Published in final edited form as:

Nat Neurosci. 2012 June ; 15(6): 871–875. doi:10.1038/nn.3102.

Visual neurotransmission in *Drosophila* requires expression of Fic in glial capitate projections

Mokhlasur Rahman¹, Hyeilin Ham², Xinran Liu¹, Yoshie Sugiura¹, Kim Orth², and Helmut Krämer^{1,3}

¹Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas, USA

²Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

³Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

Abstract

Fic domains can catalyze the addition of adenosine monophosphate to target proteins. To date, the function of Fic domain proteins in eukaryotic physiology remains unknown. We generated genetic models of the single *Drosophila* Fic domain-containing protein, Fic. Flies lacking *Fic* were viable and fertile, but blind. Photoreceptor cells depolarized normally following light stimulation, but failed to activate postsynaptic neurons, as indicated by the loss of ON transients in electroretinograms, consistent with a neurotransmission defect. Functional rescue of neurotransmission required expression of enzymatically active Fic on capitate projections of glia cells, but not neurons, supporting a role in the recycling of the visual neurotransmitter histamine. Histamine levels were reduced in the lamina of *Fic* null flies, and dietary histamine partially restored ON transients. These findings establish a previously unknown regulatory mechanism in visual neurotransmission and provide, to the best of our knowledge, the first evidence for a role of glial capitate projections in neurotransmitter recycling.

Reversible post-translational modifications have a pivotal role in eukaryotic signaling and are therefore exploited by bacterial pathogens. Studies on a *Vibrio* virulence factor, VopS, demonstrated that its Fic domain is used to modify Rho-GTPases with adenosine monophosphate (AMP)¹. This modification was first observed over 40 years ago when covalent modification of a tyrosine with AMP reversibly regulated a bacterial protein glutamine synthetase^{2,3}. Only recently have new enzymes, including the Fic domain-containing proteins and nucleosidyl transferases, been discovered that are not restricted to bacteria, but are also found in eukaryotes^{1,4,5}. The former domain is found in human Huntingtin-interacting protein E and the *Drosophila* Fic protein (Flybase: CG9523) and both

© 2012 Nature America, Inc. All rights reserved.

Correspondence should be addressed to H.K. (helmut.kramer@utsouthwestern.edu).

AUTHOR CONTRIBUTIONS

M.R. and H.K. performed the fly genetic studies. X.L. and Y.S. performed the electron microscopy studies. H.H. performed the molecular biology and biochemistry studies. H.K., M.R. and K.O. designed the study and analyzed the data. H.K. and K.O. wrote the paper. All of the authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

Supplementary information is available in the online version of the paper.

have been shown to catalyze AMPylation reactions^{5,6}, but the physiological contexts in which they operate have not been defined yet. To explore the role of these proteins, we focused on the *Drosophila* Fic protein.

RESULTS

We reasoned that a regulatory role of Fic might be revealed by genetically manipulating its expression in flies. Notably, high-level overexpression of *UAS-Fic* by the *da-Gal4* driver was lethal (Supplementary Table 1). This lethality depended on the enzymatic activity of Fic, as no phenotypes were detected following expression of the catalytically inactive Fic^{H375A} mutant, which lacks AMPylation activity⁶. Flies expressing wild-type *Fic* or mutant *UAS-Fic* transgenes at low levels under *arm-Gal4* control did not exhibit externally visible phenotypes or lethality (Supplementary Table 1). These data support the hypothesis that AMPylation may regulate important cellular processes.

To explore this proposal, we generated *Fic* loss-of-function deletion mutations by excision of a nearby P element (Fig. 1a). One of the resulting alleles, *Fic*⁵⁵, lacked the 5' UTR and the first 260 of 493 amino acids, including the highly conserved tetratricopeptide repeat domain. Western analysis confirmed the loss of Fic protein (Fig. 1b). Flies homozygous for *Fic*⁵⁵ were viable and fertile. Their behavior, however, seemed sluggish compared with wild-type flies. This deficit was quantified in a fast-phototaxis assay^{7,8}, which revealed that the *Fic*⁵⁵ mutants were not attracted to a light source, that is, they were blind (Fig. 1c). This phenotype was specific for *Fic*⁵⁵ flies, as it was also observed in flies expressing a *UAS-Fic* dsRNA transgene under *da-Gal4* control, and wild-type behavior was restored by a genomic *Fic* transgene (Fig. 1c).

This altered behavior could reflect a deficit in motor output, light reception or the processing of visual information. To distinguish between these possibilities, we measured the electrical response to a light pulse using electroretinograms (ERGs). ERGs revealed normal light-induced depolarization in wild-type and *Fic*⁵⁵ flies, arguing against a substantial defect in light reception in *Fic*⁵⁵ photoreceptors (Fig. 1d). ERGs of *Fic*⁵⁵ flies missed, however, the characteristic ON transient of the response to light. ON transients in ERGs are a reflection of synaptic activation of the postsynaptic laminar neurons^{9,10}. This defect was specific for the loss of Fic function, as it was also observed in *Fic*⁵⁵/Df(2L)BSC296 hemizygotes and after *Fic* knockdown (*UAS-Fic* dsRNA; *da-Gal4*). Furthermore ON transients were restored by a genomic *Fic* transgene or by expression of a *Fic* cDNA under the control of the low-level *arm-Gal4* driver (Fig. 1e). Similarly, expression of transgenes that encoded Fic fused at its C terminus to GFP or horseradish peroxidase (HRP) restored ON transients, indicating that these chimeric proteins are functional. Notably, expression of the catalytically inactive Fic^{H375A} mutant⁶ was not able to restore ON transients (Fig. 1e), indicating that Fic enzymatic activity is necessary for normal activity of photoreceptor synapses required for vision.

