Unique and Overlapping Functions of Formins Frl and DAAM During Ommatidial Rotation and Neuronal Development in Drosophila

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ABSTRACT The noncanonical Frizzled/planar cell polarity (PCP) pathway regulates establishment of polarity within the plane of an epithelium to generate diversity of cell fates, asymmetric, but highly aligned structures, or to orchestrate the directional migration of cells during convergent extension during vertebrate gastrulation. In Drosophila, PCP signaling is essential to orient actin wing hairs and to align ommatidia in the eye, in part by coordinating the movement of groups of photoreceptor cells during ommatidial rotation. Importantly, the coordination of PCP signaling with changes in the cytoskeleton is essential for proper epithelial polarity. Formins polymerize linear actin filaments and are key regulators of the actin cytoskeleton. Here, we show that the diaphanous-related formin, Frl, the single fly member of the FMNL (formin related in leukocytes/formin-like) formin subfamily affects ommatidial rotation in the Drosophila eye and is controlled by the Rho family GTPase Cdc42. Interestingly, we also found that frl mutants exhibit an axon growth phenotype in the mushroom body, a center for olfactory learning in the Drosophila brain, which is also affected in a subset of PCP genes. Significantly, Frl cooperates with Cdc42 and another formin, DAAM, during mushroom body formation. This study thus suggests that different formins can cooperate or act independently in distinct tissues, likely integrating various signaling inputs with the regulation of the cytoskeleton. It furthermore highlights the importance and complexity of formin-dependent cytoskeletal regulation in multiple organs and developmental contexts.

KEYWORDS planar cell polarity; formin; cytoskeleton; neural development; noncanonical Wnt signaling

In multicellular organisms, epithelia are polarized along the apical–basal axis as well as within the epithelial plane, imparting cells with a sense of direction. The latter polarity, planar cell polarity (PCP), can be propagated over hundreds of cell diameters and is evident in many vertebrate and non-vertebrate organs. In its simplest form, PCP is seen in cuticular structures of insects such as Drosophila melanogaster, where sensory bristles on the thorax or actin hairs produced by each wing cell all point in the same direction, posteriorly on the thorax and distally in the wing (Goodrich and Strutt 2011; Maung and Jenny 2011). Analogously in vertebrates, hair follicles, cilia in the lung and brain ventricles, and stereocilia in the sensory cells of the inner ear are arranged in a stereotypic manner that is crucial for proper organ function ((Montcouquiol et al. 2003; Devenport and Fuchs 2008; Tissir et al. 2010; Vladar et al. 2012; reviewed in Goodrich and Strutt 2011; Devenport 2014). More complex examples of PCP signaling include coordination of cell fate specification and ommatidial orientation in the Drosophila compound eye, the orientation of cell division, and cell migration during vertebrate gastrulation (reviewed in Jenny 2010; Goodrich and Strutt 2011; Devenport 2014). One of the main signaling pathways involved in the establishment of PCP is the noncanonical Wnt/Frizzled (Fz)-PCP pathway that was originally identified in Drosophila and is highly conserved in vertebrates. Mutations in PCP genes cause misaligned ommatidia or wing hairs in flies and severe developmental abnormalities such as
neural-tube and left-right defects, and ciliopathies in mammals (Murdoch et al. 2001; Ross et al. 2005; Borovina et al. 2010; Song et al. 2010).

Core PCP factors including the transmembrane proteins Frizzled (Fz), Flamingo [Fmi; also known as (aka) Stan], Van Gogh (Vang, aka Stbm), the adaptor proteins Dishevelled (Dsh), and Prickle are required for PCP establishment in all tissues (reviewed in Strutt and Strutt 2009; Vladar et al. 2009; Maung and Jenny 2011). Fz and Dsh transduce the PCP signal to Rho family GTPases, including RhoA and Rac, leading to changes in the cytoskeleton (Strutt et al. 1997; Boutros et al. 1998; Fanto et al. 2000; Maung and Jenny 2011). Additionally, at least in the *Drosophila* eye, Rac activates a JNK-type MAP kinase cascade leading to a transcriptional response (Fanto et al. 2000; Jenny 2010). Overall, however, the activation of Rho GTPases and their mechanism of action downstream of Dsh during PCP signaling are only poorly understood.

![Figure 1](image.png)

**Figure 1** Loss of *frl* causes a PCP-like defect in the eye. Tangential sections of adult eyes are shown with corresponding schematic representation of ommatidial orientations underneath. Anterior is to the left and dorsal is up in all sections. Black and red arrows represent dorsal and ventral chiral forms of ommatidia, and circles mark lost R-cells in all figures. (A) Control eyes expressing *Dcr2* under control of the *sevenless* (*sev*) promoter. The inset shows an enlarged single ommatidium with numbered rhabdomeres, the light-sensitive organelles of R-cells (note that only seven rhabdomeres are visible at once in a section, as R7 lies on top of R8). (B) Compared to control (A), knock down of *frl* causes ommatidial rotation defects. (C–F) *frl*Ex83 (C) and *frl*Ex88 (E) clones marked by the absence of pigment show PCP-like defects (due to the *Minute* heterozygous background, most of the sections are homozygous mutant). (D and F) Lethality as well as orientation defects of *frl*Ex83 (D) and *frl*Ex88 (F) are rescued by a genomic copy of *frl* (*FosFrl*). Note that throughout this work, all alleles of *frl* correspond to the excision alleles indicated in the background of a *FRT80* chromosome with a fosmid adding back all additional genes removed by the excisions (*FosAB*).
in Frl. (F) Coomassie staining of Gst-CT, consistent with an intramolecular interaction of the N and C termini of Frl. (G) Frl likely is autoinhibited. (A) Schematic of domain structure of Frl with amino acid number of predicted domains indicated (relative to version Frl-PC). GBD, GTPase binding domain; DID, diaphanous inhibitory domain; DD, dimerization domain; FH1 and FH2, formin homology 1/2 domains; DAD, diaphanous autoregulatory domain. Underneath: schematic and coordinates of constructs used in this study; note that FlagΔFH2 is 11 aa shorter than the Gst-ΔFH2. (B–D) Images of eyes of flies overexpressing full-length Frl (B), constitutively active Frl (FrlΔGBD-DID; C), or dominant negative Frl (FrlΔFH2; D) under the control of sev-Gal4. Note that in contrast to full-length Frl that does not affect eye formation, FrlΔGBD-DID and FrlΔFH2 cause severely rough eyes. (E) Gst pull-down experiment of in vitro translated proteins indicated on the left with Gst-fusion proteins indicated on top. While only a small amount of full-length Frl is binding to the FH2-CT domain, much stronger binding is found between the GBD-DID-DD-CC region with Gst-FH2-CT and Gst-CT, consistent with an intramolecular interaction of the N and C termini of Frl. (F) Coomassie staining of a typical pull-down gel of the assays in E with corresponding Gst proteins indicated on top.

