After extensive washes, cells were then fixed again for 10 min in 4% paraformaldehyde. Fluorescent images were captured using Zeiss LSM laser-

scanning confocal microscope (Zeiss, Thornwood, NY) and presented using Adobe Photoshop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Dlg interacts with Stbm. **(a)** Domain structures of Dlg and Stbm. **(b)** Stbm antisera, but not pre-immune serum, recognizes a 75K band in embryonic extracts (left). Western blot of two individual embryos (stage 16) derived from *Df(2R)NP4/CyO* parents (right). All control *CS* embryos expressed the 75K protein (data not shown). **(c, d)** Stbm and Dlg (arrow) co-immunoprecipitate from embryo extracts. **(e, f)** Stbm and Dlg also interact in COS-7 cells. Combinations of transfected expression plasmids are indicated. Stbm recovered from the immunocomplex, as shown in **e** and **f**, or expressed endogenously in COS-7 cells, as shown

in **e**, is indicated by arrows or an arrowhead, respectively. The size of fly Stbm expressed in the COS-7 cells (asterisk in **f**) is shown for comparison.

Figure 2.

Epithelial cells of *stbm* mutant embryos exhibit defects in membrane formation. The source of specimen and visualized proteins are indicated at the upper or lower right corners, respectively. All panels are single confocal images, except **a** and **b**, which are composites from multiple confocal sections spanning the vertical cell boundary. (**a**, **b**) Cross-section showing membrane pattern of *CS* and *stbm*⁷⁻⁶ embryos during cellularization. Arrows indicate absence of Dlg and Dlt. (**c**-**f**) Tangential sections (**c**, **e**) and cross-sections (**d**, **f**) of *CS* (**c**, **d**) and *stbm*⁷⁻⁶ (**e**, **f**) embryos. Arrows in **c** and **e** mark the cleavage furrow where confocal sections in **d** and **f** were taken, respectively. Arrowheads indicate Dlt pattern. (**g**)

*stbm*⁷⁻⁶/*stbm*¹⁵³ embryo with similar phenotype as *stbm*⁷⁻⁶ in **e**. Abnormal Dlt pattern is marked with an arrowhead. (**h**, **i**) Visualization of membrane structure with conA in *CS* (**h**) and *stbm*⁷⁻⁶ (**i**) embryos. Arrowheads in **h** and **i** indicate the presence and absence, respectively, of Dlg, Dlt and conA. Scale bar represents 10 μ m.

Figure 3.

Expression pattern of Stbm and Dlg in fly and mammalian cells. **(a)** Localization of Stbm in plasma membrane (arrowhead) and vesicle-like structures (arrow) from wing imaginal disc. **(b)** Expression of Dlg and Stbm in numerous vesicles of salivary gland duct cells. The basolateral plasma membrane slightly below the septate junction is marked with an arrowhead. **(c)** Localization of Stbm, Dlg and Hrs in cellularizing embryos. **(d)** Colocalization of Stbm and Dlg in vesicle-like structures (arrowheads) and patches of the plasma membrane (arrow). **(e, f)** Colocalization of Stbm and GM130 in vesicular structures in embryos before cellularization. Dashed line indicates surface of embryo. The region

marked with a rectangle is magnified in **f**. **(g)** Vesicles in an cellularizing embryo containing Stbm (arrow), GM130 (asterisk), or both (arrowhead). **(h)** Detection of endogenous Stbm in human cell lines TE85 and 293, mouse cell line NIH3T3, and fly embryos. **(i)** Vesicles containing Stbm, GM130 or both in a TE85 cell lacking Golgi stack structure. Nucleus (N) is outlined. **(j, k)** TE85 cells with extensive Golgi stacks stained for endogenous GM130 and endogenous Stbm (**j**) or for exogenous YFP-p58 and endogenous Stbm (**k**). Nuclei were stained with DAPI. Scale bar represents 5 μm in **a** and **b**, 10 μm in **c**, 7 μm in **d** and **e**, 4 μm in **f–i** and 25 μm in **j** and **k**.

Figure 4.

Dlg and Stbm must interact to colocalize and promote membrane formation. Various cDNAs transfected into TE85 cells are indicated at upper right corners. Nuclei (N) were visualized with DAPI (data not shown). (a) Vesicular localization of ectopically expressed fly Stbm. (b) Cytoplasmic localization of ectopically expressed YFP-SAP97. (c-f) Pattern of YFP-SAP97 in cells cotransfected with YFP-SAP97 and stbm. The regions marked with arrows in c and arrowhead in e are enlarged in d and f, respectively. Vesicles containing both YFP-SAP97 and fly Stbm are marked with arrows. (g-i) Colocalization of SAP97 and Stbm in cells transfected with fly stbm and SAP97 (g), but not with fly stbm^{PBM} and SAP97 (h) or

fly stbm and SAP97^{ΔPDZ1-2} (**i**). (**j–m**) Tangential views of the epithelial cells from wing disc expressing GFP–SAP97 alone (**j**) or together with Stbm (**k**). The UAS and Gal4 lines used to drive overexpression of Stbm or GFP–SAP97 are indicated at the upper right corners. The regions marked with rectangles in **j** and **k** are magnified and shown with the Nr_x pattern in **l** and **m**, respectively. The extent of basolateral membrane visualized by Nr_x is marked by brackets (**l**, **m**). One example of aggregated Dlg structures is indicated by an arrow (**j**). Scale bar represents 10 μm in **a–c**, **e** and **g–k**, 2.5 μm in **d** and **f**, and 5 μm in **l** and **m**.

Figure 5.

Dlg is required for plasma membrane formation in both embryos and larvae. (a) Tangential view of a *dlg*^{HF321} embryo cultured at restrictive temperature lacks Dlg at the membrane. (b) Composite images of multiple cross-sections spanning the vertical cell boundary of the same embryo, revealing mis-localization of Dlg and Dlt. (c) A similar *dlg*^{HF321} embryo, showing cytoplasmic vesicle-like structures containing Stbm. Dlg localizes diffusely in the cytoplasm or in vesicle-like structures. (d, e) *dlg*^{m52} mutant clones are marked by the absence of Myc marker. Myc is expressed on the *dlg*⁺ chromosome. A mutant clone (*dlg*⁻/*dlg*⁻) and wild-type clones (twin spots; *dlg*⁺/*dlg*⁺) are marked by yellow and red lines,

respectively. Unmarked cells are dlg^+/dlg^- . Localization of Nr x at the septate junction is more diffuse (arrowhead) or absent (arrow) in the dlg^- clone. Note the higher level of Nr x in twin spot than in dlg^+/dlg^- cells (**d**). Membrane structure of the dlg^- clone is visualized with Dlt (**e**), and a portion is magnified at the bottom left corner (**e'**). Scale bar represents 10 μ m in **a–c**, and 20 μ m in **d** and **e**.