Loss of ON transients in ERG recordings reflects the loss of synaptic transmission from photoreceptor cells. This defect might occur as a consequence of many different physiological deficiencies, such as the loss of pre- or postsynaptic proteins affecting synapse function^{11,12}, or changes in the distribution of organelles, including mitochondria or synaptic vesicles^{13,14}. When photoreceptors and their synaptic connections were evaluated by electron microscopy (Fig. 2a–f), no difference was evident in the arrangement of photoreceptor axons (Fig. 2a,b) or rhabdomeres (Fig. 2g,h) or in the synaptic ultrastructure, as mitochondria, synaptic vesicles and active zone T bars appeared unchanged (Fig. 2a–f). These findings argue against Fic function being required as a structural element of synapses.

To further narrow the focus of Fic activity, we explored which cells require its expression for normal visual neurotransmission. To test the requirement in photoreceptor cells, we generated chimeric flies¹¹ with whole-eye clones homozygous for *Fic*⁵⁵ (*ey3.5-Flp; FRT Fic*⁵⁵). These flies still exhibited ON transients (Fig. 2i), as did flies with photoreceptor-specific knockdown of Fic (*GMR-Gal4/UAS-Fic* dsRNA). Furthermore, expression of Fic in both photoreceptors and their lamina target neurons under control of the *elav-Gal4* neuronal driver¹⁵ did not restore ON transients in *Fic*⁵⁵ mutants (Fig. 2i). Together these data argue against a requirement of Fic in photoreceptors or lamina neurons.

In contrast, glia-specific expression of Fic under the control of the *repo-Gal4* driver¹⁶ was sufficient to restore ON transients (Fig. 2i). Moreover, glia-specific knockdown of Fic (*UAS-Fic* dsRNAi; *repo-Gal4*) indicated that Fic is necessary for normal ON transients in glia cells. To test the relevance of these electrophysiological responses to behavior, we evaluated flies in the fast-phototaxis assay. Consistent with the ERG recordings, loss of Fic function in whole-eye clones or its photoreceptor-specific knockdown (*GMR-Gal4/UAS-Fic* dsRNA) did not eliminate fast phototaxis. Furthermore, neuronal Fic expression (*elav-Gal4; UAS-Fic*) did not restore normal behavior in *Fic*⁵⁵ mutants (Fig. 2j). In contrast, *repo-Gal4*-driven knockdown of Fic indicated its requirement in glia cells for fast-phototaxis. Moreover, glia-specific expression of Fic (*repo-Gal4; UAS-Fic*) significantly improved fast phototaxis of *Fic*⁵⁵ mutants ($P < 0.001$; Fig. 2j), although it did not fully restore normal behavior, indicating a requirement in additional cells of the phototaxis circuits¹⁷. Notably, expression of the catalytically inactive *Fic*^{H375A} mutant in glia cells did not rescue the characteristic ON transient of the response to light (Fig. 2i) or improve the fast-phototaxis behavior of *Fic*⁵⁵ flies (Fig. 2j). Together, these data indicate that Fic enzymatic activity in glia cells is necessary and sufficient for normal synaptic transmission in the visual system.

One important role of glia cells in the *Drosophila* lamina is the recycling of histamine, the neurotransmitter released by photoreceptors^{18–20}. Histamine is synthesized in photoreceptors by histidine decarboxylase (*Hdc*)²¹. However, *Hdc* activity covers only a fraction of the histamine requirement for their normal synaptic activity; the balance of histamine is provided by recycling²². After release by photoreceptor synapses, histamine is removed from the synaptic cleft by active uptake into glia cells. There, a β -alanyl-histamine synthetase encoded by *ebony* catalyzes the condensation of histamine with β -alanine^{23,24}. The resulting product, carcinine, is extruded from glia cells and taken up by photoreceptor cells in a poorly understood process^{18,25,26}. In photoreceptors, histamine is recovered from carcinine by a β -alanyl-histamine hydrolase encoded by *tan*²⁰. Interruption of this cycle results in the loss of visual transmission, as indicated by behavioral deficits and the loss of ON transients in ERG recordings^{10,18,19,21}.

Notably, phenotypes caused by mutants interfering with histamine metabolism can be partially rescued by dietary histamine or carcinine, which as a result of the recycling pathway, can be taken up into photoreceptor cells^{18,19}. When *Fic*⁵⁵ flies were fed a diet enriched with histamine or carcinine, we observed partial restoration of ON transients (Fig. 3a), although the level of rescue was not sufficient to restore normal behavioral in the fast-phototaxis assay (Fig. 3b). To test whether histamine distribution is altered, we stained sections of wild-type and *Fic*⁵⁵ heads with antibodies to histamine²⁵. Wild-type flies exhibited some variable staining in photoreceptor cell bodies and strong staining in the layer of epithelial glia cells just proximal to photoreceptors and in photoreceptor axon terminals (Fig. 3c). In contrast, histamine immunoreactivity in epithelial glia was strongly reduced in *Fic*⁵⁵ flies (Fig. 3d), although staining was still observed in photoreceptor cell bodies. Staining in epithelial glia cells was partially restored by glia-specific expression of Fic (Fig. 3e). Specificity of staining for histamine was indicated by its absence in *Hdc*^{P218} heads²¹ (Fig. 3f). Loss of histamine staining in *Fic*⁵⁵ lamina is reminiscent of the changes in

histamine distribution in other mutants that disrupt histamine recycling in photoreceptor synapses, including *tan*, *ebony* and *Vmat*^{20,23,25,27}. Together, these data are consistent with the notion that histamine metabolism is one of the physiological processes regulated by the enzymatic activity of Fic in glia cells.