In the *Drosophila* eye, PCP is manifest in the mirror-symmetric arrangement of its ~800 ommatidia or facets dorsal and ventral to the midline (equator) (Wolf and Ready 1993; Singh et al. 2005; reviewed in Jenny 2010). This arrangement is established in third instar larvae, after ommatidial precursors emerge from the morphogenetic furrow (MF), a transient indentation that sweeps from posterior to anterior across the eye imaginal disc (Treisman and Heberlein 1998; Roignant and Treisman 2009; Jenny 2010). Fz-PCP signaling determines the R3 vs. R4 photoreceptor (R-cell) fates in the precluster consisting of the R8, R3/4, and R2/5 cells, with the precursor closer to the equator becoming R3. This in turn triggers rotation of the clusters by 90° clockwise on the dorsal and counterclockwise on the ventral side of the equator, a process requiring changes in the cytoskeleton mediated by Rho kinase (Winter et al. 2001). During pupal stages, R-cell clusters remodel to form adult ommatidia with six outer photoreceptors (R1–R6) arranged in a trapezoid around the two inner photoreceptors (R7/8; see insert in Figure 1A) that are aligned in a stereotypic parallel and antiparallel arrangement. In core PCP mutants, R3/4 fates and ommatidial rotation are randomized.

Members of the seven formin families are important regulatory proteins of the cytoskeleton characterized by the presence of two formin homology (FH) domains. The FH1 domain binds profilin in complex with G-actin, while the FH2 domain nucleates and polymerizes unbranched actin filaments (reviewed in Higgs 2005; Faix and Grosse 2006; Liu et al. 2010). Although all formins affect actin filaments, little is known about their cooperation in the regulation of the cytoskeleton. In addition to the FH1 and FH2 domains, the subfamily of diaphanous-related formins (DRFs) such as Diaphanous, Daam (Dishevelled-associated activator of morphogenesis), and Frl (formin related in leukocytes; FMNLs in humans) also contain an N-terminal Rho family GTPase binding domain (GBD), a diaphanous inhibitory domain (DID), and a dimerization domain (DD) followed by a coiled coil domain (CC; see also schematic in Figure 2A). C terminal to the FH2 domain, there is the diaphanous autoregulatory domain (DAD) that binds to the GBD-DID domain, in most cases keeping the formin in an inactive conformation (Higgs 2005; Vaillant et al. 2008; Liu et al. 2010). DRFs are usually activated by a Rho family GTPase binding to the GBD domain, thereby alleviating autoinhibition. In addition, factors binding to the C-terminal DAD domain can also contribute to DRF activation. For example, Dsh is able to activate Daam1 by binding to its C-term/DAD domain in tissue culture and during convergence and extension in *Xenopus* (Habas et al. 2001; Liu et al. 2008). Surprisingly, *Drosophila* DAAM mutants show no PCP defects in the eye or wing. Instead, DAAM is required to pattern the tracheal cuticle via the modification of the actin cytoskeleton, for sarcomeric thin filament formation, and for axon growth, the latter function under control of the Rac GTPase (Matusek et al. 2006, 2008; Molnar et al. 2014; Gombos et al. 2015). Thus, the role of formins and the extent of their cooperation in PCP signaling in fruit flies remained elusive.

Here, we find that *Drosophila* Frl (CG32138), the sole representative of the Frl formin subfamily in flies, likely contributes to ommatidial rotation during eye development, as RNAi-mediated knockdown of *frl* or eyes mutant for *frl* shock mild rotation defects. Furthermore, binding assays and in vivo overexpression studies suggest that full-length Frl is autoinhibited. Communoprecipitation and pull-down assays showed that Frl strongly binds to GTP-bound forms of Cdc42 and, more weakly, to Rac, but genetic interaction assays suggest that, in vivo, Frl is activated by Cdc42. Although Frl can bind Dsh in vitro, we found no in vivo evidence for such an
interaction during PCP establishment in the eye. Interestingly, while we found no obvious redundancy between frl and DAAM during PCP signaling in the eye, they cooperate during R-cell morphogenesis or maintenance and during axon growth in the mushroom body, a process also requiring a subset of the PCP genes (Shimizu et al. 2011; Ng 2012). Overall, our data suggest that Frl may contribute to ommatiodial rotation in the eye and, importantly, has context-dependent, redundant, and nonredundant functions with DAAM during neural development in Drosophila.

Materials and Methods

Fly strains
dsh1, sep-Gal4, sev-Gal4, FRTG13 shg63-1, FRTG13 shg1626 (Mirkovic and Mlodzik 2006), svp-lacZ07482, sev > F2 and sev-Dsh (Boutros et al. 1998), and sev-RacV12 (Luo et al. 1994) were kind gifts from M. Mlodzik (Mount Sinai Hospital) and L. Luo (Stanford University). nmoD8 (amorphic), nmoP3, and CadN14 (removing N-cadherin 1 and 2) (Prakash et al. 2005) were kind gifts from E. Verheyen (Simon Fraser University) and T. Clandinin (Stanford University). VDRCGD04412, frHMS000445 (BL no. 32447), UAS-CD42 (BL no. 28873), Df(2L)BSC148 (uncovering several genes including the N-cadherins), cdc421, cdc422, cdc425, cno2, aos27, phy3245, and sp427 were obtained from the Vienna Drosophila RNAi Center (VDRC) or Bloomington Stock Centers. racC111, racA24, racC111 racA23 FRT2A, racC111 racA23 FRT2A mt3 (all from the Bloomington Stock Center), UAS-Dcr2, and DAAMExd8 FRT19A (a null allele) and DAAMExC1 and DAAMExD (both hypomorphic alleles giving rise to viable hemizygous males) were described previously (Ng et al. 2002; Matussek et al. 2006; Dietzl et al. 2007; Ng 2012; Gombos et al. 2015).

Fly genetics

frlExd3 and frlExd8 mutations were generated by standard excision using A2–3 to remodelize the isogenized P-element insertions P[d06832] (after recombining away a second site lethal; see Supporting Information, Figure S1B). frlExd62 was generated by imprecise excision of P[HP36775]. frlExd3 and frlExd8 retain various amounts of scrambled P-element sequences (including some UAS sequences for frlExd8) at the original 5′ P end in intron 1 of frl, but delete the start codon (schematic in Figure S1B). The upstream breakpoints of frlExd3 and frlExd8 are in hsc70C and neur4 and delete 21.4 kb and 16 kb, respectively. frlExd62 deletes 11.4 kb starting in CG6833 and ending in intron 2 of frl, thus removing the first two exons of frl, including the start codon (Figure S1B). While frlExd3 and frlExd8 are of different genetic background than frlExd62, all excisions obtained delete additional upstream genes. To prevent any phenotypes that may by due to these genes, FlyFOS 027901 (FosAB, add back; Figure S1B) (Ejsmont et al. 2009) encoding all missing upstream genes but ending in intron 1 of frl was integrated into landing site VK000027 (Venken and Bellen 2005) and recombinated onto a FRT80 chromosome with each excision. frlEx FRT80 FosAB were used throughout this work as frl alleles. FlyFOS 023275 encoding frl (and other genes) integrated in VK000002 on 2L rescues lethality and eye phenotype of all frl alleles.

