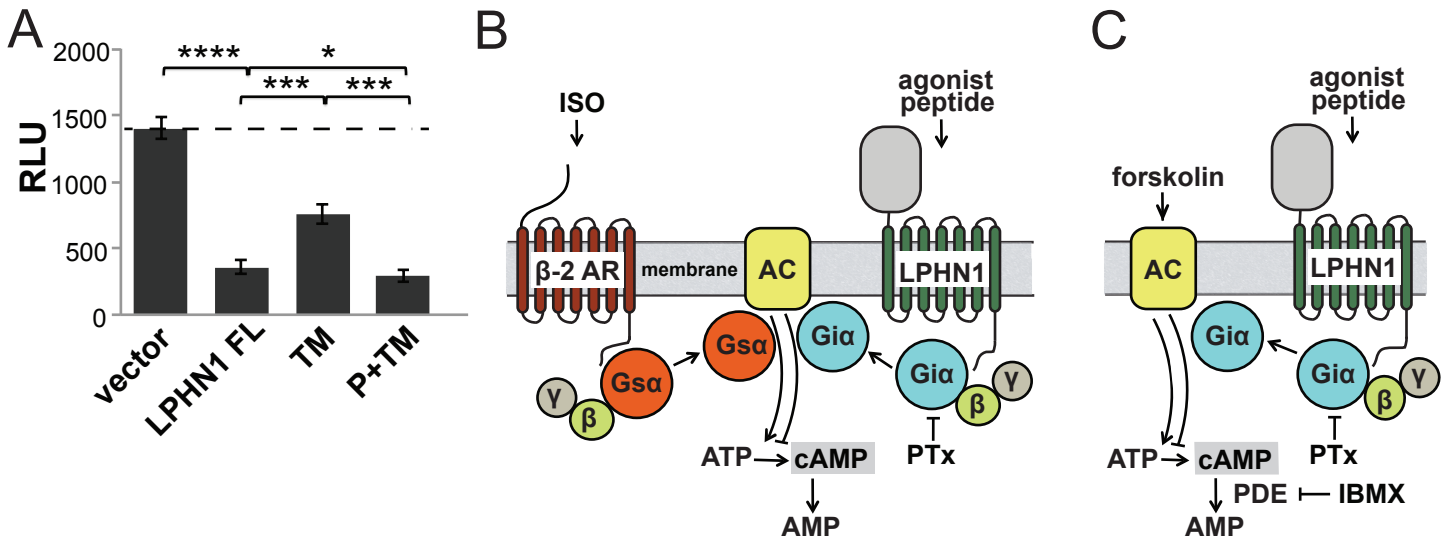
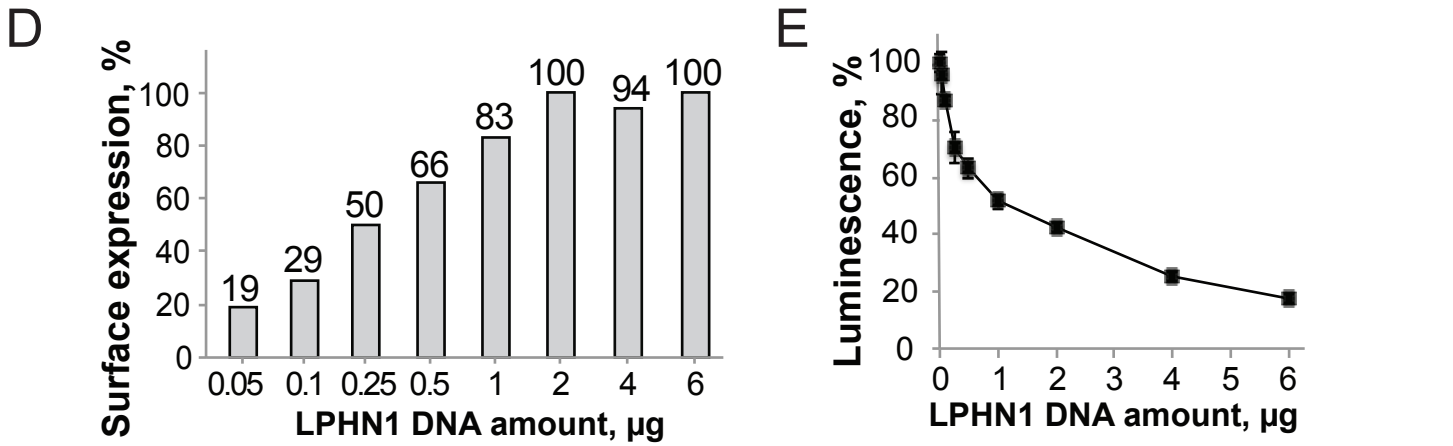
ISCI, Volume *3*

Supplemental Information

A Comprehensive Mutagenesis Screen of the Adhesion GPCR Latrophilin-1/ADGRL1

Olha Nazarko, Amanuel Kibrom, Jana Winkler, Katherine Leon, Hannah Stoveken, Gabriel Salzman, Katarzyna Merdas, Yue Lu, Pradnya Narkhede, Gregory Tall, Simone Prömel, and Demet Araç


A
RLU
2000
1500
1000
500
0
****
*
***
***
vector
LPHN1 FL
TM
P+TM
B
ISO
agonist peptide
β-2 AR
membrane
AC
LPHN1
Gsα
Giα
γ
β
ATP
cAMP
AMP
PTx
C
agonist peptide
forskolin
AC
LPHN1
Giα
ATP
cAMP
AMP
PTx
PDE
IBMX


D
Surface expression, %
100
80
60
40
20
0
19
29
50
66
83
100
94
100
0.05
0.1
0.25
0.5
1
2
4
6
LPHN1 DNA amount, µg
E
Luminescence, %
100
80
60
40
20
0
0
1
2
3
4
5
6
LPHN1 DNA amount, µg