To gain further insight into the mechanism by which histamine metabolism is affected by Fic, we analyzed its subcellular localization. Subcellular fractionation of S2 cells showed Fic partitioning with the endoplasmic reticulum marker BiP in the P2 particulate fraction, indicating that Fic may be a membrane-associated or transmembrane protein (Fig. 4a). To test whether Fic is secreted, we analyzed whether Fic was N-glycosylated, a modification that occurs in the lumen of the endoplasmic reticulum. When Fic was expressed using an *in vitro* translation system, the addition of microsomes caused a shift in mobility consistent with N-glycosylation of Fic. Mutation of Asn288 at the only predicted N-glycosylation site suppressed this shift in Fic^{N288Q} (Fig. 4b). We reasoned that if Fic is secreted into the endoplasmic reticulum lumen, then its glycosylation might be sensitive to endoglycosidase H (EndoH), which specifically cleaves the high-mannose sugars. To test this, we treated Fic protein purified from Sf21 cells or endogenously present in S2 cell lysates with EndoH. Both Fic preparations exhibited a shift in their mobility that was consistent with a loss of high-mannose modifications (Fig. 4c). To assess whether part or all of Fic was secreted, we performed a protease protection assay. *In vitro*-translated Fic was readily degraded by proteinase K, but, after translation in the presence of microsomes, a ~48-kDa protein was protected from degradation (Fig. 4c). These data suggest that Fic is a type II transmembrane protein with its single N-terminal hydrophobic stretch (Fig. 1a) functioning as a transmembrane domain and its N-glycosylation site and catalytic domain being secreted into the lumen of the secretory pathway.

To determine the localization of Fic in glia cells (Fig. 4d–r), where it is required for normal visual neurotransmission, we expressed a functionally active Fic-HRP fusion protein (Fig. 1e) either under the control of the endogenous *Fic* promoter and enhancer elements (Fig. 4d,f) or under control of the *repo-Gal4* driver (Fig. 4h,j,l–o). HRP activity of the chimeric protein was used to detect Fic-HRP by electron microscopy²⁸. Fic-HRP was expressed on the surface of glia cells and prominently enriched in capitate projections that epithelial glia cells insert into synaptic endings of photoreceptors (Fig. 4d,f,h,j,l–o). HRP activity was strongest in the head of capitate projections, as revealed by the electron-dense DAB precipitate in the extracellular space between glia cell and neuron (Fig. 4d,f,h,j,l–o). Control sections of flies lacking Fic-HRP expression showed no such DAB accumulation (Fig. 4e,g,i), instead revealing the slightly expanded extracellular space typical for capitate projections (Figs. 2e,f and 4k,p–r)^{14,29}. Prompted by this localization of Fic, we quantified the appearance of capitate projections. In *Fic*⁵⁵ mutant heads, the frequency of capitate projections was slightly reduced; we observed 4.3 ± 0.3 capitate projections per synaptic cross-section in wild type compared with 2.8 ± 0.2 in *Fic*⁵⁵ (mean $\pm$ s.d., $n = 3$ heads each, $P = 0.012$, $t = 4.32$, $F = 3.0$). However, the shape (Fig. 2e,f) and average diameter of capitate projections were not altered in *Fic*⁵⁵ mutants (wild type, 184 ± 5.8 nm; *Fic*⁵⁵, 190 ± 4.0 nm (mean $\pm$ s.d.); $n = 3$ heads each, $P = 0.4$, $t = 0.846$, $F = 2.079$), indicating that Fic activity is not necessary for their formation. Notably, capitate projections have been speculated to be the site of neurotransmitter recycling into photoreceptor cells¹⁴, although no transporter or other transmembrane protein, besides Fic, is known to specifically localize to these specialized synaptic organelles.

DISCUSSION

Our data are consistent with the idea that Fic's enzymatic activity is involved in the regulation of the distribution of the visual neurotransmitter histamine, which is critical for

the function of photoreceptor synapses and vision. Proteins in the histamine metabolism and secretion pathway that are sensitive to Fic-mediated AMPylation are not yet known. A first hint at possible targets comes from Fic's requirement in glia cells, where its target may participate in the uptake of histamine or its subsequent modification to carcinine. This latter reaction is catalyzed by Ebony, a cytoplasmic protein^{23,24}, which is therefore an unlikely substrate for the secreted Fic active site. Thus, the more likely targets in glia cells are the unknown transporters that facilitate the uptake of histamine or its recycling to photoreceptors in the form of carcinine.

Strong support for the latter model arises from the specific localization of Fic in glia cells, where this enzyme is found on the cell surface at capitate projections (Fig. 4l–o). These invaginations of glia cells into the synaptic endings of photoreceptor cells are dynamic organelles that can occur as shallow indentations (Fig. 4k,o) or with extended necks with one (Fig. 4h,m,p) or multiple heads (Fig. 4r). On the basis of this intriguing morphology, changes in their number in *tan* and *ebony* mutants affecting histamine recycling³⁰, and the close association with sites of active endocytosis, capitate projections have long been speculated to be sites of neurotransmitter recycling^{14,26}. The localization of Fic to these organelles provides, to the best of our knowledge, the first functional data to support this speculation. The localization of Fic in glia cells may reflect a direct interaction with transporters that are targeted to capitate projections and serve as targets of Fic.

