Loss- and gain-of-function analysis of the lipid raft proteins Reggie/Flotillin in *Drosophila*: They are posttranslationally regulated, and misexpression interferes with wing and eye development

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Reggie/Flotillin proteins are upregulated after optic nerve dissection and evolutionary highly conserved components of lipid rafts. Whereas many biochemical and cell culture studies suggest an involvement in the assembly of multiprotein complexes at cell contact sites, not much is known about their biological *in vivo* functions. We therefore set out to study the expression pattern and the effects of loss- and gain-of-function in the *Drosophila melanogaster* model system. We found that in flies these proteins are mainly expressed in axons at the root of fiber tracts, in places where strong fasciculation is required, e.g. at the neck of the peduncle of the mushroom bodies and in the optic chiasmata. Despite their evolutionary conservation which implies fundamental and important functions, a P-element-induced null mutant (KG00210) of *reggie1/lotillin2* (*reggie1/lot2*) in *D. melanogaster* shows no apparent phenotypic defects. This was even more surprising as we show that in this *reggie1/lot2* null mutant the paralogous Reggie2/Flot1 protein is unstable and degraded, while the transcript is still present. The requirement of Reggie1/Flot2 for Reggie2/Flot1 stabilization is confirmed by misexpression experiments. Reggie2/Flot1 can only be misexpressed when Reggie1/Flot2 is provided as well. Conversely, Reggie1/Flot2 immunoreactivity can be detected, when its transgene is misexpressed alone. Using appropriate Gal4 driver lines, misexpression of Reggie1/Flot2 alone or together with Reggie2/Flot1 in the eye imaginal disc results in a specific and severe mislocalization of cell adhesion molecules of the immunoglobulin superfamily (IgCAMs) (while DE-Cadherin is unaffected) and in differentiation defects pointing to impaired signaling. In the wing imaginal disc, global overexpression of Reggie/Flotillin proteins leads to a significant extension of the Wingless signal and severely disrupts normal wing development. Our data support the notion that Reggie/Flotillin proteins are implicated in signaling processes at cellular contact sites.

Introduction

Lipid rafts are detergent resistant membrane (DRM) microdomains of distinct lipid and protein composition, which is reflected by the insolubility of DRM/raft proteins in nonionic detergents. Raft/DRM proteins promote the aggregation and interaction of specific sets of proteins and contribute to the spatial and temporal control of signaling processes (reviewed in Simons and Toomre, 2000; Tsui Pierchala et al., 2002; Benzing, 2004). Moreover, DRM/lipid rafts are involved in vesicle trafficking, protein sorting and phagosome biogenesis (Zajchowski and Robbins, 2002; Golub et al., 2004; Helms and Zurzolo, 2004).

Certain DRM/raft microdomains depend on specific scaffolding proteins. Caveolin, for instance, is responsible for the formation of caveolae, whereas stomatins, Podocin and Reggie 1 and 2 (Schulte et al., 1997; Lang et al., 1998) also known as Flotillin 2 and 1, respectively (Bickel et al., 1997) are responsible for the formation of non caveolar flat DRM/rafts (Stuermer et al., 2001). They were initially identified as a pair of closely related proteins in a screen for antigens that are upregulated in retinal ganglion cells with regenerating axons in the goldfish after optic nerve transection (Schulte et al., 1997) and therefore named Reggie 1 and Reggie 2.

Both Reggie/Flotillin proteins are evolutionarily highly conserved and exist in most animals, including humans (Bickel et al., 1997; Galbiati et al., 1998; Malaga Trillo et al., 2002). Homologous proteins also exist in prokaryotes and plants which suggests an important and ancient role in cellular development and maintenance (Stuermer and Plattner, 2005). In vertebrates, Reggies/Flotillins are most strongly expressed in the nervous system (Bickel et al., 1997; von Philipsborn et al., 2005). Reggies/Flotillins are present in a multitude of cells in culture (Volonte et al., 1999; Stuermer et al., 2001; Rajendran et al.,

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2003) and are consistently enriched in the DRM/lipid raft fraction (Bickel et al., 1997; Rajendran et al., 2003; Deininger et al., 2003; Stuermer et al., 2004). Moreover, Reggies/Flotillins oligomerize (Neumann Giesen et al., 2004) to form microdomain scaffolds (Stuermer and Plattner, 2005) for the spatial assembly of certain GPI anchored and transmembrane proteins and for communication with intracellular signal transducing molecules (Stuermer et al., 2001; Stuermer et al., 2004) much like Caveolin or Podocin.

Compelling evidence for the morphogenetic function of DRM scaffolding proteins has been found in the kidney. The kidney slit diaphragm is dependent on the ordered arrangement of Ig superfamily proteins, Neph and Nephrin, which possess large intracellular domains involved in the communication with Podocin and additional signaling molecules on the cytoplasmic face of the plasma membrane (Benzing, 2004). It was shown that Podocin contributes to the formation of this highly specialized DRM domain which enables Neph and Nephrin to interact along and across foot processes. Mutations in the Podocin encoding gene (Boute et al., 2000) lead to severe kidney dysfunction caused by the absence of the slit diaphragm.

Furthermore, Reggie/Flotillin proteins appear to coordinate the assembly of multiprotein complexes in certain cellular domains (Stuermer et al., 2001, 2004). For example, Reggies/Flotillins cluster at cell contact sites in vivo and in vitro (Stuermer and Plattner, 2005) and are upregulated in mouse fibroblast cell cultures with increasing confluency (Lopez Casas and del Mazo, 2003). They pattern the activation zone in lymphocytes, the cap, and represent platforms for the assembly of cell surface proteins such as Thy 1, F3 and PrP^c (Stuermer et al., 2001, 2004). Reggies/Flotillins thus seem to participate in cell recognition and adhesion (von Philipsborn et al., 2005; Harris and Siu, 2002) probably through the coordination of the relevant cell adhesion/recognition molecules and intracellular signaling molecules (Stuermer and Plattner, 2005). Emerging evidence suggests further that Reggies/Flotillins communicate with the actin cytoskeleton (Kioka et al., 2002; Neumann Giesen et al., 2004; Stuermer et al., 2004).