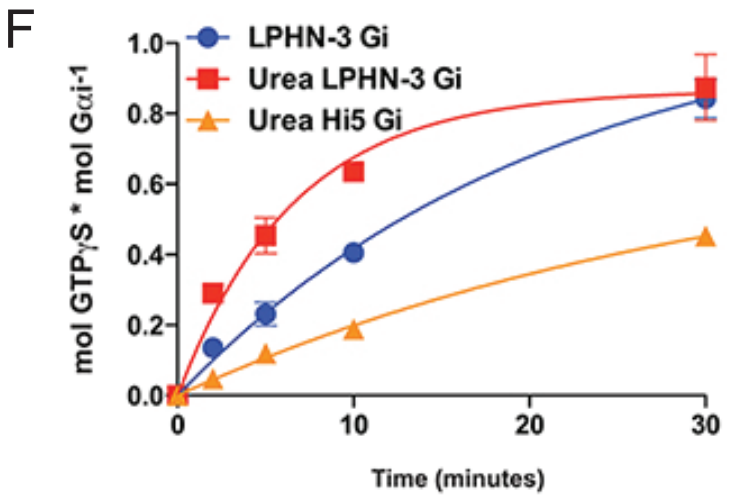
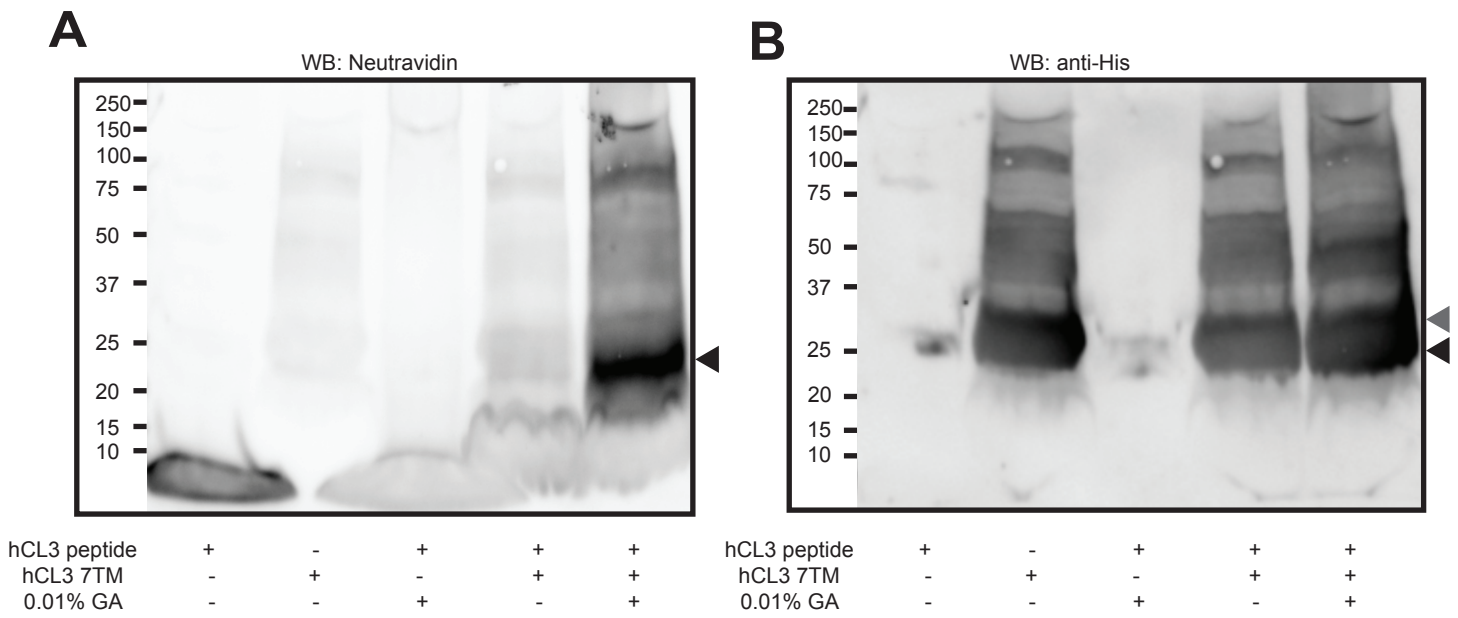

F
LPHN-3 Gi
Urea LPHN-3 Gi
Urea Hi5 Gi
mol GTPγS * mol Gαi-1
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (minutes)