What function could Fic-mediated modification of these transporters serve? One model comes from classic studies in which the active site of the enzyme glutamine synthetase is regulated by reversible modification of tyrosine residue with AMP². In a more recent example, AMPylation regulates Rab1 activation during *Legionella pneumophila* invasion into cells. Early during invasion, Rab1 is activated by the effector protein SidM, which subsequently catalyzes Rab1 AMPylation⁴. This modification transiently locks Rab1 in the activated GTP-bound state, which promotes the fusion of secretory vesicles with the vacuole containing the *Legionella* bacteria. Only release of a second bacterial effector, SidD, removes the AMP residue and thereby unlocks Rab1 so that it can be inactivated^{31,32}. Similarly, AMPylation may modify transporter activity at capitate projections depending on changes in metabolites or other cellular signals. Although Fic domains have been found to use cytidine diphosphorylcholine as a substrate for modifying proteins with phosphocholine³³, structural predictions support the hypothesis that most Fic domains will use ATP³⁴. The requirement of a phosphotransferase activity adds another unexpected layer of complexity to visual neurotransmission.

ONLINE METHODS

Antibodies and reagents

Antibodies against a C-terminal peptide of Fic were raised and affinity purified by Open Biosystems. Other antibodies used were against BiP (MAC 143, Babraham Institute), histamine (ImmunoStar), Histone2B (Millipore), tubulin (D, M1A, Sigma). Other reagents were histamine (Sigma) and carcinine (Bachem).

Molecular biology

Full-length *Fic* cDNAs encoding wild-type or N288Q mutant Fic were cloned into pET23a using BamHI and NotI sites for *in vitro* transcription/translation experiment or into a modified pBacPAK8 vector with His/FLAG tags inserted at the N terminus (provided by B. Li, University of Texas Southwestern) using KpnI and NotI sites for purification from Sf21 cells. Transgenic vectors for Fic expression in flies under *UAS* control were generated in pUAS⁺ by inserting cDNAs encoding wild-type Fic, Fic^{H375A} (ref. 6), or versions encoding Fic fused at its C terminus to GFP or HRP²⁸. For expression of Fic-HRP under endogenous

control, PCR fragments encompassing 2.2 kb of Fic upstream region and the 1.8-kb Fic coding region, the HRP coding region, or 3 kb of the Fic 3' region were combined in a modified pAttb transformation vector. For all transgenes, expression of proteins of the expected sizes was confirmed by western blots (data not shown).

Fly work

Flies were maintained using standard conditions. The *Fic* dsRNA line KK105634 was obtained from the Vienna *Drosophila* RNAi Center. The Bloomington stock center provided *repo-Gal4*, *da-Gal4*, *GMR-Gal4* and *elav-Gal4* driver lines, Df(2L)BSC296, and the P element insertions k11101 and k07502b. Both of these insertion lines were listed as lethal in Flybase. We found, however, that they were viable over each other or over a deficiency of the area (Df(2L)BSC296). Thus, P element k11101 was mobilized using delta 2/3 transposase. Candidate deletions were identified by altered eye color and chromosomes with deletions in the *Fic* gene were mapped by PCR. All three deletions identified were homozygous viable. For genomic rescue, Pacman vector Cm^R-BW CH321-69B6 containing the *Fic* gene was inserted at chromosomal position 65B2 (Best Gene). The same landing site was used for insertion of the transgene encoding genomic Fic-HRP. ERGs were recorded as described previously³⁶, with at least 20 flies per genotype and five traces per fly. Phototaxis was measured in a T maze with one arm exposed to ultraviolet light and another in the dark. Flies were placed in the center chamber of the T maze and had the choice between ultraviolet light and the dark. After 30 s, the percentage of flies that had moved toward the light was determined. At least 300 flies of each genotype were tested. In each set of experiment, around 45 flies were placed in the center of the chamber and tested three times each. To test nutritional supplements, we placed a yeast paste including histamine (25%, wt/wt) or carcinine (12%, wt/wt) on top of normal food in vials and newly enclosed wild-type adults were allowed to feed for 5 d on this histamine- or carcinine-enriched food.

Western blotting

Western blots were performed as described³⁷. For detection of endogenous Fic from flies, the protein was first immunoprecipitated using antibody to Fic linked to magnetic beads. Proteins were eluted using SDS loading buffer, separated by SDS-PAGE and detected on western blots with Fic primary antibodies and IRdye 800Cw-labeled Protein-A on an Odyssey Imager (Li-Cor).

Subcellular fractionation

S2 cells were harvested and hypotonically lysed in HNMEK lysis buffer (20 mM HEPES (pH 7.6), 150 mM NaCl, 2 mM MgCl₂, 2 mM EDTA, 10 mM KCl, 0.5 mM EGTA, 1 mM DTT and protease inhibitor cocktail tablet from Roche) on ice for 20 min. The cells were homogenized using a glass douncer and the total lysate was centrifuged at 500g for 10 min to pellet nuclei (P1). The supernatant was recovered and centrifuged at 10,000g for 10 min to pellet the particulate fraction enriched in organelles such as endoplasmic reticulum and Golgi (P2). The supernatant was then centrifuged at 100,000g for 1 h to separate membrane (P3) and cytosolic fraction (S3). Samples were analyzed by SDS-PAGE and western blotting.