In the present study, we applied contemporary genetic techniques in combination with conventional cell biological methods to investigate the role of Reggie/Flotillin proteins in *Drosophila*. Available data indicate that *Drosophila reggie2/flo1* mRNA is predominantly expressed in the embryonic nervous system (Galbiati et al., 1998). We show here that Reggie/Flotillin proteins are also strongly expressed in neural tissues and that this expression is restricted to specific axonal sites. We furthermore demonstrate that *Drosophila* Reggies/Flotillins partition into the DRM/lipid raft fraction of embryonic membranes.

A null mutant for *reggie1/flo2* is viable and lacks a detectable external or internal mutant phenotype. This was surprising since we could show that this mutant is also depleted of Reggie2/Flo1 protein, whereas the transcript is still present. The Reggie2/Flo1 protein can furthermore only be misexpressed together with Reggie1/Flo2. We used misexpression of Reggies/Flotillins to study the interference with lipid raft/DRM domain dependent functions during imaginal disc development. Our data show that Reggies/Flotillins interfere with the distribution of cell adhesion and signaling molecules in imaginal discs.

Results

Structure and expression of Drosophila Reggie/Flotillin genes

The *Drosophila* genome contains two unlinked Reggie/Flotillin genes on chromosomes I (*flo2*; 12F5 13A1) and II (*flo1*, 52B1 2) which are orthologous to vertebrates *reggie1* and *reggie2*, respectively. Western blot and RT PCR analysis demonstrate that transcripts and translation products of both paralogues are expressed during all developmental stages of the fly. Extensive database analysis identified two *reggie1/flo2* EST populations, differing in 39 additional base pairs genomically encoded by a short exon. These transcripts represent the splice form *reggie1b/flo2b*, as opposed to the shorter *reggie1a/flo2a* form. Alternative splicing has not been reported in *reggie/flotillins* from other species, and the amino acid sequence encoded by this exon appears to represent an insertion specific to *Drosophila* (Fig. 1A). Furthermore, we showed the expression of both splice variants throughout development by RT PCR analysis (Figs. 1B, C). Comparison between the expression levels of both splice variants revealed that *reggie1b/flo2b* is strongly expressed during embryonic and larval development, whereas *reggie1a/flo2a* predominates in adult flies (Fig. 1C). Developmental Western blot analysis with Reggie specific antibodies also showed the expression of both *reggie/flotillin* genes throughout all developmental stages (Figs. 1D, E).

This expression pattern is consistent with in situ hybridization results reported previously (Galbiati et al., 1998). During later developmental stages, both Reggies/Flotillins show a complex expression pattern in the optic lobes and central brain (Fig. 2C) where antibody staining is restricted to axon fascicles. Expression was strongest in regions where axons or axon bundles from different directions join each other. This is particularly evident in the mushroom body (MB) (Fig. 2D), a paired structure in the insect central brain that plays an important role in higher order sensory integration and learning (Ito et al., 1997; Zars, 2000). Each of the two *Drosophila* MBs consists of about 2500 neurons called Kenyon cells (Ito et al., 1997) which are the descendants of four neuroblasts and which accordingly form four separate clusters. Labeling by anti Reggie/Flotillin antibodies is only observed where axons of the Kenyon cells from each cluster fasciculate and project into the peduncle (Fig. 2D). In the optic lobes, fiber tracts between the neuropils were labeled, but not the synaptic regions of the neuropils. Labeled fiber tracts comprise the outer and the inner optic chiasm and the neck of the fiber tracts connecting the lobula with the central brain (Figs. 2E, F). The serpentine layer in the medulla contains axons of large tangential neurons, which are weakly labeled (Figs. 2E, F). Reggie/Flotillin expression is clearly neuronal (as opposed to glial) as is exemplified in the optic chiasm of adult flies, where anti Reggie/Flotillin antibodies label the axon tracts and not the glial cells (Figs. 2G I).

KG00210 is a null mutant for Reggie1/Flo2 and is in addition depleted of Reggie2/Flo1

Based on the structural conservation, complex expression patterns and the developmental regulation, we expected to find phenotypic disturbances in *reggie/flotillin* loss of function or overexpression mutants. However, database surveys initially revealed no known mutant alleles for either Reggie1/Flo2 or

Fig. 1. (A) Schematic representation of the *Drosophila reggie1/flo2* locus. The alternatively spliced *Drosophila* specific exon is marked. The *reggie1a/flo2a* transcript (i.e. the isoform without the differentially spliced exon) is represented by the BDGP cDNA LD02571 and the *reggie1b/flo1b* transcript (containing the differentially spliced exon) by the BDGP cDNA LD07095. The amino acid sequence of the *Drosophila* specific exon is shown below the scheme. The size of exons and introns is indicated. (B, C) RT-PCR using primers specific for *reggie1/flo2* and *reggie2/flo1*, respectively, shows mRNA expression during all developmental stages. (D,E) Western blotting analysis shows the presence of a protein corresponding to the expected molecular weight of Reggie/Flotillin (47 kDa) during all developmental stages. The 44 kDa band (*) seen in embryo lysates is due to unspecific staining of abundant yolk proteins. The PVDF membrane was stripped and reprobbed with an antibody against β -Tubulin to obtain a measure for protein concentration. The relative intensities of Reggie/Flotillin bands in embryos are less strong than at later developmental stages where the stronger bands reflect the enrichment of Reggie/Flotillin proteins in brain and head lysates.