To generate mutant clones in the mushroom body (MB), we used the mosaic analysis with a repressible cell marker (MARCM) technique (Lee et al. 1999). For frl mutant clones, elav-Gal4, UAS-mdCD8::GFP, hsFLP/+; FRT80 tubP-Gal80/ frlExd3 FRT80B FosAB, elav-Gal4, UAS-mdCD8::GFP, hsFLP/+; FRT80B, tubP-Gal80/ frlExd8 FRT80B FosAB, and elav-Gal4, UAS-mdCD8::GFP, hsFLP/+; FRT80B, tubP-Gal80/ frlExd62 FRT80B FosAB, and for wild-type control clones elav-Gal4, UAS-mdCD8::GFP, hsFLP/+; FRT80B tubP-Gal80/ FRT80B pupae were heat shocked at 37°C for 30 min at 50 hr after puparium formation (APF). The morphology of the MB clones was analyzed in flies 3–7 days old.

Immunohistochemistry

Third instar eye discs were fixed and stained as described (Freeman et al. 1992; Weber et al. 2000). For analysis of mushroom bodies, adult brains were dissected in cold PBS, fixed in 4% paraformaldehyde (diluted in PBS) at room temperature (RT) for 20 min. Primary antibodies were applied overnight at 4°C. After the secondary antibodies and standard washing steps, samples were mounted in PBS:glycerol (1:4). Confocal images were captured on an Olympus FV1000 LSM microscope, images were edited with Adobe Photoshop 7.0CE and Olympus FV10-ASW. Antibodies used were rabbit anti-Sal (1:50; a kind gift from C. Desplan (New York University), mouse α-β-Gal (1:200; Promega), rat α-Elav (1:200; DSHB 7E8A10), rabbit α-GFP (1:1000; Life Technologies), and mouse α-FasII (1:50; DSHB 1D4) as primary antibodies, and the corresponding Alexa-488 or Alexa-546 coupled secondary antibodies (Life Technologies).

Eye analysis

Mutant eye clones were induced with eyFLP (Newsome et al. 2000) or hsFLP (1 hr 15 min at 38°C at 0–72 hr after egg laying) using the Minute technique (Morata and Ripoll 1975) as indicated. Adult heads were embedded and sectioned as described (Jenny 2011). Ommatidia in three to six eyes were scored for quantifications with an ommatidium considered misrotated if misaligned by >20°. For DAAM frl double mutant analysis, an eyFLP insertion on the third chromosome was recombined onto a W+ [70c] FRT80 chromosome (note that the location of the eyFLP transgene on chromosome 3 is unknown), which was then combined with a FRT19A (W+). FRT19A; W+ [70c] FRT80 eyFLP males were crossed with DAAMExd8 FRT19A/FM6; frlEx FRT80 FosAB/TM6B virgins.

Plasmids

All PCR amplified fragments were sequence verified. Frl constructs are based on cDNA clone AT04875 (encoding protein variant PC). pSca-Frl was assembled in multiple steps from a N-terminal PCR product starting with CG32138_CT_for_Mfe to an internal BmgBI site, a BmgBI/BsrGI fragment of AT04875,
and a C-terminal PCR product starting at the BsrGI site and ending with CG32138_rev2_Xba. pSca-FlagΔGBD-DID and pSca-FlagΔFH2 correspond to PCR products amplified with primers CG32138_DeltaGBD_for and CG32138_rev2_Xba, and CG32138_for_MfeNco and CG32138_DeltaFH2_rev_Xba, respectively. The constitutively active FlagΔGBD-DID thus starts with amino acid 346 ([MK]GGH...) and the dominant negative FlagΔFH2 ends with amino acid 677 (GAMT; see also schematic in Figure 2A).

**Gst fusion constructs**: Gst-Dsh fragments are as in Jenny et al. (2005). pGexKG-GBD-DID and pGexKG-GBD-DID-DD-CC were cloned as PCR fragments using primers FrIGBD_Xba_f and FrIGBD_Hind3_r, FrIGBDplusFH3_Hind3_r, respectively in the Xba/HindIII sites of pGexKG (see schematic in Figure 2A). The FH2 domain with and without Cterm was cloned as PCR product with primers FH2plusC_Bam_F and CterXhoR_Frl and FH2minusC_Xho_R into the BamHI/XhoI sites of pGexAT1. pGexAT1-Cterm was cloned as PCR product amplified with primers cterBamF_Frl and cterXhoIR_Frl into the BamHI/XhoI sites of pGex4T1. pGex-3X-Rac encoding Drosophila Rac1 was a kind gift from S. Parkhurst (Fred Hutchinson Cancer Research Center). Drosophila Cdc42 was amplified with Cdc42_Gex_for_Bam and Cdc42_Gex_rev_Sal and cloned into the corresponding sites of pGex4T1.

**In vitro translation constructs**: Full-length Frl was in vitro translated from cDNA clone AT04875 using SP6 polymerase. The GBD-DID, GBD-DID-DD-CC, DD-CC, FH2, and FH2-CT domains were cloned as PCR products (see schematic in Figure 2A) using primers FrIGBD_Nsi_f/FrIGBD_Nco_r, FrIGBD_Nsi_f/FrIGBDplusFH3_Nco_r, FrI FH3_for_Nsi/ FrIGBDplusFH3_Nco_r, FrIFH2plusC_Nsi_f/FrIFH2minusC_Nco_r, FrIFH2plusC_Nsi_f/FrIFCherm_Nsi_r, respectively into the Nsi/I/Not sites of pβTH (Jenny et al. 2003).