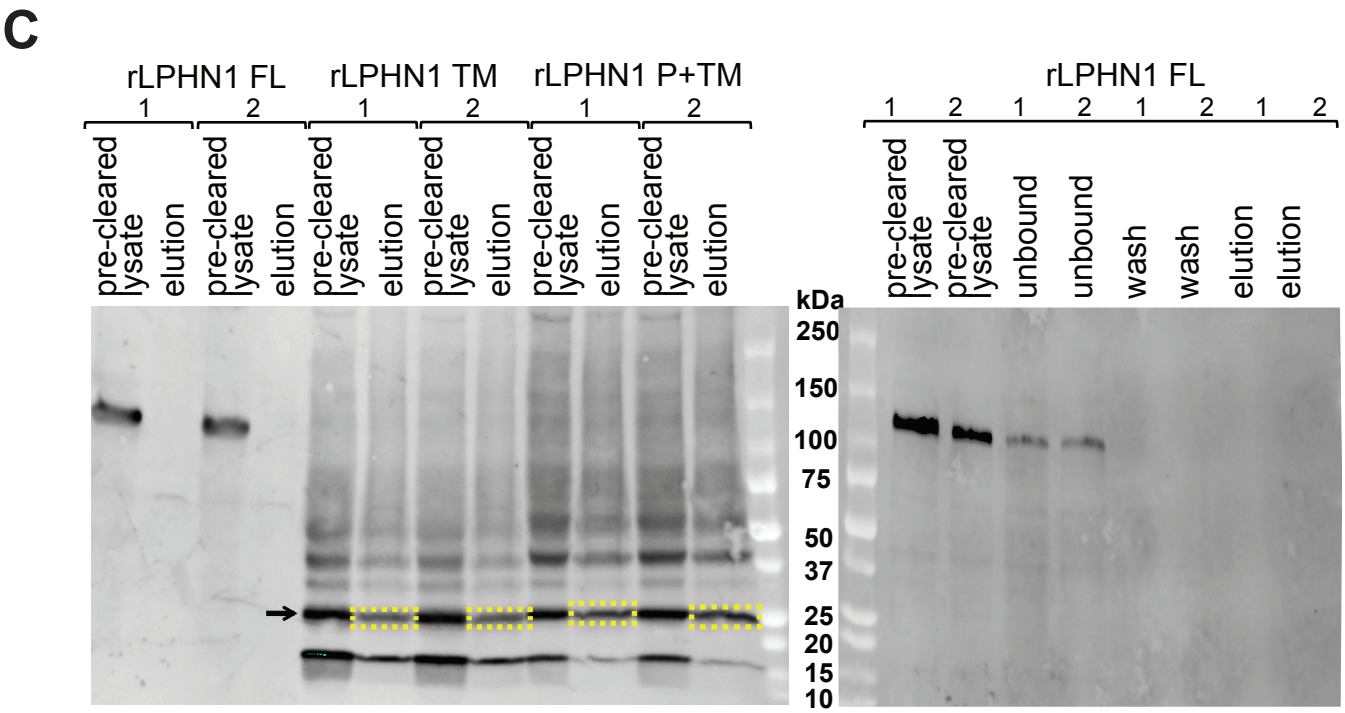
Figure S1

Figure S1, Related to Figure 1. Development of the cAMP assay to monitor Latrophilin activity

(A) Lphn1 overexpression decreases basal cAMP level in HEK293 cells. Cells were transfected with empty vector or different Lphn1 constructs and cAMP level was measured by the Glosensor assay. (B-C) Schematic representation of the modified cAMP assays to increase cAMP level by activation of co-transfected B2-adrenergic receptor with isoproterenol (B) or by activation of endogenous adenylate cyclase with forskolin (C). (D) The cell-surface expression of Lphn1 on non-permeabilized HEK293 cells quantified by flow cytometry using an N-terminal FLAG tag as a function of DNA concentration. (E) Transiently transfected HEK293 cells display DNA amount-dependent cAMP signaling of Lphn1 determined by β2-adrenergic receptor cAMP assay.

(F) G protein coupling of Lphn3. High-Five cell membranes with Lphn3 were subject to urea treatment to induce ECR-shedding and compared to membranes with no receptor or no treatment. Membranes were reconstituted with purified heterotrimeric G proteins, including the αi subunit and the βγ subunits. The receptor-mediated G protein activation kinetics were measured using the [35S]-GTPγS binding assay for Gαi. Data are presented as mean ± S.E.M.


A
WB: Neutravidin
250
150
100
75
50
37
25
20
15
10
hCL3 peptide + - + + +
hCL3 7TM - + - + +
0.01% GA - - + - +
B
WB: anti-His
250
150
100
75
50
37
25
20
15
10
hCL3 peptide + - + + +
hCL3 7TM - + - + +
0.01% GA - - + - +


C
rLPHN1 FL
rLPHN1 TM
rLPHN1 P+TM
1 2 1 2 1 2
pre-cleared lysate
elution
rLPHN1 FL
1 2 1 2 1 2 1 2
pre-cleared lysate
unbound
wash
elution
kDa
250
150
100
75
50
37
25
20
15
10

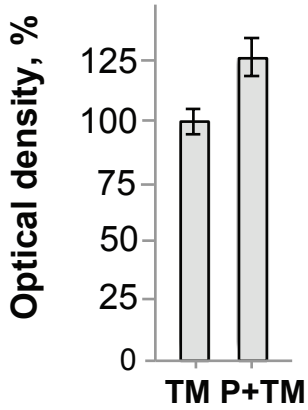

D
Optical density, %
125
100
75
50
25
0
TM P+TM

Figure S2

Figure S2, Related to Figure 2, *Stachel* peptide binds and activates the TM domain of Latrophilin

(A) Synthesized human Lphn3 *Stachel* peptide can be crosslinked to purified human Lphn3 TM in the presence of glutaraldehyde. Western blot of Lphn3 peptide, Lphn3 TM, and mixture of Lphn3 peptide and Lphn3 TM in presence or absence of 0.01% glutaraldehyde blotted with (A) labeled NeutrAvidin to detect biotinylated *Stachel* peptide, black arrow, or (B) anti-His antibody to detect His-tagged hLphn3 7TM, black and gray arrows indicate Lphn3 TM domain, respectively.

(C) Western blot for quantification of cell-surface expressed Lphn1 constructs. HEK293 cells were transfected with full length, TM or P+TM Lphn1 constructs. Cell-surface expressed proteins were biotynilated 48 hrs after transfection, pulled-down with streptavidin beads and eluted with urea. Eluted protein bands are highlighted with yellow dashed frames. Though, full length Lphn1 did not elute from streptavidin beads and thus cannot be quantified, it is clear that some was surface-expressed and biotinylated as it was depleted upon incubation with streptavidin beads (right). Numbers indicate repeats for the same construct.

(D) Optical density of eluted protein bands (yellow dashed frames from B). Bar heights represent the mean of two measurements. Error bars indicate ??.