Reggie2/Flo1. To obtain mutants for either Reggie2/Flo1 or Reggie1/Flo2, we made use of the ongoing Berkeley *Drosophila* Genome Project Gene Disruption Project (Bellen et al., 2004; <http://flypush.imgen.bcm.tmc.edu/pscreen>). Several homozygous viable transposon insertion lines were identified. Of these, line KG00210 carries a P element insertion in exon 5 of the *reggie1/flo2* gene. To ascertain whether *reggie1/flo2* gene expression is affected by this P element, we performed an RT-PCR analysis on isolated RNA from adult fly heads. No *reggie1/flo2* mRNA could be detected in mutant flies (Fig. 3A). Interestingly, however, the P element mutant turned out to be not only deficient for the corresponding Reggie1/Flo2 translation product, but also for the paralogous Reggie2/Flo1 protein (Figs. 3B, C), whereas the *reggie2/flo1* mRNA was still detectable. Reggie2/Flo1 protein levels were downregulated to trace amounts as judged by Western blot analyses (Fig. 3C). Surprisingly, however, homozygous mutants displayed no obvious external morphological phenotype. Furthermore, no structural defects in the embryonic nervous system could be detected using immunofluorescence with the neuronal markers mab 22C10 (anti Futsch) and mab 1D4 (anti FasII). The

inner and the outer optic chiasms of the optic lobes, where Reggies/Flotillins are most strongly expressed (Figs. 2E, F), also showed no obvious defects, as judged by examination of horizontal paraffin sections of mutant adult fly heads (Fig. 3D). In these sections, the central brain including the mushroom bodies and the central complex was also undistinguishable from wild type. Viability, fertility, behavior and overall activity of the mutant flies showed no apparent abnormalities.

Reggies/Flotillins are constituents of Drosophila DRM domains/lipid rafts

In order to establish the membrane association and partitioning of Reggies/Flotillins, membranes isolated from *Drosophila* embryos were subjected to isopycnic centrifugation on either sucrose or Optiprep density gradients of lower osmolality after detergent extraction with Triton X 100. Both Reggies/Flotillins are present in the Triton X 100 insoluble DRM fractions of wild type embryos (Fig. 4A). These fractions also contain Fasciclin 1, a GPI linked protein that identifies the lipid raft fractions in *Drosophila*

Fig. 2. Reggie/Flotillin immunostaining in the *Drosophila* nervous system. (A, B) In stage 16 embryos, the stereotypical pattern of longitudinal tracts and commissures is labeled with antibodies against Reggie1/Flo2 (A) and Reggie2/Flo1 (B), respectively. (C) Frontal view of the right hemisphere of an adult brain, stained with an antibody against Reggie2/Flo1. (D) Adult mushroom body expressing membrane-targeted GFP (*OK107-GAL4; UAS-mCD8-GFP*) and labeled with anti-Reggie2/Flo1 antibodies (red). Overlapping areas appear in yellow. Two orthogonal projection views are shown (left). Cartoon of a mushroom body (Davis, 2001) with three cross-sectional views depicted to the right showing Reggie2/Flo1 immunoreactivity at the level of the root of the peduncle (right). (E, F) Horizontal view of optic lobes in adult flies stained with antibodies against Reggie1/Flo2 (E) and Reggie2/Flo1 (F), respectively. Reggie/Flotillin immunoreactivity is strongest in the optic chiasms where axon bundles cross between the different neuropils. s, serpentine layer of the medulla; (G–I) Reggie/Flotillin is expressed in neurons. The outer optic chiasm of a *repo-GAL4;UAS-mCD8-GFP* adult brain is shown. Glial cells are marked with GFP in green (G, I), Reggie1/Flo2 immunoreactivity is shown in red (H, I). Note that little overlap is detectable in the merged images (yellow, I). as, axis of symmetry; c, calyx; cb, central brain; m, medulla; mbc, mushroom body cortex; ol, optic lobe; p, peduncle of the mushroom body; Xo, outer optic chiasm; Xi, inner optic chiasm; X3, connection between lobula and central brain. Scale bars, 20 μ m.

(Rietveld et al., 1999) but lacks Na^+/K^+ ATPase, a marker for non raft fractions (Chamberlain, 2004). Recently, *Drosophila* Wnt 1 (Wingless) was shown to be recruited to lipid rafts in a cell culture overexpression paradigm (Zhai et al., 2004). Here, we show that a

fraction of endogenous Wingless is present in the DRM fraction. In the *Reggie/Flotillin* mutant KG00210 line, the fractionation pattern of Fasciclin 1 and Wingless was unchanged, despite the complete absence of both Reggies/Flotillins (Fig. 4B). Using the Optiprep

Fig. 3. Analysis of the *reggie1/flo2* P-element mutant KG00210. (A) RT-PCR using specific primers reveals that no *reggie1/flo2* mRNA is detected in the mutant (KG), whereas *reggie2/flo1* mRNA is present. RT-PCR with primers specific for the ribosomal protein rp49 served as control. (B, C) Western blots of lysates of *reggie1/flo2* mutant flies at different developmental stages. The blots were probed with antibodies specific for Reggie1/Flo2 (B) and Reggie2/Flo1 (C), respectively as indicated (arrowheads). For loading control, the blots were stripped and reprobed with an antibody against β -Tubulin. (B) No Reggie1/Flo2 was detected. The asterisk (*) marks non-specific labeling of the abundant embryonic yolk proteins and serves as an internal control (compare to wild-type data in Fig. 1D). (C) Reggie2/Flo1 is drastically reduced in the mutant (compare Fig. 1E; the wild-type data of Fig. 1E and the mutant data here represent two halves of one image of the same Western blot). Immunoreactive bands of lower molecular weight are also seen, indicative of Reggie2/Flo1 protein degradation. (D) Horizontal autofluorescent paraffin sections from adult wild-type (WTB) and *reggie1/flo2* mutant (KG00210) heads. The optic lobes with the outer and inner optic chiasm are shown. No structural differences between wild type and mutant were detected. Xo, outer optic chiasm; Xi, inner optic chiasm. Scale bar, 20 μ m.

gradient for purification (Rietveld et al., 1999), we could also show that the IgCAM Rst accumulates in the DRM fraction containing the Reggies/Flotillins (Fig. 4C).