**Cell culture plasmids**: pRK5_Myc_RhoA Q63L, pRK5_Myc_Rac1 Q61L, pCR_Myc_Rac1 N17, pDNA3_Myc_Cdc42 Q61L, and pCR_Myc_Cdc42 N17 encoding Myc tagged constitutively active and dominant negative human Rho GTPase versions were kind gifts from D. Cox (Developmental and Molecular Biology, Albert Einstein College of Medicine) (Cox et al. 1997; Abou-Kheir et al. 2008; Cammer et al. 2009). pCS2(105)-Dsh-Flag was described previously (Yanfeng et al. 2011). pCaspTubPA-Frl was generated by introducing the KpnI/XbaI fragment of pSca-Frl into the corresponding site of pCaspTubPA. pAcFlag3 was made by inserting oligos triple flag upper and lower into the KpnI/EcoRI sites of a version of pAc5.1 (Invitrogen) in which the internal BamHI had previously been destroyed. pAcFlag3-Frl was cloned by utilizing a MfeI/XbaI fragment of pCaspTubPA-Frl into the EcoRI/XbaI sites of pAcFlag3. pCS2(105)-Flag3-FrlΔFH2 was cloned by inserting a KpnI/blunt/Nael fragment of pAcFlag3-Frl into the blunt EcoRI site of pCS2(105). Note that this construct is 11 aa shorter than the GST version and ends at amino acid 666. pCsMT-Frl and pCsMT-Flag3-Frl were made by inserting NcoI/XbaI fragments of pSca-Frl and pSca-FlagΔFH2 into the corresponding sites of pCsMT (six N-terminal Myc tags). pCsMT-FlagΔGBD-DID was cloned as a HindIII/blunt/XbaI fragment of pSca-FlagΔGBD-DID into the NcoI/blunt/XbaI sites of pCsMT.

**Transgens**: pUAST versions of Frl, FrlΔGBD-DID, and FrlΔFH2 were made by cloning the MfeI/XbaI fragments of the corresponding pSca constructs into the EcoRI/XbaI sites of pUAST (Brand and Perrimon 1993). Transgens were injected by Genetic Services (Sudbury, MA) and Rainbow Transgensics (Camarillo, CA).

**Coimmunoprecipitation and pulldown assays**

Coimmunoprecipitations were done with a modified version of Jenny et al. (2005). Briefly, HEK293 cells were transfected with 3 μg of each plasmid using polyethylenimine (PEI). The cells were collected after 48 hr and washed with ice cold 1× PBS and lysed in 1 ml lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA supplemented with 1:1000 dilution of 1 mM benzamidine, and 1:100 dilutions of 10 μM leupeptin, and 1 μM peptatin). Cleared supernatants were precipitated with 1 μg of mouse anti-FlagM2 antibody (Sigma) that was bound to 20 μl of 50% Protein G beads overnight at 4°C. Immunoprecipitates were washed three times with cold wash buffer (lysis buffer with 0.1% Triton) and resuspended in 15 μl of 2× Laemmli buffer. Samples were separated on a 12% SDS gel and Western blot analysis was performed using standard protocols.

GST fusion protein expression and purification and GST pull-down assays for Frl and Dsh interactions were performed as described (Jenny et al. 2003) using 1 μg of GST fusion protein and 3 μl of a standard 50 μl TNT coupled transcription/translation reaction (Promega). Gst-Cdc42 and Gst-Rac pull-downs were modified from Magie et al. (1999). Per reaction, 1 μg of respective Gst protein was bound to 15 μl of Gst Sepharose beads (Amersham) in PD buffer (Jenny et al. 2003). After three washes, beads were washed twice more with exchange buffer (EB) (50 mM Heps pH 7.08, 50 mM NaCl, 5 mM EDTA, 0.1 mM EGTA, 0.1 mM DTT) and resuspended in 100 μl EB supplemented with either 100 μM GMP-PNP or GDP and 25 mM MgCl2 for nucleotide loading and incubated for 30 min at 30°C while rocking. Excess EB was then removed. A total of 3 μl in vitro translated Frl-GBD-DID-DD-CC were precleared with 10 μl GST-Sepharose beads in 100 μl HEPES PD buffer (20 mM Heps 7.5, 250 mM NaCl, 0.5% Triton, 10 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1 μM pepstatin, 10 μM leupeptin, 1 mM benzamidine) for 30 min at 4°C. The precleared Frl-GBD-DID-DD-CC was then combined with the nucleotide-loaded Gst protein beads with an additional 400 μl HEPES PD buffer and rotated for 45 min at 4°C followed by three washes in Heps PD buffer. Proteins were eluted by addition of 15 μl 2× SDS loading buffer and resolved on 12% SDS PAGE gels. After Coomasie staining, the gels were dried and exposed and scanned on a phosphorimager (Fuji 9000). Signals were quantified using MultiGauge V3.0.

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Reagent availability

All strains and plasmids constructed and used are available from the indicated Drosophila stock centers or upon request.

Oligonucleotides

CG32138_for_MfeNco, TATCAATTGGCCACCATGGGCGCGCG
CG32138_rev2_Xba, ATATCTAGACTAACAATC

CG32138 ΔFH2_rev_Xba, ATATTCTAG ATTAGCTGAGGCGCACTCG
FrGBD_Xba_f, GATCTCTAGACTAAGGACGAGCACGAGAATC;
FrGBD_Hind3_r, GAATCAAGCTTCTACTTACAGACAGATGCG

CG32138-TCAAATCG; CG32138_rev2_Xba, ATATCTAGACTAACAATC

CG32138_for_MfeNco, TATCAATTGGCCACCATGGGCGCGCG

CG32138 ΔGBD_for, AT

CAATTCGGGATACGGAGCGCGCCACCGACGG
FrGBD_Hind3_r, GAATCAAGCTTCTACTTACAGACAGATGCG

Flies are sectioned eyes of animals expressing CA- and DN-Frl under the control of the DAD domain, but more weakly to the C terminus only

frl

C terminus (and used as frl mutants throughout this work). Loss of frl in a Minute heterozygous background to more easily obtain large mutant areas (Morata and Ripoll 1975) caused weak rotation defects in all alleles with frl

frl

Figure 1, C and E and not shown; note that wild-type FRT80 M clones show no defects (n = 293). Importantly, viability and the eye phenotype were rescued by FlyFOS 023275, which encodes the complete frl gene (FosFrl; Figure 1, D and F; Figure S1B and not shown). Frl may thus contribute to ommatidial rotation in the Drosophila eye.

Dominant negative Frl causes ommatidial rotation defects

Constitutively active (CA) and dominant negative (DN) DRF forms can be constructed by deleting the N-terminal regulatory GBD and DID domains (ΔGBD-DID) or FH2 domain and C terminus (ΔFH2), respectively (Habas et al. 2001; Liu et al. 2008; Vaillant et al. 2008). To test whether Frl may be regulated by autoinhibition, we made transgenic flies expressing full-length Frl, CA-Frl (FrlΔGBD-DID), and DN-Frl (FrlΔFH2) under the control of UAS-Gal4 (Figure 2A). While the eyes of flies overexpressing full-length Frl under control of sev-Gal4 (sev > Frl) looked normal (Figure 2B; several independent transgenic lines showed similar results), eyes of flies expressing the putative CA-Frl (Figure 2C) or DN-Frl (Figure 2D) were severely rough. These results are consistent with Frl, like other DRFs being autoinhibited. Indeed, Gst pull-down experiments showed that an in vitro translated GBD-DID-DD-CC fragment strongly bound to the Gst-FH2-CT (including the DAD domain), but more weakly to the C terminus only fused to Gst (Figure 2E; schematic in Figure 2A). Consistent with an intramolecular interaction, full-length Frl only very weakly bound to Gst-FH2-CT. Neither the GBD-DID nor the DD-CC domains alone were sufficient to bind to Gst-FH2-CT nor were the in vitro translated FH2 or FH2-CT fragments able to bind to Gst-GBD-DID (Figure 2, E and F shows a Coomassie stained gel of a typical pull-down; note that we were unable to purify soluble Gst-DD-CC). Thus, the absence of a GOF phenotype of full-length Frl and the ability of N- and C-terminal fragments of Frl to interact indicate that Drosophila Frl is likely to be autoinhibited.