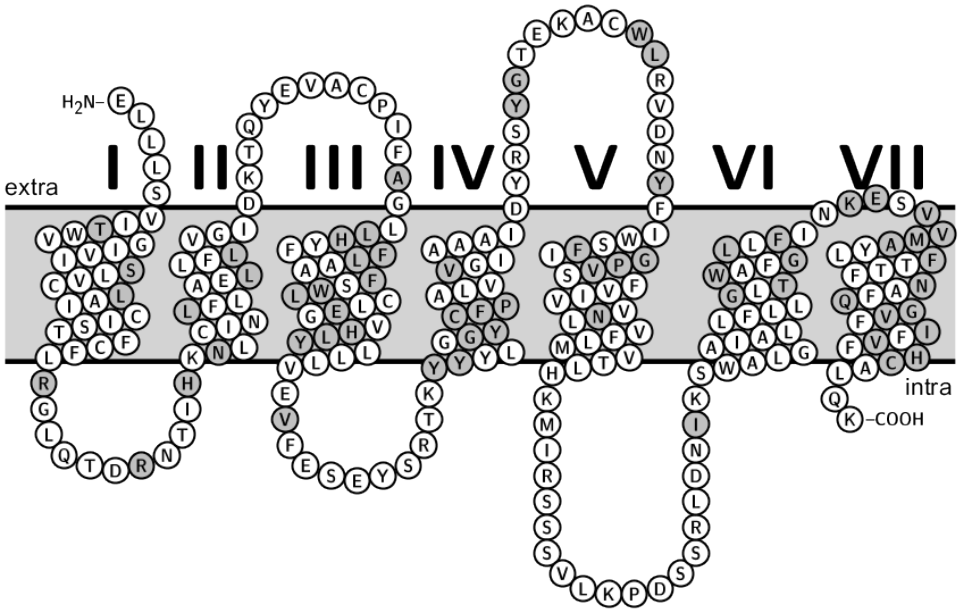


Figure S3, Related to Figures 3 and 5. Snake plot showing all mutants that were cloned. All mutations are represented by grey color on the snake diagram.

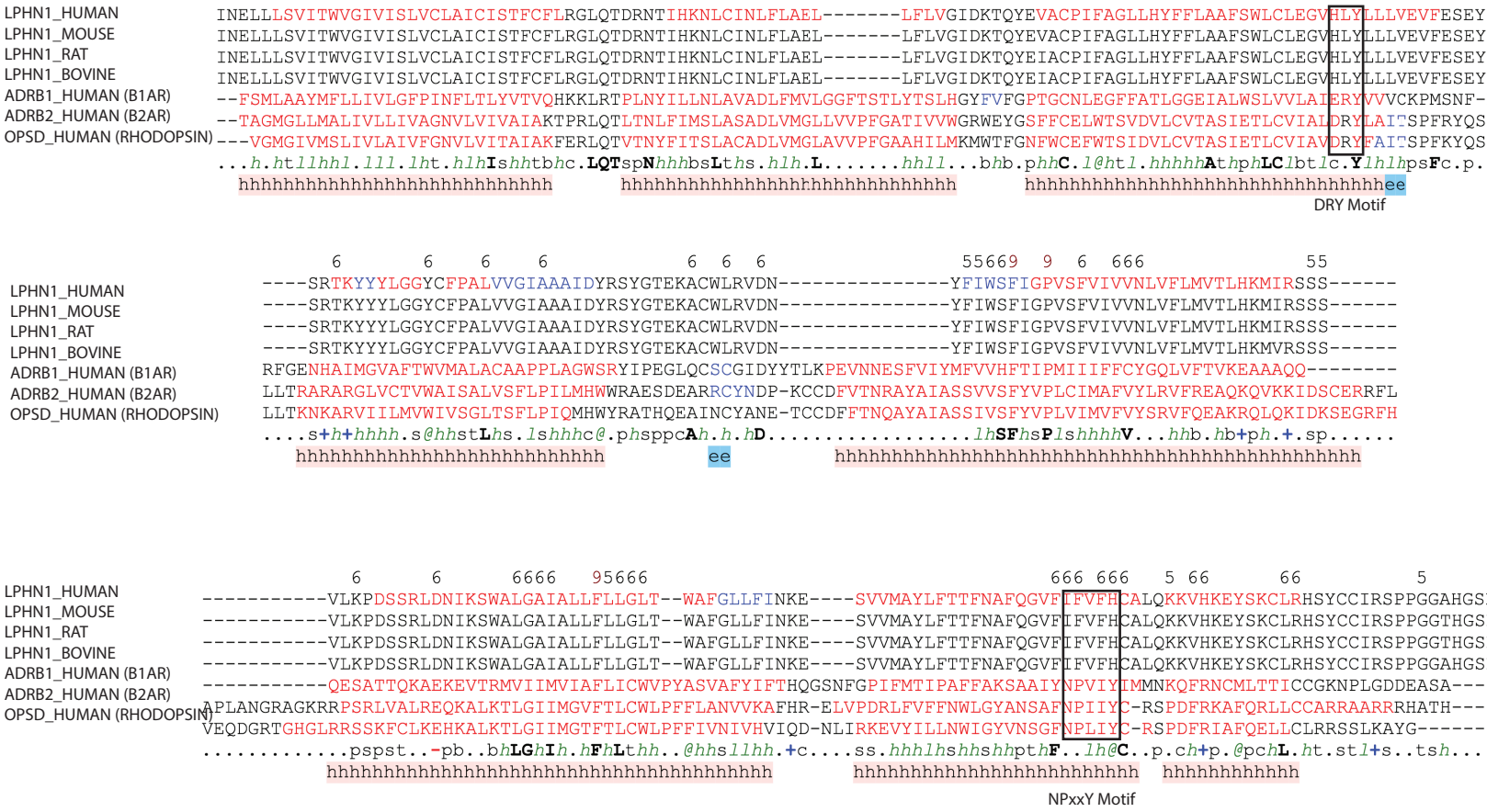

LPHN1_HUMAN
LPHN1_MOUSE
LPHN1_RAT
LPHN1_BOVINE
ADRB1_HUMAN (B1AR)
ADRB2_HUMAN (B2AR)
OPSD_HUMAN (RHODOPSIN)
DRY Motif
NPxxY Motif

Figure S4

Figure S4, Related to Figures 3 and 5. Multiple sequence alignment comparing Lphns and rhodopsin family GPCRs. PROMALS3D multiple sequence and structure alignment server was used for alignment. Four mammalian Lphn1 constructs from the Adhesion family (O88917 AGRL1_RAT_A, Q80TR1 AGRL1_MOUSE, O94910_AGRL1_HUMAN, O97831_AGRL1_BOVIN) and three human Rhodopsin family constructs, beta 1 and 2 adrenergic and rhodopsin (P08100 OPSD_HUMAN, P08588 ADRB1_HUMAN, P07550 ADRB2_HUMAN) were used for alignment.