Reggie2/Flo1 protein misexpression requires Reggie1/Flo2

The analysis of the KG00210 *reggie1/flo2* null mutant unexpectedly revealed that, in addition to Reggie1/Flo2, Reggie2/Flo1 was also no longer detected on Western blots (Fig. 3C). Instead,

immunoreactive bands of lower molecular weight appeared on Western blots of lysates from pupal brains and adult heads, which are not observed in lysates of wild type flies. This finding indicates that protein degradation rather than transcriptional or translational downregulation accounts for the loss of Reggie2/Flo1 (Fig. 3C). To support this hypothesis, we overexpressed both genes either alone or together using the GAL4AS system (Brand and Perrimon, 1993). If Reggie1/Flo2 were necessary for the expression or maintenance of its paralogue, ectopic expression of *reggie2/flo1*

Fig. 4. Detection of Reggie1/Flo2, Reggie2/Flo1, Fasciclin-1 and Wingless in *Drosophila* lipid raft fractions. (A–C) Embryonic membrane preparations were solubilized in 1% Triton X-100 and floated by density gradient centrifugation. (A, B) Sucrose density gradient, (C) Optiprep density gradient. Twelve fractions of equal volumes were collected from the top and subjected to Western blot analysis using the antibodies as indicated. (A, B) Detection of Fasciclin-1, a GPI-linked protein, serves to identify the fractions containing the detergent-insoluble proteins (fractions 3–6). Detection of Na^+/K^+ -ATPase serves to identify the non-raft fractions. (A) Wild-type embryos. Both Reggies/Flotillins and fractions of Fasciclin-1 and Wingless are found in the DRM fractions. (B) KG00210 embryos, mutant for *reggie1/flo2*. No Reggie/Flotillin is detected, whereas the Fasciclin-1 and Wingless partitioning pattern is unaltered. (C) Wild-type embryos. Using Optiprep as the density medium (see Experimental methods), we were able to show that a fraction of the IgCAM Roughest (Rst) copurifies with the Reggie/Flotillin proteins.

would conceivably not lead to a significant increase of protein levels, whereas expression of *reggie1/flo2* in combination with the paralogue would. Using the *da GAL4* driver line (Wodarz et al., 1995) and Western blot analysis of embryo lysates, we demonstrated that Reggie1/Flo2 can be overexpressed alone whereas Reggie2/Flo1 requires the coexpression of its paralogue for stable expression (Fig. 5A). To confirm this finding, we used immunofluorescence analysis. The *twist GAL4* line was chosen as the driver line (Baylies and Bate, 1996) since this line displays a well documented GAL4 expression pattern in the embryonic somatic mesoderm where no endogenous *reggie/flotillin* expression is found. Reggie1/Flo2

protein was readily detected in the embryonic mesoderm following *twist GAL4* induced ectopic expression of Reggie1/Flo2 (Fig. 5B). As predicted by the mutant analysis, we failed to reveal ectopic Reggie2/Flo1 in the analogous experiment using an *UAS reggie2/flo1* transgenic line (Fig. 5C). However, transformant flies expressing both transgenes showed ectopic protein expression of both Reggies/Flotillins (Figs. 5D, E). This confirms that Reggie1/Flo2 is necessary for the stabilization of Reggie2/Flo1, whereas Reggie1/Flo2 does not require Reggie2/Flo1.

Reggie/Flotillin misexpression interferes with signaling processes in imaginal discs

Overexpression of either Reggie1/Flo2 or both Reggies/Flotillins in imaginal discs using the GAL4 enhancer trap line *Mz1369* revealed that excessive Reggie/Flotillin protein is detrimental for the development of the corresponding tissues and organs (Fig. 6). Overexpressing flies display compound phenotypic defects including abnormal eyes (Figs. 7 and 8), ocelli, bristles and wings (Fig. 9). Similar to the results obtained with the *da GAL4* and *twist GAL4* driver lines, overexpression of Reggie2/Flo1 alone under the transcriptional control of *Mz1369 GAL4* failed to result in corresponding protein accumulation and hence did not lead to phenotypic defects. The types of defects caused by *Mz1369 GAL4* driven misexpression of Reggie1/Flo2 alone or in tandem with Reggie2/Flo1 were largely indistinguishable, although flies overexpressing both proteins displayed a tendency towards more pronounced defects. In the eye disc, immunofluorescent staining with an antibody against DE Cadherin (Oda et al., 1994) revealed that misexpression of Reggies/Flotillins led to severe defects comprising a variable number of cone cells and primary pigment cells per ommatidium, errors in number and positioning of the bristle complexes and mild cell sorting defects (Fig. 7B). We speculated that these defects would be reflected in a changed expression pattern of interacting cell adhesion molecules that are known to be instrumental to the development of the regular ommatidial pattern, e.g. the Ig superfamily protein Sticks and Stones (SNS) and its heterophilic binding partners Roughest (Rst) and Kin of irre (Kirre) (Galletta et al., 2004; Strunkelnberg et al., 2001). In the eye imaginal disc, these molecules are usually seen at the apical membrane of primary pigment cells (SNS) or interommatidial cells (Rst and Kirre), respectively (Figs. 7C, E, G). In the overexpression mutants, however, antibody staining of these proteins in midpupal eye discs revealed a significant and excessive accumulation of strongly immunoreactive multivesicular bodies in interommatidial cells (Figs. 7D, F, H). Double labeling with anti Kirre and anti SNS antibodies revealed that these IgCAMs were contained in the same vesicular bodies (Fig. 8). The abnormal intracellular accumulation of the cell adhesion molecules Rst, Kirre and SNS resulting from *reggie/flotillin* misexpression is even more remarkable considering the phenotypically wild type localization of DE Cadherin in these overexpression mutants, indicating that the overexpression selectively affects Rst, Kirre and SNS (Fig. 7B).