To examine the eye phenotypes in more detail, we analyzed sectioned eyes of animals expressing CA- and DN-Frl under the control of sep-Gal4, which is weaker than sev-Gal4, as it uses the enhancer and promoter of the sep gene instead of a heat-shock minimal promoter (Basler and Hafen 1990; Basler et al. 1991; Strutt and Strutt 2002). Overexpression of sep > CA-Frl caused loss of R-cells (Figure 3A). In contrast, overexpression of sep > DN-Frl consistently also caused rotation

Results

Loss of frl affects ommatidial rotation

The Drosophila genome encodes one gene each for six of the seven families of formins, including CG32138, the representative of the Frl/Fnnl subfamily (Higgs 2005). To test whether Frl may be involved in PCP signaling in the Drosophila eye, we knocked down frl in the eye using RNAi under the control of sevenless-Gal4 (sev) (Basler et al. 1991), which at the time of photoreceptor recruitment is expressed mainly in R3/R4. Compared to sev > Dcr2 that was coexpressed to enhance knockdown (Figure 1A) (Dietzl et al. 2007), RNAi-mediated knockdown of frl showed mild, but clear PCP-like defects (Figure 1B and Figure S1A). We then generated frl mutants by imprecise excision of P-elements, HP36775 and d06832, located upstream of frl or within the first intron of frl, respectively (see Materials and Methods and Figure S1B). To prevent potential defects caused by additional deletion of upstream genes annotated in FlyBase (FlyBase Consortium 2003), we integrated a genomic rescue DNA fragment encoding all upstream genes, but terminating after exon 1 of frl (FlyFOS 027901; FosAB for add back; Figure S1B) (Ejsmont et al. 2009) on the right arm of chromosome 3. The FosAB transgene was then recombined onto each frl excision that had previously been recombined with FRT80. All three frl excision FRT80 FosAB chromosomes are thus mutant only for frl and are homozygous lethal (and used as frl mutants throughout this work). Loss of frl in a

frl

Figure 1, E and not shown; note that wild-type FRT80 M clones show no defects (n = 293). Importantly, viability and the eye phenotype were rescued by FlyFOS 023275, which encodes the complete frl gene (FosFrl; Figure 1, D and F; Figure S1B and not shown). Frl may thus contribute to ommatidial rotation in the Drosophila eye.
defects in adult eyes (Figure 3B; note that stronger lines also lead to gaps that may be due to the loss of R-cell clusters; Figure 5G). Coexpression of CA-Frl (using several independent transgenic lines) with DN-Frl did not alter the phenotype of DN-Frl (not shown), most likely due to inadequate expression levels. The phenotype of DN-Frl is stronger than of frl mutant clones, which could be due to expression of DN-Frl being more acute than a loss of frl in mutants, which may be able to partially compensate for its loss. Alternatively, DN-Frl may also inhibit other forms (see below). Importantly, however, the defects arose at the time of PCP signaling in third instar larvae, as, compared to control (Figure 3C), misrotating omittalial clusters and clusters lacking R-cells were seen in eye discs stained for seven-up (svp)-lacZ, which is most strongly expressed in the R3/4 pair and more weakly in R1/6 during the early rotation period (Figure 3D) (Mlodzik et al. 1999; Fanto et al. 1998; Fanto et al. 2000; Gaengel and Mlodzik 2003; Jenny et al. 2003). Similar results were obtained upon staining of third instar eye discs with antibodies against Salm (Spalt-major) and testing and clusters lacking R-cells were seen in eye discs stained for sev-Gal4 (Jenny et al. 1998; Vaillant et al. 2000; Gaengel and Mlodzik 2003; Jenny et al. 2003). Thus, CA-Frl affects R-cell morphogenesis (or maintenance), and DN-Frl, similar to frl mutants, implicates Frl in omittalial rotation in the eye.

Frl is regulated by Cdc42 in vivo

DRFs can be activated by GTP-bound Rho family GTPase binding to the N-terminal GBD-DID domain (Habas et al. 2001; Vaillant et al. 2008; Liu et al. 2010). To determine which of the Rho family GTPases could bind Frl, we cotransfected Myc-tagged constitutively active human Rho, Rac, and Cdc42 and dominant negative Rac and Cdc42 with Flag-tagged FrlΔFH2 (schematic in Figure 2A) into HEK293 cells. Immunoprecipitation of Flag-FrlΔFH2 revealed a strong interaction with CA Cdc42 Q61L, but not with its DN version, Cdc42 N17 (Figure 4A). Upon prolonged exposure, we reproducibly also found a weaker coprecipitation of CA Rac Q61L (Figure 4A). No interaction was identified with RhoA, nor between Dsh (as a negative control) and any of the GTPases. To confirm Frl interactions, we used Drosophila Cdc42 and Rac1 fused to Gst loaded with the nonhydrolyzable GTP analog GMP-PNP or GDP to pull down in vitro translated 35S-methionine-labeled FrlGBD-DID-DD-CC. FrlGBD-DID-DD-CC bound to Cdc42 with about an eightfold increase of the active, GTP-bound form of Cdc42 over GDP-Cdc42 (Figure 4B; quantified in Figure 4D). Again, weaker binding was found for GST-Rac1 with about a fourfold enrichment over GDP-Rac1 (Figure 4C; quantified in Figure 4E). These findings are consistent with human FMNL1/2 or zebrafish Fmn13 activation by Cdc42 (Block et al. 2012; Kuhn et al. 2015; Richards et al. 2015; Wakayama et al. 2015) and mouse Frl1/Fmn1 showing a weak preference for Rac-GTP over Rac-GDP, although the latter failed to bind Cdc42 (Yayoshi-Yamamoto et al. 2000).