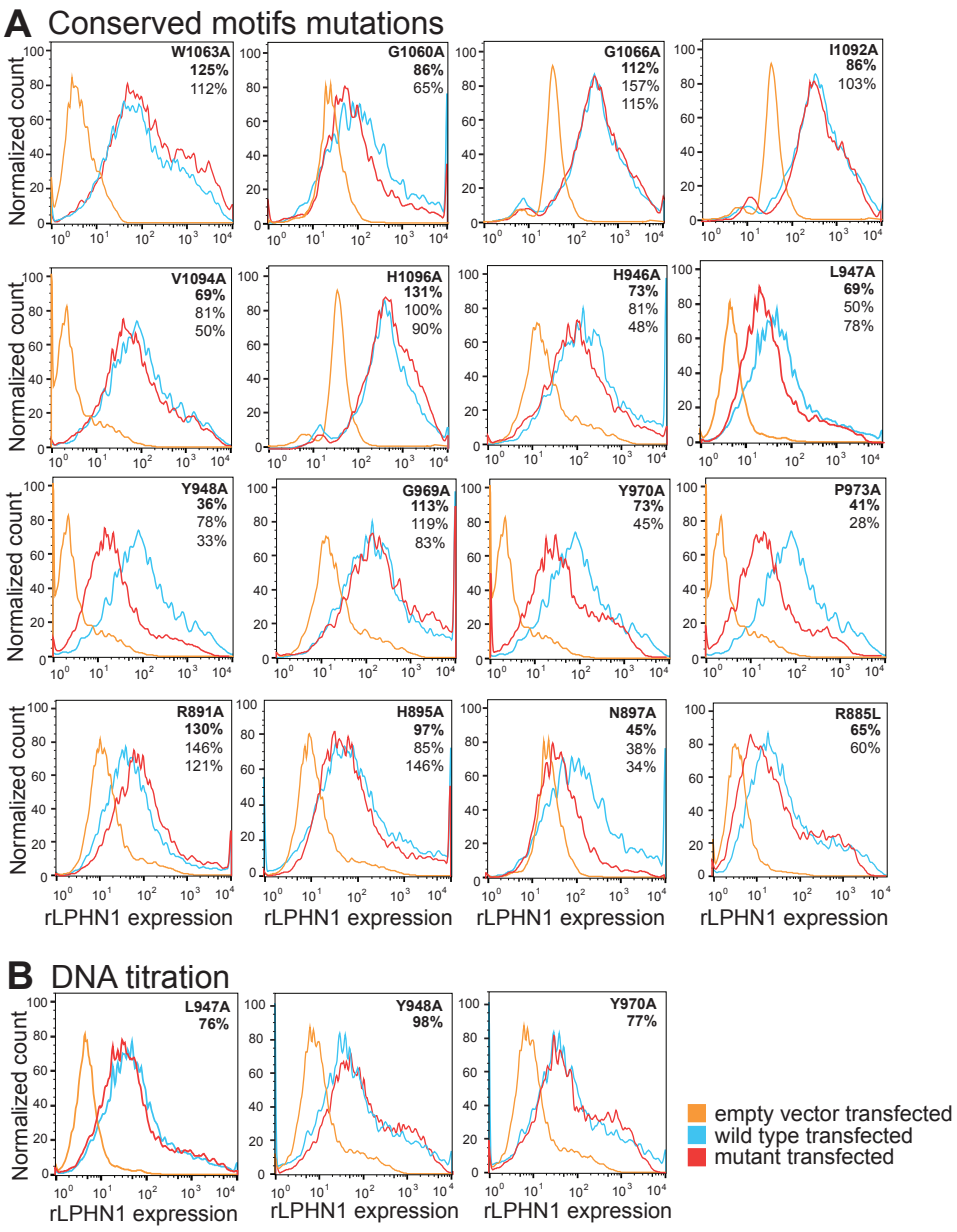

A Conserved motifs mutations
W1063A
125%
112%
G1060A
86%
65%
G1066A
112%
157%
115%
I1092A
86%
103%
V1094A
69%
81%
50%
H1096A
131%
100%
90%
H946A
73%
81%
48%
L947A
69%
50%
78%
Y948A
36%
78%
33%
G969A
113%
119%
83%
Y970A
73%
45%
P973A
41%
28%
R891A
130%
146%
121%
H895A
97%
85%
146%
N897A
45%
38%
34%
R885L
65%
60%
Normalized count
rLPHN1 expression
B DNA titration
L947A
76%
Y948A
98%
Y970A
77%
empty vector transfected
wild type transfected
mutant transfected

Figure S5

Figure S5, Related to Figures 3 and 5. Expression data for Lphn1 mutants.

(A) Surface expression of Lphn1 conserved motif mutants presented as overlay of typical flow cytometry histograms. HEK293 cells transfected with N-terminally FLAG-tagged full-length Lphn1 constructs (wt or mutant) or empty vector were stained with anti-FLAG antibody without permeabilization. Lphn1 expression (i.e. FLAG signal) for cells transfected with each mutant is directly compared to cells transfected with wild-type Lphn1 and empty vector. (B) DNA amount experiments for L947A, Y948A and Y970A mutants. Lphn1 wt DNA amount used for transfection was decreased 2.5-fold to achieve expression level comparable with mutants.