The severe phenotype resulting from *reggie/flotillin* overexpression in wing imaginal discs somehow resembled a *wingless* overexpression phenotype (Neumann and Cohen, 1996), including blistering. Since Wingless activity was recently shown to depend on a lipid raft dependent pathway (Zhai et al., 2004), we analyzed the Wingless expression pattern in *reggie/flotillin* overexpressing wing imaginal discs of L3 larvae. Immunofluorescent anti Wing

Fig. 5. Misexpression of Reggie/Flotillin in the embryo. (A) Western blot analysis of Reggie/Flotillin misexpression using *da-GAL4* as a driver line. Reggies/Flotillins are detected in lysates of 16–22 h old embryos with the antibodies indicated. Detection of Actin served as a loading control. Note that Reggie1/Flo2 is detected when misexpressed alone or together with its paralogue Reggie2/Flo1 whereas no Reggie2/Flo1 was detected when misexpressed alone. (B–E) Misexpression in the embryonic mesoderm using *twist-GAL4* as a driver line. All images are lateral views of the posterior part of stage 11/12 embryos. Anterior is to the left. (B) Misexpression of *reggie1/flo2*, (C) *reggie2/flo1* or (D, E) *reggie1/flo2* and *reggie2/flo1* combined. Detection with antibodies against Reggie1/Flo2 (B, D) and Reggie2/Flo1 (C, E), respectively. Reggie1/Flo2 immunoreactivity is readily observed when the corresponding gene is expressed alone (B), whereas the presence of Reggie2/Flo1 cannot be shown when its gene is misexpressed alone (C). Only when coexpressed with Reggie1/Flo2 the presence of the Reggie2/Flo1 protein can be detected (E). Scale bar, 50 μ m.

Fig. 6. Misexpression of Reggie/Flotillin in imaginal discs. (A–F) *Mz1369;UAS-reggie1/flo2;UAS-reggie2/flo*. (A–D) Adult flies show a variable phenotype affecting bristles, ocelli, eyes, wings, abdominal segments and thoracic structures consistent with underlying imaginal disc abnormalities. (E, F) Wings exhibit a highly variable but severe mutant phenotype, including blistering and melanotic cells.

less staining revealed a broadening of the Wingless immunoreactive domain in the *reggie/flotillin* misexpression genotype, demonstrating the ability of Reggie/Flotillin to interfere with DRM domain/lipid raft based signaling processes (Fig. 9).

Discussion

The present study set out to investigate the functional genetics of a highly conserved set of proteins with a presumed pivotal role in lipid raft formation in *Drosophila*. We investigated the expression of two paralogous Reggie/Flotillin proteins in wild type and mutant *Drosophila* and analyzed the effects of both loss and gain of function on the development of the fly.

Immunofluorescence staining of stage 16 embryos revealed that Reggie1/Flo2 and Reggie2/Flo1 are, as in zebrafish (von Philipsborn et al., 2005), expressed in the embryonic central nervous system, where commissures were more intensely labeled than connectives (Figs. 2A, B). Postembryonically expression of both proteins is strongest in the nervous system as well and restricted to axon shafts (Fig. 2) at positions where fasciculation with other axons occurs, e.g. in the optic chiasms or at the neck of the peduncle. This supports the notion that DRM proteins provide platforms for cell adhesion and cell cell communication (Harris and Siu, 2002; Benzing, 2004; Stuermer and Plattner, 2005). It is not yet known which cell adhesion molecules are involved in the axonal fasciculation domains, but the assumption that they should be connected to Reggie/Flotillin rafts is a good starting point for their identification (Fig. 4A).

The lipid raft association of the *Drosophila* Reggies/Flotillins was previously reported (Galbiati et al., 1998). We show here that lipid raft formation in *Drosophila* is not dependent on these proteins (Fig. 4B). It is noteworthy that reported raft proteins such as Fasciclin 1 or Wingless are only fractionally recruited into the DRM/raft fraction (Rietveld et al., 1999; Zhai et al., 2004), even when Reggie/Flotillin rafts are absent (Fig. 4B). Depending on the conditions of DRM isolation, we also demonstrate the partial association of Rst with DRM/rafts (Fig. 4C). This is consistent with the view that Rst (and the other large IgCAMs) participates in a raft associated complex for recognition/adhesion. *Drosophila*

Rst, Kirre and SNS are the homologues of the vertebrate IgCAMs Nephs and Nephrin, which are required for the formation of specific cell contact sites in the vertebrate kidney and which are dependent on the patterned expression of the DRM/raft protein Podocin (reviewed in Benzing, 2004). Considering the complex expression pattern (Figs. 1, 2) and the evolutionarily highly conserved structure of the Reggies/Flotillins, it came as a surprise that we were unable to detect any external or internal mutant phenotypes in the KG00210 mutant, which lacks the Reggie1/Flo2 protein and in addition is depleted of the Reggie2/Flo1 protein (Fig. 3).

Although a more detailed analysis might reveal mutant phenotypes that escaped our attention, the apparent lack of a severe phenotype was unexpected. Possible explanations include compensatory upregulation of other DRM proteins with overlapping or redundant function such as stomatins, of which there appear to be three homologues in the fly genome. In addition, Western blotting experiments revealed a minor fraction of undegraded Reggie2/Flo1 protein. It is conceivable that this residual Reggie2/Flo1 protein is sufficient to suppress an apparent mutant phenotype. The absence of such mutant phenotypes for Reggie/Flotillins is reminiscent of the Caveolin 1 knock out mouse, which displays only very mild defects (Razani et al., 2001), possibly due to compensatory upregulation of other scaffolding proteins.