To test whether the interactions between Frl and Cdc42 and Rac were physiologically relevant, we tested for genetic interactions between Frl and Rho GTPases. Similar to sep-Gal4 driven overexpression of CA-Frl, overexpression of Cdc42 caused R-cell loss (compare Figure 5, A and E with Figure 3A; quantified in Figure 5D). Importantly, this R-cell loss was partially, but significantly suppressed by removal of one gene dose of any of the three frl excision alleles (Figures 5, B and C and not shown; quantified in Figure 5D) and completely suppressed by coexpression of DN FrlΔFH2 (Figure 5F; quantified in Figure 5I), suggesting that cdc42 can act as an upstream activator of frl. In addition, coexpression of Cdc42 with sep > DN-Frl also fully suppressed the rotation phenotypes of sep > DN-Frl (compare Figure 5F with 5G; quantified in Figure 5I), while removing one gene dose of cdc42 significantly enhanced sep > DN-Frl (Figure 5H; quantified in Figure 5I). We did not detect a genetic interaction between cdc42 and frl in frl Minute clones in the background of hemizygous males of the hypomorphic cdc42<sup>5G</sup> allele (not shown; however, see below for a genetic interaction between cdc42 and frl in the mushroom body). These data are consistent with potential Frl activation by Cdc42 in vivo, whereby DN-Frl competes with endogenous Frl for Cdc42. Thus overexpressed Cdc42 would not only activate endogenous Frl, but would also titrate DN-Frl. Similarly, removal of one gene dose of cdc42 would shift the balance toward DN-Frl and thus would enhance the phenotype of dominant negative Frl. Our in vitro binding and in vivo genetic interaction data suggest that Cdc42 can activate Frl in the eye.

In contrast, we did not observe genetic interactions between frl and rac in vivo. Activated Rac (sev-Rac<sup>V12</sup>) was shown to cause PCP phenotypes in the eye that can be modified by removal of one gene dose of PCP pathway components (Fanto et al. 2000). However, the frl excision alleles did not consistently suppress or enhance sev-Rac<sup>V12</sup> (Figure S2), nor was overexpression of Rac<sup>V12</sup> and DN-Frl synergistic, suggesting that Rac binding to Frl may not be relevant in the eye.

Dsh binds Frl in vitro

The core PCP protein Dsh activates the form xDaam1 during PCP signaling in Xenopus gastrula by associating with the DAD domain (Habas et al. 2001; Liu et al. 2008). Similar to xDaam1 (Habas et al. 2001), we found that in vitro translated full-length Frl, but not the Frl-FH2 domain bound to Gst-tagged full-length Dsh and its basic PDZ region (Figure 6A; the core PCP protein Pk served as positive control) (Jenny et al. 2003). In vitro translated Frl-FH2-CT also bound to Gst-Dsh-bPDZ over background (Figure 6A). We confirmed this interaction by cotransfecting HEK293 cells with Dsh-GFP and Myc-tagged Frl, FrlΔGBD-DID, and FrlΔFH2, respectively. Consistent with the above results, FrlΔGBD-DID lacking the N-terminal region of Frl, but not FrlΔFH2 lacking the C-terminal part was able to coprecipitate Dsh-GFP (Figure 6B). The inability of full-length Frl to coprecipitate Dsh may indicate that in tissue culture cells (or a lysate thereof), Dsh on its own may not be able to overcome the autoinhibitory interaction of Frl.

We then tested for genetic interactions between frl and dsh. First, we tested if frl alleles could dominantly modify the PCP
phenotype of males hemizygous for the viable, PCP-specific allele $\text{dsh}^1$ (Fahmy and Fahmy 1959; Boutros et al. 1998; Axelrod 2001). Neither removal of one gene dose of $\text{frl}$ by $\text{frl}^{\text{ExS83}}$ nor $\text{frl}^{\text{ExS62}}$ was able to modify the PCP phenotype of $\text{dsh}^1$ males (Figure 6, C–E; quantified in Figure 6F; note that $\text{dsh}^1$ can be suppressed by the core PCP allele $\text{pk}^3$) (Jenny et al. 2005). Similarly, the phenotype of homozygous mutant $\text{frl}^{\text{ExS83}}$ or $\text{frl}^{\text{ExS62}}$ clones in $\text{dsh}^1$ hemizygotes looked similar to $\text{dsh}^1/Y$ alone (Figure 6F) and the phenotype of $\text{frl}^{\text{ExS83}}$ or $\text{frl}^{\text{ExS62}}$ clones was not enhanced in females lacking one gene dose of $\text{dsh}$ (not shown). We then tested whether the GOF PCP phenotypes of $\text{sev} > F a$ or $\text{sev} - \text{Dsh}$ (Boutros et al. 1998) could be modified by removal of a gene dose of $\text{frl}$ or a deficiency uncovering the $\text{frl}$ locus. However, similar to $\text{DAAM}$ (Matussek et al. 2006), $\text{frl}$ showed no such dominant interaction. Thus, despite $\text{frl}$ binding to Dsh in $\text{in vitro}$, we were not able to find $\text{in vivo}$ evidence to support a functional requirement for such an interaction during PCP signaling in the eye and $\text{frl}$ may thus act downstream of $\text{Cdc42}$ rather than the core PCP factors during eye polarization.

We then tested whether $\text{DN-Frl}$ genetically interacts with other components known to be required for ommatidial rotation (reviewed in Jenny 2010; Goodrich and Strutt 2011; Devenport 2014). We did not observe a dominant genetic interaction of $\text{DN-Frl}$ with EGF pathway-related genes [argos ($\text{aos}$), sprouty ($\text{spy}$), phyllopod ($\text{phyl}$), or canoe; not shown] or the rotation-specific kinase nemo ($\text{nmo}$; Figure S3) or E-cadherin ($\text{shg}$; Figure S3). In contrast, simultaneous removal of one gene dose of both $\text{Drosophila}$ N-cadherin genes ($\text{CadN1/2}$) known to control the speed and extent of ommatidial rotation (Mirkovic and Mlodzik 2006) enhanced $\text{DN-Frl}$, again suggesting that $\text{DN-Frl}$ affects ommatidial rotation (Figure 7; quantified in Figure S3; note that $\text{CadN1/2}$ may also show a trend to enhance R-cell loss).

**Frl cooperates with DAAM during axon growth in the mushroom body**

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that develop in a stereotypic way (Lee et al. 1999). Each Kenyon cell extends a neurite that splits into a dendritic and an axonal part. The axons extend anteroventrally forming a prominent fiber tract called the peduncle (Figure 8D). In adults, after reaching the anterior side, axons of the α neurons run medially and form the α lobe, whereas axons of the α/β and α/neurons bifurcate and their dorsal branches form the α and α′ lobes while their medially growing branches form the β and β′ lobes (see schematic in Figure 8D). After initial neurite outgrowth, axonal extension toward the appropriate target areas requires the core PCP genes vang, fz, dsh, fmi, and pk (Shimizu et al. 2011; Ng 2012). In addition, we recently showed that DAAM functions as cytoskeletal effector of the PCP guidance system in the MBs. In ~70% of the DAAM mutant lobes, MB axons either fail to form, terminate their growth before reaching the tip of the neuropile lobes, or fail to project into the proper lobes, indicating that while DAAM is required for axon formation in the MB, additional forms might cooperate with DAAM for proper MB development (Gombos et al. 2015).