In vitro translation with microsomes

Full-length Fic protein was generated *in vitro* using T_NT Quick Coupled Transcription/Translation Systems (Promega) following the manufacturer's instructions. To analyze glycosylation, we added canine pancreatic microsomal membranes (Promega) to the reaction. Briefly, 0.25 µg of pET23a-Fic (wild type or N288Q) was mixed with TNT Quick

mastermix, [^{35}S]methionine (10 mCi ml $^{-1}$), microsomal membranes and nuclease-free water to a final volume of 25 μl and incubated at 30 °C for 1 h.

The protease protection assay was modified from a previously described protocol³⁸. Briefly, 9 μl of *in vitro*-translated reaction was mixed with either 1 μl of proteinase K (10 mg ml $^{-1}$) or buffer (100 mM potassium acetate, 2 mM magnesium acetate, 50 mM HEPES, pH 7.4) and incubated on ice for 60 min. Proteolysis was terminated by treatment with 0.2 μl of 0.25 M PMSF followed by the addition of 50 μl of preheated PK-kill buffer (1% SDS (wt/vol), 0.1 M Tris, pH 8.0). All reactions samples were boiled for 1 to 2 min at 100 °C and analyzed by SDS-PAGE and autoradiography.

EndoH treatment

Recombinant Fic purified from Sf21 cells and the endogenous Fic from S2 cells were treated with EndoH (New England Biolabs), as described by the manufacturers. N-terminal His/Flag-tagged Fic was expressed in Sf21 cells using a baculovirus expression system (Clontech) according to the manufacturer's instructions and affinity purified using nickel beads (Sigma). For EndoH treatment, 5 μg of recombinant Fic or 50 μg of S2 subcellular fraction enriched in endogenous Fic was denatured at 100 °C for 10 min with denaturing buffer. 2 μl of EndoH and G5 reaction buffer were added to the reaction and incubated at 37 °C for 1 h. Following SDS-PAGE, recombinant Fic was analyzed by Coomassie Blue staining and endogenous Fic was detected by western blotting.

Histology

Histamine immunohistochemistry was performed as described previously²⁵. In short, fly heads were fixed for 4 h in ice-cold solution of 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (wt/vol, Sigma) in 0.1 M phosphate buffer, washed overnight in 25% sucrose in phosphate buffer (wt/vol), embedded in optimal cutting temperature compound, frozen in liquid nitrogen and sectioned at 20- μm thickness on a cryostat microtome (Hacker-Bright, England). Sections were incubated overnight with antibodies to histamine (1:500, Immunostar, cat# 22939). Secondary antibodies were labeled with Alexa488 (1:500, Molecular Probes, cat# A-11008). Images were captured using Zeiss LSM510 confocal microscope with a 20 $\times$ NA 0.75 or a 63 $\times$ NA 1.4 lens on an inverted confocal microscope (LSM510 Meta; Carl Zeiss) at 21–23 °C.

Electron micrographs of 3-d-old fly heads and detection of Fic-HRP by HRP activity was carried out as described previously²⁸. Size and frequency of capitate projections were determined from images of cross sections of photoreceptor synaptic endings. At least 100 synapses were scored in three lamina each for mutant and wild type. All digital images were imported into Photoshop (Adobe) and adjusted for gain, contrast and gamma settings.

Statistics

One-way ANOVA was used to determine statistical significance of differences in ERGs recordings or phototaxis assays, followed by Tukey's multiple comparison test. For all ANOVA tests, *F* values were larger than 150. For these measurements, the Kolmogorov-Smirnov test indicated a normal distribution ($\alpha = 0.05$). All bar graphs resulting from these comparisons show means $\pm$ s.d. Numbers of ERG measurements on individual flies and the number of phototaxis tests (each using 45 to 50 flies per genotype) are indicated in bar graphs for each genotype or supplemental condition. Sample sizes were chosen to be close to previously published examples¹⁴. Average diameters and frequency of capitate projections in wild type and *Fic*⁵⁵ were compared by two-tailed Student's *t* test. Statistical significance was determined using the Prism software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to R. Hiesinger for help and advice with the ERG measurements and the use of his ERG setup, W. Pak, B. Hoveman and the Bloomington Stock center for fly lines, M. Buszczak for antibodies, C. Gilpin from the Molecular and Cellular Imaging Facility at UT Southwestern, S.H. Kim and B. Tu for technical help, and to E. Kavalali and A. Haberman for helpful discussions. This work was supported by grants to H.K. from the US National Institutes of Health National Eye Institute (EY10199 and EY021922) and Visual Science Core grant EY020799. K.O. and H.H. are supported by grants from the US National Institutes of Health (Allergy and Infectious Disease, R01-AI056404 and R01-AI087808) and the Welch Foundation (I-1561). K.O. is a Burroughs Wellcome Investigator in Pathogenesis of Infectious Disease and a W.W. Caruth Jr. Biomedical Scholar.