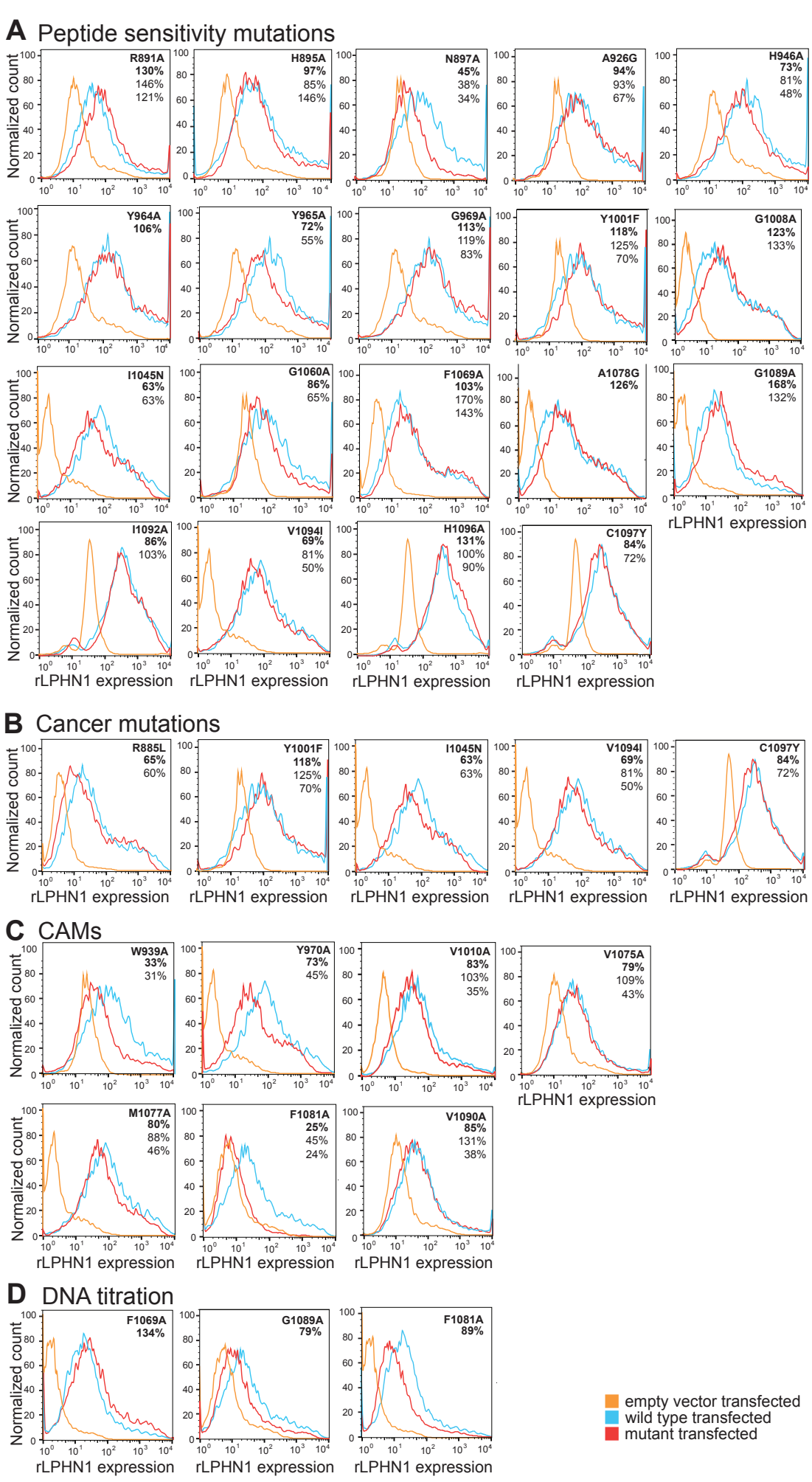

A Peptide sensitivity mutations
R891A 130% 146% 121%
H895A 97% 85% 146%
N897A 45% 38% 34%
A926G 94% 93% 67%
H946A 73% 81% 48%
Y964A 106%
Y965A 72% 55%
G969A 113% 119% 83%
Y1001F 118% 125% 70%
G1008A 123% 133%
I1045N 63% 63%
G1060A 86% 65%
F1069A 103% 170% 143%
A1078G 126%
G1089A 168% 132%
I1092A 86% 103%
V1094I 69% 81% 50%
H1096A 131% 100% 90%
C1097Y 84% 72%
Normalized count
rLPHN1 expression
B Cancer mutations
R885L 65% 60%
Y1001F 118% 125% 70%
I1045N 63% 63%
V1094I 69% 81% 50%
C1097Y 84% 72%
C CAMs
W939A 33% 31%
Y970A 73% 45%
V1010A 83% 103% 35%
V1075A 79% 109% 43%
M1077A 80% 88% 46%
F1081A 25% 45% 24%
V1090A 85% 131% 38%
D DNA titration
F1069A 134%
G1089A 79%
F1081A 89%
empty vector transfected
wild type transfected
mutant transfected

Figure S6

Figure S6, Related to Figures 3 and 5. Expression data for Lphn1 mutants.

Surface expression of Lphn1 (A) peptide response mutations, (B) cancer-associated mutations and (C) constitutively active mutants presented as overlay of typical flow cytometry histograms. HEK293 cells transfected with N-terminally FLAG-tagged full-length Lphn1 constructs (wt or mutant) or empty vector were stained with anti-FLAG antibody without permeabilization. Lphn1 expression (i.e. FLAG signal) for cells transfected with each mutant is directly compared to cells transfected with wild-type Lphn1 and empty vector. (D) DNA amount experiments for F1069A, G1089A and F1081A mutants. Lphn1 WT DNA amount used for transfection was decreased 2.5 fold to achieve expression level comparable with F1081A mutant. F1069A and G1089A DNA amount were decreased 1.5 times to achieve expression level comparable with WT.

Table S1, Related to Figures 3 and 5. Complete signaling and expression data.

Table encodes full raw data. Annotated for each mutation is the location of the mutation, the justification for performing the mutation, the Cell-Surface Expression, Signaling (Raw and Normalized), whether there is an observed peptide response and possible references. Basal activity means cAMP level without addition of ligand peptide and it was calculated by taking the luminescence value of wildtype over the luminescence value of the mutant. A value of <1 means mutant is more active than WT, while a value of >1 means mutant is less active than WT. The slashes between values separate individual experiments. Each individual experiment is at least three repeats. Colors match to the residue colors in main figures.

Transparent Methods

Lphn1 homology modeling

The modeling was done using the MEMOIR: Membrane protein modeling pipeline. This server, maintained by the University of Oxford, combines four different protein software programs iMembrane for membrane annotation, MP-T for sequence alignment, Medeller for coordinate generation, and Completionist for loop modeling.