Evidence for posttranslational regulation of Reggies/Flotillins is provided by the *reggie1/flo2* KG00210 mutant which represents a null allele at the transcriptional level. Surprisingly, in this mutant lacking Reggie1/Flo2, the Reggie2/Flo1 protein (but not its mRNA) is apparently degraded as inferred from the appearance of small Reggie2/Flo1 immunopositive bands on Western blots. This implies that Reggie2/Flo1 requires Reggie1/Flo2 for its stabilization. In fact, misexpressed Reggie2/Flo1 was only detectable when Reggie1/Flo2 was coexpressed. Several other examples for such protein protein interaction dependent stabilization mechanisms have been published: in *C. elegans*, the stomatin like protein UNC 24 is needed for the stabilization of the stomatin homologue UNC 1 (Sedensky et al., 2001). In the mouse, the Caveolin 1 knock out mouse shows a significant reduction of Caveolin 2 levels (Drab et al., 2001; Razani et al., 2001).

Fig. 7. Misexpression of Reggie/Flotillin in midpupal eye imaginal discs. On the left, wild-type controls are shown (A, C, E, G), on the right, the misexpression genotype *Mz1369;UAS-reggie1/flo2;UAS-reggie2/flo1* (B, D, F, H). (A, B) DE-Cadherin staining reveals cell boundaries. *Reggie/Flotillin* misexpression leads to a severe disturbance of the ommatidial pattern. Asterisks point to two ommatidia, the left one containing three primary pigment cells and an increased number of cone cells, while the neighboring ommatidia on the right contains only a single primary pigment cell and three cone cells instead of the normal number of four (B). (C, D) Rst, (E, F) Kirre and (G, H) Sns immunoreactivity. For all three members of the immunoglobulin superfamily, *Reggie/Flotillin* misexpressing eye discs contain massive accumulation of immunoreactive vesicles in interommatidial cells.

Recently, a similar interdependence was shown for mitochondrial prohibitin and prohibitone, which form membrane bound ring complexes (Berger and Yaffe, 1998; Tatsuta et al., 2005). Interestingly, these examples all concern protein pairs that, like Reggie/Flotillin, are known to reside in lipid rafts or caveolae, form multimers and thus scaffold for complex protein protein interactions (Simons and Toomre, 2000; Stuermer and Plattner, 2005). However, *Drosophila* Reggie1/Flo2 and Reggie2/Flo1 are not mutually dependent on each other since it is possible to stably express Reggie1/Flo2 in the absence of Reggie2/Flo1. Similarly, the Caveolin 2 knock out mouse has an almost unaltered Caveolin 1 protein content (Razani et al., 2002). The underlying mechanism for the unilateral dependence between two highly similar paralogous polypeptides remains as yet unknown. Obligate heterodimerization alone provides an insufficient explanation for the lability of one but not the other partner. Ubiquitination consensus sites or PEST sequences are ostensibly absent from Reggie/Flotillin proteins.

The biochemically defined DRM distribution of Fasciclin 1 and Wingless remained unaltered in the mutant (Fig. 4B). The lack of any visible phenotype in the mutant reminds of many reported cases where loss of function mutations affecting cell adhesion and signaling result in weak phenotypes, while ectopic and increased expression causes severe abnormalities (e.g. Thor et al., 1999; Certel et al., 2000; Torroja et al., 1999; Wills et al., 1999). Thus, ectopic expression of a gene in a particular cell type may produce phenotypes, even though it is not normally expressed in this cell.

Gain of function analysis has been successfully exploited in a screen for proteins that are involved in motor axon guidance and synaptogenesis (Kraut et al., 2001). In the absence of loss of function phenotypes, defects caused by overexpression of genes, therefore, provide useful hints to the function of the gene product (Duffy, 2002).

Such overexpression of *UAS reggie1/flo2* or of *UAS reggie1/flo2;UAS reggie2/flo1* with pan neuronal GAL4 drivers lead to embryonic death, and GAL4 drivers with an expression restricted to imaginal structures caused severely deformed flies (Fig. 6). This is consistent with an interference of overexpressed Reggies/Flotillins with morphogenesis and organogenesis. How the DRM proteins regulate these events is only vaguely understood except for the consensus that they communicate with the cytoskeleton (Baumann and Saltiel, 2001; Neumann Giesen et al., 2004; Stuermer et al., 2004).

Our results show that misexpression of the DRM/lipid raft proteins Reggie1/Flo2 and Reggie2/Flo1 in imaginal discs leads to cell differentiation defects. A possible explanation for the

Fig. 8. Misexpression of Reggie/Flotillin in eye imaginal discs (*Mz1369;UAS-reggie1/flo2;UAS-reggie2/flo1*) leads to a massive accumulation of vesicular bodies in interommatidial cells that are immunoreactive for Kirre (green) and SNS (red) as seen by the yellow color in the merged image (right).

Fig. 9. Effects of misexpression of the two Reggie/Flotillins (green fluorescence) on the Wingless signal (red fluorescence) in the wing imaginal discs. (A–C) *Mz1369* driven misexpression of *reggie2/flo1* fails to result in Reggie2/Flt1 protein overexpression (compare Fig. 5) and shows a normal Wingless immunoreactivity pattern. (D–F) *Mz1369* driven misexpression of *reggie1/flo2* in the wing imaginal discs results in Reggie1/Flt2 overexpression and in a significant expansion of the Wingless signal.

aberrations seen in the eye imaginal discs could be provided by the specific effect on the IgCAMs of the Rst family. Rst and Kirre, as well as their ligand SNS are found inside the interommatidial cells in multivesicular bodies (Figs. 7D, F, H; Fig. 8). The proper positioning of DE Cadherin in the overexpression mutant demonstrates that Reggie/Flotillin misexpression disturbs positioning of only a subset of adhesion proteins in the eye imaginal disc (Fig. 7B).