References

1. Yarbrough ML, et al. AMPylation of Rho GTPases by *Vibrio* VopS disrupts effector binding and downstream signaling. *Science*. 2009; 323:269–272. [PubMed: 19039103]
2. Brown MS, Segal A, Stadtman ER. Modulation of glutamine synthetase adenylation and deadenylation is mediated by metabolic transformation of the P II regulatory protein. *Proc Natl Acad Sci USA*. 1971; 68:2949–2953. [PubMed: 4399832]
3. Broberg CA, Orth K. Tipping the balance by manipulating post-translational modifications. *Curr Opin Microbiol*. 2010; 13:34–40. [PubMed: 20071215]
4. Müller MP, et al. The *Legionella* effector protein DrrA AMPylates the membrane traffic regulator Rab1b. *Science*. 2010; 329:946–949. [PubMed: 20651120]
5. Worby CA, et al. The fic domain: regulation of cell signaling by adenylation. *Mol Cell*. 2009; 34:93–103. [PubMed: 19362538]
6. Kinch LN, Yarbrough ML, Orth K, Grishin NV. Fido, a novel AMPylation domain common to fic, doc, and AvrB. *PLoS ONE*. 2009; 4:e5818. [PubMed: 19503829]
7. Benzer S. Behavioral mutants of *Drosophila* isolated by countercurrent distribution. *Proc Natl Acad Sci USA*. 1967; 58:1112–1119. [PubMed: 16578662]
8. Melzig J, et al. Genetic depletion of histamine from the nervous system of *Drosophila* eliminates specific visual and mechanosensory behavior. *J Comp Physiol A*. 1996; 179:763–773. [PubMed: 8956497]
9. Alawi AA, Pak WL. On-transient of insect electroretinogram: its cellular origin. *Science*. 1971; 172:1055–1057. [PubMed: 5573957]
10. Heisenberg M. Separation of receptor and lamina potentials in the electroretinogram of normal and mutant *Drosophila*. *J Exp Biol*. 1971; 55:85–100. [PubMed: 5001616]
11. Stowers RS, Schwarz TL. A genetic method for generating *Drosophila* eyes composed exclusively of mitotic clones of a single genotype. *Genetics*. 1999; 152:1631–1639. [PubMed: 10430588]
12. Pantazis A, et al. Distinct roles for two histamine receptors (hclA and hclB) at the *Drosophila* photoreceptor synapse. *J Neurosci*. 2008; 28:7250–7259. [PubMed: 18632929]
13. Stowers RS, Megeath LJ, Gorska-Andrzejak J, Meinertzhagen IA, Schwarz TL. Axonal transport of mitochondria to synapses depends on miltion, a novel *Drosophila* protein. *Neuron*. 2002; 36:1063–1077. [PubMed: 12495622]
14. Fabian-Fine R, et al. Endophilin promotes a late step in endocytosis at glial invaginations in *Drosophila* photoreceptor terminals. *J Neurosci*. 2003; 23:10732–10744. [PubMed: 14627659]
15. Lin DM, Goodman CS. Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron*. 1994; 13:507–523. [PubMed: 7917288]
16. Sepp KJ, Schulte J, Auld VJ. Peripheral glia direct axon guidance across the CNS/PNS transition zone. *Dev Biol*. 2001; 238:47–63. [PubMed: 11783993]
17. Zhu Y, Nern A, Zipursky SL, Frye MA. Peripheral visual circuits functionally segregate motion and phototaxis behaviors in the fly. *Curr Biol*. 2009; 19:613–619. [PubMed: 19303299]

18. Gavin BA, Arruda SE, Dolph PJ. The role of carcinine in signaling at the *Drosophila* photoreceptor synapse. *PLoS Genet.* 2007; 3:e206. [PubMed: 18069895]
19. Melzig J, Burg M, Gruhn M, Pak WL, Buchner E. Selective histamine uptake rescues photo- and mechanoreceptor function of histidine decarboxylase-deficient *Drosophila* mutant. *J Neurosci.* 1998; 18:7160–7166. [PubMed: 9736639]
20. Wagner S, et al. *Drosophila* photoreceptors express cysteine peptidase tan. *J Comp Neurol.* 2007; 500:601–611. [PubMed: 17154266]
21. Burg MG, Sarthy PV, Koliantz G, Pak WL. Genetic and molecular identification of a *Drosophila* histidine decarboxylase gene required in photoreceptor transmitter synthesis. *EMBO J.* 1993; 12:911–919. [PubMed: 8096176]
22. Borycz JA, Borycz J, Kubow A, Kostyleva R, Meinertzhagen IA. Histamine compartments of the *Drosophila* brain with an estimate of the quantum content at the photoreceptor synapse. *J Neurophysiol.* 2005; 93:1611–1619. [PubMed: 15738275]
23. Richardt A, Rybak J, Stortkuhl KF, Meinertzhagen IA, Hovemann BT. Ebony protein in the *Drosophila* nervous system: optic neuropile expression in glial cells. *J Comp Neurol.* 2002; 452:93–102. [PubMed: 12205712]
24. Richardt A, et al. Ebony, a novel nonribosomal peptide synthetase for beta-alanine conjugation with biogenic amines in *Drosophila*. *J Biol Chem.* 2003; 278:41160–41166. [PubMed: 12900414]
25. Romero-Calderón R, et al. A glial variant of the vesicular monoamine transporter is required to store histamine in the *Drosophila* visual system. *PLoS Genet.* 2008; 4:e1000245. [PubMed: 18989452]
26. Edwards TN, Meinertzhagen IA. The functional organisation of glia in the adult brain of *Drosophila* and other insects. *Prog Neurobiol.* 2010; 90:471–497. [PubMed: 20109517]
27. Borycz J, Borycz JA, Loubani M, Meinertzhagen IA. tan and ebony genes regulate a novel pathway for transmitter metabolism at fly photoreceptor terminals. *J Neurosci.* 2002; 22:10549–10557. [PubMed: 12486147]
28. Sunio A, Metcalf AB, Kramer H. Genetic dissection of endocytic trafficking in *Drosophila* using a horseradish peroxidase-bridge of sevenless chimera: hook is required for normal maturation of multivesicular endosomes. *Mol Biol Cell.* 1999; 10:847–859. [PubMed: 10198042]
29. Stark WS, Carlson SD. Ultrastructure of capitate projections in the optic neuropil of *Diptera*. *Cell Tissue Res.* 1986; 246:481–486. [PubMed: 3098431]
30. Meinertzhagen, IA.; Wang, Y. *Drosophila* mutants tan and ebony have altered numbers of capitate projections, glial invaginations into photoreceptor terminals. In: Elsner, N.; Wässle, H., editors. *Neurobiology: From Membrane to Mmind*. Vol. II. Georg Thieme Verlag; Stuttgart: 1997. p. 457
31. Tan Y, Luo ZQ. Legionella pneumophila SidD is a deAMPylase that modifies Rab1. *Nature.* 2011; 475:506–509. [PubMed: 21734656]
32. Neunuebel MR, et al. De-AMPylation of the small GTPase Rab1 by the pathogen *Legionella pneumophila*. *Science.* 2011; 333:453–456. [PubMed: 21680813]
33. Mukherjee S, et al. Modulation of Rab GTPase function by a protein phosphocholine transferase. *Nature.* 2011; 477:103–106. [PubMed: 21822290]
34. Engel P, et al. Adenylation control by intra- or intermolecular active-site obstruction in Fic proteins. *Nature.* 2012; 482:107–110. [PubMed: 22266942]
35. Rost B, Yachdav G, Liu J. The Predict Protein server. *Nucleic Acids Res.* 2004; 32:W321–326. [PubMed: 15215403]
36. Williamson WR, Wang D, Haberman AS, Hiesinger PR. A dual function of V0-ATPase a1 provides an endolysosomal degradation mechanism in *Drosophila melanogaster* photoreceptors. *J Cell Biol.* 2010; 189:885–899. [PubMed: 20513768]
37. Akbar MA, Ray S, Kramer H. The SM protein Car/Vps33A regulates SNARE-mediated trafficking to lysosomes and lysosome-related organelles. *Mol Biol Cell.* 2009; 20:1705–1714. [PubMed: 19158398]
38. Sharma A, Mariappan M, Appathurai S, Hegde RS. *In vitro* dissection of protein translocation into the mammalian endoplasmic reticulum. *Methods Mol Biol.* 2010; 619:339–363. [PubMed: 20419420]