We therefore tested whether frl also affects MB axons and, if so, whether it cooperates with DAAM. We induced positively marked single cell clones that lack frl in α/β neurons using the MARCM technique (see Materials and Methods for details) and assessed axon defects in adult brains. Compared to wild-type axons that bifurcate and project to the ends of the α/β lobes (counterstained with FasII in Figure 8E), 14% of frl Ex83, 16.7% of frl Ex88, and 21.6% of frl ExK62 mutant neurons showed premature axon growth arrest (but no bifurcation defects; Figure 8, F and G, and not shown; quantified in Figure 8H), suggesting that Frl is required for correct growth of MB axons, but may not be required for or act redundantly with other genes for their correct guidance. We then tested for genetic interaction between frl and DAAM. Hemizygous males of the hypomorphic alleles DAAMEx1 and DAAMEx4 are viable and show moderate MB axon growth and guidance defects (Gombos et al. 2015). Importantly, the growth phenotypes of DAAMEx1 and DAAMEx4 were dominantly and strongly enhanced by the removal of one gene dose of frl by all three alleles (quantified in Figure 9, A and B for the α and β lobes, respectively). Furthermore, these phenotypes in DAAMEx1 and DAAMEx4 hypomorphs are also enhanced by RNAi-mediated knockdown of frlTRIPEHIS004453 expressed under control of OK107-Gal4 in the β lobe (Figure 9D) and for DAAMEx4 also in the α lobe (Figure 9C). Distinct from DAAM (Gombos et al. 2015), but consistent with the lack of genetic interaction between
and dsh during PCP signaling in the eye, removal of one gene
dose of frl did not alter the axon growth or guidance phenotype of
the PCP-specific dsh<sup>1</sup> allele (quantified in Figure 9, E and F).
Collectively, our data suggest that frl and DAAM are both required
for proper axonal development of the MB neurons. Their strong
dominant genetic interaction indicates a cooperative mode of
action between these two formins in this tissue, although they
are likely controlled by different signals.

We also tested whether frl interacted with cdc42 in the MB.
We found that hemizygous males of the hypomorphic allele

Figure 5 Frl can be activated by Cdc42 in vivo. (A–C) The R-cell loss of sep >
Cdc42 (A) is dominantly suppressed by re-
moval of one gene dose of each frl allele
(B; frl<sup>Ex83</sup>; C; frl<sup>Ex162</sup> and not shown; quanti-
fication in D). (D) Quantification (percentage
ommatidia with wild-type R-cell complement)
of sep > Cdc42 lacking one gene dose of the
alleles indicated underneath. (E–H) sep >
Cdc42 induced R-cell loss (E shows the
phenotype of a different chromosomal insertion
than used in A) is fully suppressed by coex-
pression of dominant negative sep > DN-Frl
(ΔFH2) (F; quantified in I). Conversely, the
phenotype of sep > DN-Frl (G; note that this
stronger line also causes gaps in between
ommatidia) is suppressed by coexpression of
sep > Cdc42 (F), but dominantly enhanced
by cdc42<sup>1</sup>, a null allele (H; quantified in I). (I)
Quantification of PCP and R-cell loss pheno-
types of indicated genotypes (gaps: whole
ommatidia missing). t-test; ***P < 0.001;
**P < 0.02.
**cdc42** show growth and guidance defects in the α and β lobes (quantified in Figure 9, G and H). Importantly, all three *frl* alleles dominantly enhance the growth phenotype of *cdc42* hemizygotes (Figure 9, G and H), strongly suggesting that Cdc42 controls Frl also in this context.

**Discussion**

In this report, we have analyzed the role of the diaphanous-related formin Frl during ommatidial rotation in the *Drosophila* eye and axon outgrowth during mushroom body development. RNAi-mediated knockdown or mutations in *frl* cause mild ommatidial rotation defects. Biochemical and genetic interaction experiments are consistent with Frl being autoinhibited and activated by Cdc42 *in vivo*. Significantly, we showed that Frl and DAAM, members of two distinct formin subfamilies, cooperate in R-cell morphogenesis or maintenance and have distinct and overlapping functions in axon growth in the mushroom body, for which a subset of PCP genes is also known to be required (Shimizu *et al.* 2011; Ng 2012).

**Activation of Frl**

Overexpression of full-length Frl does not cause a phenotype in the eye, indicating that Frl, similar to other DRFs, likely...
Figure 7 The phenotype of DN-Frl (A) is dominantly enhanced by concurrent removal of one gene dose of both N-cadherins (CadNA14; B). Above: tangential eye sections; below: schematics. See Figure S3 for quantification.

needs to be activated. Many DRFs such as mDia2, Daam, or mFrl2 are autoinhibited by binding of the C-terminal DAD domain to the GBD-DID domains (Higgs 2005; Otomo et al. 2005, 2010; Vaillant et al. 2008; Liu et al. 2010). Consistent with a need for activation, the GBD-DID containing N-terminal region of Frl can bind the FH2-CT and the CT domain much more efficiently than full-length Frl (Figure 2E), although for unknown reasons, Frl-GBD-DID is not sufficient for this interaction in vitro. In addition, constitutively active Frl and dominant negative Frl cause severe rough eye phenotypes and, when expressed in a more restricted way using sep-Gal4, loss of R-cells and PCP-like phenotypes, respectively (note that although a “weaker” transgenic line mostly shows rotation defects (Figure 3B), we cannot exclude that additional morphological defects also contribute to the ommatidial rotation defects caused by DN-Frl). These data are thus consistent with Frl being autoinhibited like most DRFs (Campellone and Welch 2010; Bogdan et al. 2013).

Drosophila Frl strongly binds to Cdc42 with a significant preference for the active vs. the inactive form (Figure 4), suggesting that Frl is activated by Cdc42. Consistently, we observed that overexpression of Cdc42 and the CA-Frl cause a similar R-cell loss and that the R-cell loss upon Cdc42 expression is dominantly suppressed by removal of one gene dose of all frl alleles (and by DN-Frl; Figure 5). Conversely, we find that ectopic activation of Cdc42 suppressed the PCP defects of DN-Frl, likely by competing with DN-Frl and thus allowing a higher activation of endogenous Frl by Cdc42. Overall, our data strongly suggest that Frl is activated by Cdc42 in vivo, which is also supported by our finding that the phenotype of a hypomorphic cdc42 mutation in the mushroom body is enhanced by all three frl alleles (Figure 9, G and H). Our observations are consistent with recent data demonstrating that Cdc42 activates human FMNL2 (Fr13) during cell migration in culture and that Cdc42 activates Fml3 (Fr12) to promote filopodia formation during angiogenic sprouting (Block et al. 2012; Kuhn et al. 2015; Richards et al. 2015; Wakayama et al. 2015).