Site-directed mutagenesis

Site-directed mutagenesis was performed using QuickChange approach. Constructs for site-directed mutagenesis were generated from full length rat Lphn1 on a PCMV5 vector. The mutants were verified through sequencing at the UChicago DNA Sequencing Facility.

Cell culture and transfection

HEK293 and HEK293T cell lines (ATCC; generous gifts from S. Koide lab) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; 11965092) supplemented with 10% FBS (F0926; Sigma) at 37°C in 5% CO_2 humidified incubator.

For both cAMP signaling assay and western blots, HEK293 cells were plated in 6-well plates and incubated until 60% confluent. For forskolin cAMP assays, HEK293 cells were co-transfected with 350 ng Lphn, 350 ng 22F Glosensor reporter plasmid (E2301, Promega; a generous gift from R. Lefkowitz lab), and 2.8 µL transfection reagent Fugene 6 (PRE2693; Promega). For β2-adrenergic receptor cAMP assays, the cells were co-transfected with the same reagents along with 9 ng β2-adrenergic receptor. For western blot and flow cytometry experiments, HEK293 cells were transfected with 2µg Lphn and 8µL Fugene 6.

For SRE signaling assays, HEK293T cells were plated in a 96-well plate and incubated until 35-50% confluent. Cells were co-transfected with 2.5 ng Lphn, 10 ng Dual-Glo lucerifase reporter plasmid, and 0.3 µl transfection reagent LipoD293 (SL100668; SignaGen Laboratories). The Dual-Glo luciferase reporter plasmid was cloned from plasmids (FJ376737 and FJ773212) purchased from Promega.

cAMP assay

24 hrs after transfection, HEK293 cells were detached and re-plated in a white 96-well plate (50,000 cells per well) and incubated for another 24 hrs. For pertussis toxin inhibition, during the last 5 hrs of

incubation the transfected HEK293 cells were treated with 500 ng/mL pertussis toxin (*Bordetella pertussis*; PHZ1174; Life Technologies) to specifically block Gi coupling. Two types of cAMP assay were performed: β2-adrenergic receptor assay and forskolin assay.

For β2-adrenergic receptor assay: After the 24 hr incubation period, the medium was changed to 100 µL CO_2-independent **Opti-MEM** I Reduced-Serum Medium (31985070, Life Technologies). Following a 30 min incubation period, 1 µL Glosensor substrate (Promega, PRE1290) and 11 µL FBS (Sigma-Aldrich, F0926) were added to the cells. After pre-equilibration for 10-15 min, basal cAMP signal was measured. Cells were then treated with either 100 µM agonist peptide (synthesized by GenScript) or DMSO for 5 min, then activated with 50 nM isoproterenol (I650450, Sigma).

For forskolin assay: After the 24 hr incubation period, the medium was changed to 100 µL CO_2-independent **Opti-MEM** I Reduced-Serum Medium (31985070, Life Technologies) supplemented with 0.3 mM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; I5879; Sigma). Following a 30 min incubation period, 1 µL Glosensor substrate (Promega, PRE1290) and 11 µL FBS (Sigma-Aldrich, F0926) were added to the cells. After pre-equilibration for 10-15 min, basal cAMP signal was measured. Cells were then treated with either 100 µM agonist peptide (synthesized by GenScript) or DMSO for 5 min, then activated with 0.5 µM forskolin (*Coleus forskohlii,* F6886, Sigma).

For both assays, measurements were taken using Synergy HTX BioTeck plate reader at 25°C, GAIN 200 in 5 min intervals. Data from the 15 min time point were analysed in Excel and presented as average values from 3 repeats with standard errors for each sample.

SRE assay

Assay was performed as previously described (cite Salzman, et al., Neuron 2016). Breifly, HEK293T cells were co-transfected with a Dual-Glo SRE luciferase reporter plasmid and Lphn3. Prior to lysis, cells were subject to serum starvation for 15 hours. Firefly and renilla luminescence signals were measured on Synergy HTX BioTeck plate reader. The firefly:renilla ratio for each well was calculated and normalized to empty vector. Data are presented as average values from 3 repeats with standard errors.

Western blot

HEK293 cells were transfected in 6 well plates with 2 µg of receptor of interest or empty vector incubated 48 hrs, and washed with ice-cold PBS (1mM $MgCl_2$, 0.1mM $CaCl_2$), and stored at -80C. Cells

were quickly thawed at 37C, resuspended on ice in PBS+0.01% BSA+protease inhibitors, and subjected to centrifugation @6500xg for 5 min. The pellet was resuspended in 500 µl solubilization buffer (20mM HEPES, pH 7.4; 150 mM NaCl; 2 mM MgCl; 0.1 mM EDTA, 2 mM CaCl2; 1% Triton X-100; protease inhibitors) and rotated at 30 min and centrifugated at 20,000xg, for 15 min. Supernatant was mixed with 6x loading buffer and subject to western blotting. Expressed proteins were detected with mouse anti-FLAG antibodies 1:2000 (F3165; Sigma).

Biotinylation of membrane proteins

HEK293 cells were transfected in 6 well plates with 2 µg of receptor of interest or empty vector and incubated 48 hrs, then washed twice with ice-cold PBS (1mM $MgCl_2$, 0.1mM $CaCl_2$) and incubated for 30 min, 4°C with Sulfo-NHS-LC-Biotin reagent (21335; Life Technologies), final concentration 0.5 mg/ml, to allow biotinylation of membrane proteins. Biotinylating reagent was then washed out with ice-cold PBS (1mM $MgCl_2$, 0.1mM $CaCl_2$) and cell lysate was obtained after treatment of cells with solubilization buffer (with 1% Triton X-100) for 1 hr. Biotinilated proteins were captured with streptavidin paramagnetic beads (PR Z5482; Promega), eluted from beads with 4.5 M urea and 3x SDS-PAGE loading buffer, and subject to western blotting. Expressed Lphn1 FL, TM and P+TM proteins were detected with mouse anti-FLAG antibodies 1:2000.