Figure 1.

Fic is required for visual neurotransmission. (a) Map of *Fic* gene and protein, including predicted³⁵ transmembrane domain (TM), potential N-glycosylation site Asn288, tetratricopeptide repeat domain and FIC domain with active site at His375 (ref. 6). *Fic⁶⁸*, *Fic¹²¹* and *Fic⁵⁵* lack the 5' UTR and N-terminal 125, 150 or 260 amino acids, respectively. (b) Coomassie blue-stained gel with lysates from wild-type, *Fic⁵⁵* and *Fic* RNAi flies and western blot detecting FIC protein after enrichment by immunoprecipitation with antibody to FIC (wild type: Ore-R; w^{1118} ; *Fic⁵⁵*; *Fic⁵⁵*; w^{1118} ; RNAi: *UAS-Fic* dsRNA; *da-Gal4*). RNAi-induced knockdown of FIC protein was $84 \pm 7\%$ ($n = 4$). Full-length blots are presented in Supplementary Figure 1. (c) Fast-phototaxis assays revealed a difference in behavior between wild-type and *Fic⁵⁵* or *Fic* knockdown flies. A genomic *Fic* transgene restored normal behavior. Numbers indicate individual phototaxis tests using 45–50 flies each. *** $P < 0.001$ and ns indicates $P > 0.05$ compared with wild type. (d) ERG recordings of wild-type, *Fic⁵⁵* and *Fic⁵⁵; Fic* rescued flies showed photoreceptor depolarization in response to light. Only *Fic⁵⁵* flies missed the ON transient, indicated by the red arrows. (e) Quantification of ON transients from ERG recordings of indicated genotypes with respect to *Fic* allele, *Fic* transgenes (*Fic_Tg*) and *Gal4* drivers (*Gal4_Tg*). Numbers indicate individual ERG measurements. ** $P < 0.001$ and ns indicates $P > 0.05$ compared with *Fic⁵⁵*. Genotypes (from left to right): Ore-R, w^{1118} ; *Fic⁵⁵*, w^{1118} ; *Fic⁵⁵/Df(2L)BSC296*, w^{1118} ; *UAS-Fic* dsRNA; *da-Gal4*, w^{1118} ; *Fic⁵⁵*; *Bac-gen-Fic*, w^{1118} ; *Fic⁵⁵*; *arm-Gal4/UAS-Fic*, w^{1118} ; *Fic⁵⁵*; *arm-Gal4/UAS-Fic^{H375A}*, w^{1118} ; *Fic⁵⁵*; *arm-Gal4/UAS-Fic-HRP*, w^{1118} ; *Fic⁵⁵*; *arm-Gal4/UAS-Fic-GFP*. Bars represent mean $\pm$ s.d.

Figure 2.