Rst/Kirre are expressed in founder cells during embryo muscle development, whereas their ligand SNS is expressed in fusion competent myoblasts, i.e. on opposing membranes in different cell types (Strunkelnberg et al., 2001; Bour et al., 2000). A similar scenario applies during development of the eye where Rst and Kirre are expressed in interommatidial cells, while SNS is expressed in primary pigment cells. The fact that we found Kirre/SNS immunoreactive vesicles in interommatidial cells suggests either of two scenarios. The extracellular domain of Rst/SNS may be cleaved off or shed upon binding to its ligands Rst/Kirre followed by receptor/ligand complex internalization by the interommatidial cells. Alternatively, the entire receptor/ligand complex comprising both transmembranous partners Rst/Kirre and SNS may be internalized without cleavage (Fig. 8). A conceivably similar mechanism for the internalization of a transmembrane ligand is known from the internalization of Bride of sevenless/Sevenless complexes into the R7 cell during photoreceptor development (Cagan et al., 1992). Since intracellular vesicles containing the aforementioned IgCAMs are also seen in developing wild type eyes, albeit to a much lesser extent, it is intriguing to speculate that the normal pathway of Rst/Kirre SNS receptor/ligand internalization is controlled by a lipid

raft dependent mechanism. Massive overexpression of the Reggie/Flotillin lipid raft proteins may promote this internalization process. Flotillin lipid rafts in vertebrates have already been implicated in phagocytotic processes (Bared et al., 2004), and one model for the internalization of transmembrane receptor/ligand complexes is based on receptor mediated phagocytosis (Cagan et al., 1992). Further analyses are required to determine the influence of Reggie/Flotillin on membrane internalization in normal flies.

In the wing disc, Reggie/Flotillin misexpression leads to an enlargement of the Wingless (Wg) expression domain (Fig. 9) and consequently to abnormal wings (Fig. 6). Wg is a secreted signaling molecule which acts as a morphogen in the *Drosophila* wing imaginal disc. Wg is normally produced along the dorso ventral border of the wing disc, and it has been suggested that membrane exovesicles termed argosomes provide a vehicle for the movement of the Wg protein across cells (Greco et al., 2001). Lipid rafts are likely to be involved in the formation of argosomes (Vincent and Magee, 2002) and other forms of unconventional release such as lipid bodies (Reuter et al., 2004). Recently, the lipid raft association of Wg before secretion was shown (Zhai et al., 2004). Argosomes resemble exosomes, and Reggie/Flotillin has been shown to be present on the surface of exosomes, but also in association with lysosomes and lipid bodies of various vertebrate cell types in culture (De Gassard et al., 2003). In this context, the severe wing phenotype caused by Reggie/Flotillin overexpression in wing imaginal discs could be the result of an increased release of Wg loaded argosomes resulting in abnormal dorso ventral patterning.

Experimental methods

Fly strains and conditions of culture

Stocks were maintained at 25°C on standard cornmeal molasses agar food. Staging of embryos was performed according to Campos Ortega and Hartenstein (1997). Stocks used in this study were: wild type Berlin, *UAS reggie1*, *UAS reggie2*, *UAS reggie1;UAS reggie2*, *UAS mCD8 GFP* (Lee et al., 1999; Bloomington Stock Center), *yellow white*, KG00210 (kindly provided by Dr. A. Spradling, HHMI/Carnegie Institute, Baltimore, MD; in the meantime available from Bloomington Stock Center), *OK107 GAL4* (Connolly et al., 1996; Bloomington Stock Center), *repo GAL4* (kindly provided by Dr. A. Giangrande, IBMC CNRS, Strasbourg, France), *twist GAL4* (Baylies and Bate, 1996), *da GAL4* (Wodarz et al., 1995) and *Mz1369 GAL4* (obtained from G. Technau, University of Mainz, Germany).

To obtain *UAS reggie1* and *UAS reggie2* fly strains, the BDGP cDNAs LD02571 (i.e. the isoform without the differentially spliced exon) and CK02126, respectively, were subcloned into the pUAST vector, and *yellow white* flies were transformed with the resulting plasmid (Brand and Perrimon, 1993). In order to overexpress both Reggies/Flotillins in parallel, doubly homozygous flies with the genotype *yw;UAS reggie1AS reggie1;UAS reggie2AS reggie2* were generated using standard genetic crosses.

Immunohistochemistry and microscopy

Immunohistochemistry was performed as previously described (Hiesinger et al., 1999; Strunkelnberg et al., 2001). For Western blotting and immunostaining, we used antibodies generated against recombinant goldfish Reggies expressed in bacteria, which were shown to recognize epitope tagged bacterially expressed *Drosophila* Reggies/Flotillins (data not shown). Antibodies and dilutions used were: anti goldfish Reggie2 (R168, 1:1), anti goldfish Reggie1 (R722, 1:500 1:1000), anti Roughest (24A5; 1:20), anti DE Cadherin (DCAD2; 1:100), anti Kirre (126intra; 1:200), anti SNS (M2; 1:200) and anti Wingless (4D4; 1:10). The generation of anti Kirre and anti SNS antisera will be published elsewhere (Bonengel et al., in preparation). Secondary antibodies were Cy3 (Jackson Immuno Research; West Grove, PA), Alexa488 or Alexa568 (Molecular Probes; Eugene, OR) labeled. A Leica (Nussloch, Germany) TCS^{4D} confocal microscope equipped with an ArKr laser was used for data acquisition. Data were processed using Amira software (Indeed, Berlin, Germany) and Adobe Photoshop (Adobe, San Jose, CA).