Originally, mouse Frl1 was shown to bind to Rac1 but not to RhoA or Cdc42 with a slight preference for the active, GTP-bound form over the GDP-Rac1 in lysates of a P388D1 macrophage cell line (Yayoshi-Yamamoto et al. 2000), although no functional data supporting this interaction were presented. Despite the weak, but reproducible physical interaction between Rac1 and Frl in communoprecipitation and GST pull-down experiments, we were unable to identify genetic interactions between rac and frl in the eye. Thus, the physiological relevance of Rac binding to Frl remains an open question.

Generally, the involvement of Rho-family GTPases in PCP signaling is controversial and less well understood (reviewed in Schlessinger et al. 2009). Loss of cdc42 in the mouse inner ear has recently been shown to cause misalignment of sensory stereocilia similar to loss of core PCP genes such as Vangl2 (Montcouquiol et al. 2003; Kirjavainen et al. 2015). Additionally, while Daam1 activates Rho and Rac, but not Cdc42 in Xenopus tissue undergoing gastrulation (Habas et al. 2001), DN-Cdc42 can suppress the convergence and extension phenotypes caused by overexpressed Wnt11 or Fz7, but not Dsh (Djiane et al. 2000; Choi and Han 2002), indicating that Cdc42 may act in a parallel pathway to classical PCP signaling downstream of Dsh. In Drosophila, the involvement of Rho GTPases had originally been identified in rhoA mutant clones (Strutt et al. 1997) but had mainly been characterized using genetic interaction assays with PCP GOF scenarios and overexpression of constitutively active or dominant negative GTPase variants (Strutt et al. 1997; Boutros et al. 1998; Fanto et al. 2000). Unfortunately, eye clones of cdc42 null mutant cells cannot be recovered (Genova et al. 2000; Muñoz-Descalzo et al. 2007), but a hypomorphic cdc42 allele shows mild rotation defects in the eye similar in strength to loss of frl (2.9 ± 3.8%), and the mild PCP phenotype of rac1 and its paralogs rac2 and mtl in triple mutant clones are enhanced in a cdc42 hypomorphic background, suggesting that these GTPases contribute to PCP signaling in a partially redundant manner (Muñoz-Descalzo et al. 2007). Cdc42 also acts together with Mtl (Muñoz-Descalzo et al. 2007; Muñoz-Soriano et al. 2011), which is regulated by EGFR signaling that is known to control ommatidial rotation (Brown and Freeman 2003; Gaengel and Mlodzik 2003; Strutt and Strutt 2003). While we present evidence that Cdc42 regulates Frl during PCP signaling in the eye, we failed to detect...
genetic interactions between 
sep > DN-Frl and EGFR signaling pathway components such as aos, cno, phyl, or sprouty. In support of Cdc42 affecting PCP signaling independent of Dsh, we found that similar to cdc42 (Boutros et al. 1998), frl alleles are unable to suppress sev > Fz or sev-Dsh (not shown). Interestingly, however, we found that sep > DN-Frl is enhanced by removal of a gene dose of both N-cadherins (Figure 7) that are known to control speed and extent of ommatidial rotation downstream of the Fz-PCP and EGFR pathways (Mirkovic and Mlodzik 2006), suggesting a complicated relationship between genes involved in rotation.

**Frl and DAAM cooperate during neural development**

Daam1 was originally identified in a two-hybrid screen with a fragment of Dsh containing its central PDZ domain and later was shown to be activated by Dsh interacting with the C terminus of Daam1, thereby regulating PCP signaling during convergence and extension in Xenopus (Habas et al. 2001; Liu et al. 2008). An interaction between Dvl3 and Daam2 in chicken was also identified and is considered crucial for the cofactor function of Daam1 during D/V patterning of the chick neural tube by canonical Wnt signaling (Lee and Deneen 2012). Because Drosophila DAAM has no unique function during PCP signaling in Drosophila, we initially hypothesized that Frl may cooperate with DAAM in that process. Indeed, we found that, similar to xDaam1, Frl can interact with the PDZ region of Dsh in vitro in a manner that is dependent on the FH2-CT region (Figure 6, A and B). However, we were unable to find in vivo evidence supporting such an interaction. Neither did we detect an obvious...
redundant function of frl and DAAM in PCP signaling in the eye in double mutants (although the double mutant clones are small only). Intriguingly, however, DAAM frl double mutant R-cells show abnormal, small rhabdomeres in adult eye sections, a phenotype that is not seen in either single mutant, suggesting that frl and DAAM indeed can exhibit overlapping functions in neurons (Figure 8, B and C).

It has recently been shown that DAAM is required for axon growth and pathfinding in the mushroom body, where it genetically interacts with the core PCP genes including fz, vang, and dsh, as well as Rac1 (Gombos et al. 2015). Similar to DAAM mutants, frl single mutants display an axon growth phenotype (Figure 9), and heterozygosity for frl enhances the axon growth phenotype of hypomorphic cdc42\(^2\) mutant.
males, consistent with Cdc42-activating Frl. However, distinct from DAAM, frl mutants do not affect axon guidance. Nevertheless, we again found evidence for cooperation between DAAM and Frl, as frl strongly dominantly enhanced the growth phenotype of DAAM hypomorphic mutants. Again, however, no genetic interaction was found between dsh and frl, indicating that, in contrast to DAAM, Frl is likely regulated in a core PCP-independent manner. Thus, both formins cooperate during and are required for precise formation of the MB axons. However, they probably are under distinct regulatory control and thus may integrate various signaling inputs with the regulation of the cytoskeleton. Overall, our data suggest that Frl and DAAM, members of distinct formin subfamilies, can act together in specific cellular processes, likely reflecting the need for precise control of the complex and context-dependent regulation of the actin cytoskeleton by various upstream signaling systems.

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Unique and Overlapping Functions of Formins Frl and DAAM During Ommatidial Rotation and Neuronal Development in Drosophila

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sev > ey > Dcr2, frl^{HMS00445}
**Figure S2.** *fri* does not genetically interact with *sev-Rac*[^12]. Quantification of PCP phenotypes of *sev-Rac*[^12] lacking one gene dose of or overexpressing the indicated gene. The stronger defects due to co-expression of *sevRac*[^12] with dominant negative *sep>FrlΔFH2* is likely an additive effect. T-Test; ***p<0.001; **p<0.02; *p<0.05.

[^12]: RacGTPase family member encoded by the *Rac* gene, which plays a critical role in the establishment of planar cell polarity (PCP).
Figure S3. Dominant negative frl is dominantly enhanced by simultaneous removal of a gene dose of both N-Cadherins. Quantification of PCP phenotypes of sep>DN-Frl (ΔFH2) lacking one gene dose of the indicated alleles. Note that (A) and (B) were separate experiments and thus each have their own baseline and that CadN^Δ14 may also show a trend to enhance R-cell loss (although this is statistically not significant). The deficiency BSC148 uncovers a larger genomic region including both N-Cadherin genes. T-Test; *p<0.05.