Visual neurotransmission requires Fic enzymatic activity in glia cells. (a–h) Electron micrographs of sections of wild-type (a,c,e,g) and *Fic⁵⁵* (b,d,f,h) lamina reveal the presence of mitochondria (M), capitate projections (CP), synaptic vesicles (SV) and active zone T bars (yellow arrowheads). We noticed occasional endoplasmic reticulum-like tubules in *Fic⁵⁵* synapses (arrows in d). Scale bars in c and d represent 2 μ m (a,b) and 0.5 μ m (c,d). Collages of micrographs depict capitate projections in wild-type (e) or *Fic⁵⁵* (f) lamina. Texas Red-X phalloidin staining of wild-type (g) and *Fic⁵⁵* (h) ommatidia. Scale bars represent 0.2 μ m (e,f) and 10 μ m (g,h). (i,j) Quantification of ON transients from ERG recordings (i) and fast phototaxis response (j) of flies with the indicated genotypes with respect to *Fic* allele, *Gal4* drivers (*Gal4* Tg) and *Fic* transgenes (*Fic* Tg), and Flipase transgene (*Flp* Tg). *** P <0.001 and ns indicates P > 0.05 compared with *Fic⁵⁵*. Genotypes: (a,c,e,g) Ore-R, (b,d,f,h) *w¹¹¹⁸;Fic⁵⁵* (i,j, from left to right) Ore-R, *w¹¹¹⁸;Fic⁵⁵*, *ey3.5-Flp;FRT^{40A} Fic⁵⁵/FRT^{40A} GMR-Hid cl*, *w¹¹¹⁸;Fic⁵⁵;elav-Gal4/UAS-Fic*, *w¹¹¹⁸;Fic⁵⁵;elav-Gal4/UAS-Fic^{H375A}*, *w¹¹¹⁸;Fic⁵⁵;repo-Gal4/UAS-Fic*, *w¹¹¹⁸;Fic⁵⁵;repo-Gal4/UAS-Fic^{H375A}*, *w¹¹¹⁸;GMR-Gal4/UAS-Fic-dsRNA*, *w¹¹¹⁸;UAS-Fic-dsRNA;repo-Gal4*. All bar graphs show mean $\pm$ s.d. Numbers in bars indicate the number of individual ERG measurements for each genotype (i) or individual phototaxis tests for each genotype using 45–50 flies each (j).

Figure 3.

Fic is required for recycling of the histamine neurotransmitter. **(a,b)** Quantification of ON transients from ERG recordings **(a)** and fast phototaxis response **(b)** of wild-type or *Fic*⁵⁵ flies fed food supplemented with histamine (HA) or carcinine (CA) or without supplement (-). ****P* < 0.001 compared with *Fic*⁵⁵. Bar graphs show mean ± s.d. Numbers in bars indicate the number of individual ERG measurements for each genotype **(a)** or individual phototaxis tests for each genotype with 45–50 flies each **(b)**. **(c–f)** Frozen sections of fly heads stained for histamine. Histamine is synthesized in photoreceptor cells (P), which show variable levels of staining in wild-type **(c)** and *Fic*⁵⁵ **(d)**, but not *Hdc*^{P218} **(f)** flies. In wild-type **(c)**, but not in *Fic*⁵⁵ **(d)** or *Hdc*^{P218} **(f)**, heads²¹, histamine was enriched in glia cells (red arrows) just proximal to the photoreceptor cells and in synapses of the lamina and medulla (yellow arrows). This staining was partially restored in *Fic* mutants following expression of *Fic* in glia cells **(e)**. Genotypes: **(a,b)** Ore-R; *w*¹¹¹⁸; *Fic*⁵⁵ **(c)** *w*¹¹¹⁸ **(d)** *w*¹¹¹⁸; *Fic*⁵⁵ **(e)** *w*¹¹¹⁸; *Fic*⁵⁵; *repo-Gal4/UAS-Fic* **(f)** *w*¹¹¹⁸; *Hdc*^{P218}. Scale bars represent 50 μm.

Figure 4.

Fic active site is extracellular and localizes to capitate projections. **(a)** Differential centrifugation of S2 cell homogenates at 500g (P1), 10,000g (P2) and 100,000g (P3). Fic co-fractionated with endoplasmic reticulum marker BiP, but not nuclear histone 2B (H2B), or cytosolic tubulin (Tub) and actin (Act). **(b)** *In vitro*-translated Fic was shifted in its mobility (double arrow) with microsomes (micros.). This shift depends on the presence of the only predicted N-glycosylation site of Fic at Asn288. Proteinase K (Prot. K) degrades Fic in the absence of microsomes, but in their presence a ~48-kDa fragment was protected from degradation. WT, wild type. **(c)** EndoH treatment of recombinant Fic purified from Sf21 cells or Fic endogenous to S2 cells caused a mobility shift indicating the presence of high mannose N-glycans. Full-length blots are presented in Supplementary Figure 1. **(d-r)** Electron micrographs of lamina cartridges from flies expressing genomic *Fic-HRP* (**d,f**) or under *repo-Gal4* control (**h,i,l-o**) revealed the presence of *Fic-HRP* on glial cells surrounding laminar cartridges and at capitate projections. HRP activity was enriched in the extracellular space between glia and photoreceptor terminals at the head of capitate projections (for example, arrowheads in **l-o**), regardless of whether they assumed flat (**o**) or deeply invaginated shapes (**h,l,m**). Electron micrographs of wild-type flies lacking *Fic-HRP* expression (**e,g,j,k,p-r**) confirmed the specificity of the DAB precipitate and revealed the slightly extended extracellular space typical of capitate projections (arrowheads in **p-r**). Scale bars represent 1 μ m (**d-h,j**) and 200 nm (**i,k-r**). Genotypes: w^{1118} ; *Fic-HRP* (**d,f**); w^{1118} ; *repo-Gal4/UAS-Fic-HRP* (**h,j,l-o**); Ore-R (**e,g,i,k,p-r**).