Flow cytometry

HEK293 cells were transfected with 2 µg of DNA /well in 6-well plates using Fugene6. After 48 hrs of incubation, cells were detached with citric saline solution and washed with PBS and PBS+0.1% BSA (Bovine serum albumin, A3803, Sigma). The pellet was then stained with mouse anti-FLAG M2 primary antibody, 1:1000 (F3165) in PBS+0.1% BSA for 30 min (with rotation at room temperature), washed twice with PBS+0.1% BSA and incubated with anti-mouse FITC, 1:100 (F0257) secondary antibody for another 30 min and washed twice again. Pellets were resuspended in PBS+0.1% BSA. Flow cytometry data were collected on Guava Easycyte and BD Accuri C6 flow cytometers. Data were analyzed in FlowJo.

GTPγS assay

Protocol was implemented as previously described (Stoveken et al., 2015), the only difference being that for the present study, insect cell membranes were pre-incubated with G proteins for 5 minutes before starting the assay.

Snake-plot and helix box diagrams

Snake-plot and helix box diagrams as well as coloring of mutations were done using the Protter visualization tool.

Receptor-peptide crosslinking experiments

Purified hLphn3 7TM (40uM) and biotinylated hLphn3 peptide (80uM) were incubated for 30 minutes at 4°C. Glutaraldehyde (Sigma-Aldrich #G5882, 0.01%) was added to the sample and incubated for 5 minutes at room temperature. The crosslinking reaction was quenched with 0.5M Tris pH 8.0. The sample was subjected to SDS-PAGE and transferred to PVDF membrane for western blotting. Peptide was detected with NeutrAvidin DyLight 650 (Invitrogen #84607, 1:2000 dilution) and hLphn3 was detected with mouse anti-His primary antibody (Qiagen #34660, 1:2000 dilution) and donkey anti-mouse Alexa Fluor 488 secondary antibody (Invitrogen #A-21202, 1:5000 dilution).

C. elegans experiments

Generation of plasmids and transgenes in C. elegans

For generation of constructs for *in vivo* analyses recombineering was employed (Dolphin and Hope, 2006; Tursun et al., 2009) and accompanying protocols were modified as previously described (Langenhan et al., 2009; Prömel et al., 2012) to construct the respective Lphn transgenes using cosmids, PCR-amplified targeting cassettes and positive antibiotic selection. To introduce the point mutations into a cosmid containing the complete *lat-1* locus, a three-step strategy was followed. Firstly, an intermediate plasmid was constructed by inserting a FRT-kanamycin resistance gene (kanR)-FRT cassette amplified from pIGCN21(Lee et al., 2001) into the intronic region of *lat-1* between exon 6 and 7 of cosmid pTL2 (Langenhan et al., 2009) and the entire exons 5, 6-FRT-kanR-FRT-exon 7 segment was retrieved into an outward-PCR-amplified pUC18 backbone (ThermoFisher Scientific). This intermediate vector was subject to QuikChange site-directed mutagenesis (Stratagene) introducing the respective point mutation in exon 6 of *lat-1* (CTA to GCT for L790A, CAC to GCT for H792A, TTC to GCT for F763A). A 2.5 kb fragment containing the respective point mutation was amplified, gel-purified and used as a recombineering targeting cassette on cosmid pSP5 containing the entire *lat-1* locus with a *gfp* fused to the second intracellular loop of the receptor (Langenhan et al., 2009). pSP5 had been transformed into SW105 cells beforehand (Warming et al., 2005), bacteria were made recombination-competent and the targeting cassette transformed for recombineering.

C. elegans strains

C. elegans strains were cultured and manipulated according to standard protocols (Brenner, 1974). Wild-type worms were Bristol, N2. The allele *lat-1(ok1465)* was generated by the *C. elegans* Gene

Knockout Consortium and provided by the Caenorhabditis Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The extrachromosomal transgenes *aprEx196[lat-1::gfp(L790A) (pFF9) rol-6(su1006) pBSK]*, *aprEx197[lat-1::gfp(H792A) (pFF10) rol-6(su1006) pBSK]* and *aprEx199[lat-1::gfp(F763A) (pFF11) rol-6(su1006) pBSK]* were generated for this study. The following transgenes have been previously described: *aprEx77[lat-1::gfp (pSP5) rol-6(su1006) pBSK]* (Langenhan et al., 2009) and *aprEx47[lat-1(1-581) (pTL20) rol-6(su1006) pBSK]* (Prömel et al., 2012).

Generation of transgenic lines

All transgenic strains with stably transmitting extrachromosomal arrays were generated by DNA microinjection as described (Mello and Fire, 1995; Mello et al., 1991). Cosmids were injected at a concentration of 1 ng/µl together with the coinjection marker pRF4*[rol-6(su1006)+]* (100 ng/µl) and pBluescript II SK+ vector DNA (Stratagene) as stuffer DNA to achieve a final concentration of 120 ng/µl. DNA was injected into the syncytical gonad of *lat-1(ok1465)/mIn1[mIs14 dpy-10(e128)]* hermaphrodites. Transgenic progeny were isolated and stable lines selected. Multiple independent transgenic lines were established for each transgene tested.

Microscopy

For analysis of transgene expression young adult hermaphrodites were mounted in M9 onto a 2% agarose pad. Images were acquired with an Olympus Fluoview FV1000 confocal microscope.

Lethality and fertility rescue assays

Lethality and fertility rescue assays were conducted as previously described (Langenhan et al., 2009). For the lethality rescue assay fifty L4 hermaphrodites were transferred into wells of a 72-well flat-bottom Terasaki plates (Greiner Bio-One) containing *E. coli* OP50 in M9 and allowed to lay eggs for 24 h at 22 °C. Five to ten eggs were transferred into fresh wells with corresponding solutions and incubated at 22 °C. The number of dead/surviving embryos was scored 24 hours later, the number of adult animals 48 hours later.

For the fertility rescue assay ten L4 hermaphrodites were allowed to lay eggs at 22 °C on separate NGM plates seeded with *E. coli* OP50. Every 24 h hermaphrodites were transferred onto fresh plates until egg-laying ceased and embryos were scored. Experiments were conducted at least in independent triplicates. Data were examined using ANOVA with Bonferroni post-hoc test.