RT PCR

Total RNA from embryos, 3rd instar larvae, pupae and adults was isolated using Qiagen RNeasy (Qiagen, Hilden, Germany) or Trizol (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. Reverse transcription was carried out with Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) as described by the manufacturer. First strand cDNA was used as a template in PCR reactions using Taq polymerase (Amersham Biosciences, Freiburg, Germany) and the following primer pairs specific for *reggie1/flo2* (Fig. 1: reg1forward1 5' AACCGATGTCCAGCGACTGT 3' and reg1reverse1 5' GGAATTGCTCGCTGGCAGTG 3'; Fig. 3: reg1forward2 5' AAGTAGAGGCGACGAGGAACGACTCA 3' and reg1reverse2

5' AGGCCAGGTACTGCACATCATCGTAG 3'), *reggie2/flo1* (Fig. 1: reg2forward1 5' CCGCAGCTTCCTCAACGAT 3' and reg2reverse1 5' TCCARGACCACGCGCTGCTT 3'; Fig. 3: reg2forward2 5' AACAAGGCTACCTGTGGATCGGATAA 3' and reg2reverse2 5' GATGTGGTTGATCTCTGCCTCTGATT 3') and rp49 (rp49forward 5' GACTTCATCCGCCACCAGTGC 3' and rp49reverse 5' CACCAGGAAGTCTTGAATCCG 3'). PCR conditions were: 94°C for 4 min followed by 40 cycles at 94°C for 1 min, annealing for 1 min and 72°C for 1 min and finally 72°C for 5 min. Annealing temperatures were 55°C for reg1forward1/reverse1, 63°C for reg1forward2/reverse2, 60°C for reg2forward1/reverse1, 65°C for reg2forward2/reverse2 and 59°C for rp49forward/reverse.

Isolation of detergent insoluble membrane fractions from embryos

Preparation of Drosophila embryonic membranes

Flies were allowed to lay eggs on apple juice agar supplied with fresh yeast for 14 h. After an additional 6 h of incubation, embryos were collected into a wire mesh bottom container. Embryos were washed with 0.9% NaCl + 0.1% Triton X 100 to remove debris and yeast before being dechorionated in 6% NaClO in PBS. Dechorionated embryos were washed with water, collected into 2 ml tubes and stored at -80°C. 1 mg of frozen embryos was resuspended in 2 ml TNE buffer (100 mM Tris (pH 7.5), 150 mM NaCl and 0.2 mM EDTA) containing 0.3 M sucrose, 0.1 mM DTT, 1 mM phenyl methyl sulfonyl fluoride (PMSF) and protease inhibitor cocktail (BD Pharmingen, San Diego, CA). Embryos were homogenized using a Wheaton homogenizer. The embryo homogenate was centrifuged at 4500 rpm for 10 min at 4°C to remove debris and to pellet nuclei (SS34 rotor). The post nuclear supernatant was transferred to a SW28 tube, adjusted to 1.4 M sucrose by addition of 14 ml of 1.55 M sucrose in TNE buffer and overlaid with 11 ml of 1.22 M sucrose in TNE buffer and 9 ml of 0.1 M sucrose in TNE buffer and then centrifuged for 4 h at 24,500 rpm at 4°C. Membranes were observed floating above the 1.22 M/0.1 M sucrose interphase. This fraction (2 ml) was collected, diluted three fold in TNE buffer and pelleted for 30 min at 17,500 rpm (SS34 rotor) at 4°C. The membrane fraction was washed once with 3 ml TNE buffer.

Purification of detergent insoluble membranes using a sucrose gradient

The membrane pellet was resuspended in 500 µl TNE buffer and transferred to a SW41 tube. An equal volume of cold 2% Triton X 100 in TNE buffer was added, and the membranes were solubilized on ice for 30 min. One ml 85% sucrose in TNE buffer (w/v) was added and overlaid with 6 ml of 35% sucrose in TNE buffer and 3.5 ml 5% sucrose in TNE buffer. The gradient was centrifuged overnight at 35,000 rpm at 4°C (SW41 rotor). Twelve fractions were collected by aspiration from the top and analyzed SDS polyacrylamide gel electrophoresis and Western blotting (Figs. 4A, B).

Purification of detergent insoluble membranes using an Optiprep gradient

The membrane fraction derived by differential centrifugation was resuspended in 200 µl TNE buffer. An equal volume of cold 2% Triton X 100 in TNE buffer was added, and the membranes were solubilized on ice for 30 min. Following solubilization, 800 µl Optiprep (Axis shield, Oslo, Norway) was added to achieve a final concentration of 40% Optiprep. 1 ml of the suspension was transferred to SW65 tubes and overlaid with 1 ml of 30, 20 and

5% Optiprep, respectively, and finally with 1 ml of TNE buffer. The gradient was centrifuged for 6 h at 46,900 rpm (SW41 rotor) at 4°C. Fractions were collected and analyzed as described above (Fig. 4C).

Fly lysates

Dechorionated embryos, larval and pupal brains and adult heads were homogenized in 1× SDS electrophoresis sample buffer and boiled for 5 min. An aliquot corresponding to three heads or brains, respectively, was loaded per lane of a SDS polyacrylamide gel.

Western blotting

The sucrose gradient fractions and fly head lysates were fractionated on 10% SDS polyacrylamide gels and blotted onto Hybond P membranes (Amersham Biosciences, Freiburg, Germany) according to standard methods (Ausubel et al., 1994). Non specific binding sites were blocked with 3% skim milk powder (Fluka, Buchs, Switzerland) in PBST (PBS containing 0.1% Tween (v/v)) for 30 min at room temperature followed by incubation with the primary antibody for 1 h at room temperature and washed four times with PBST for 10 min each. HRP conjugated secondary antibodies (Sigma) were used at the recommended dilution in PBST and incubated for 45 min at room temperature. Antigen antibody complexes were detected by chemiluminescence using a commercial ECL kit (Amersham Biosciences, Freiburg, Germany). Primary antibodies and the dilutions used were: R722 (anti goldfish Reggie1; 1:1000), R168 (anti goldfish Reggie2; 1:10), 21D11 (anti Roughest; 1:40), E7 (anti β Tubulin, 1:250), JLA20 (anti Actin; 1:200), f5H7 (anti Fasciclin 1, 1:500; kindly provided by Dr. M. Hortsch, Univ. of Michigan, Ann Arbor, MI), 1B2 (anti Na⁺/K⁺ ATPase, 1:8000), 4D4 (anti Wingless; 1:50) and 21D11 (anti Roughest; 1:40).

Paraffin sections

Horizontal paraffin sections of adult fly heads were prepared as described by Jäger and Fischbach (1987) and viewed with a confocal microscope.

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