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Supplemental Information

Lypd6 Enhances Wnt/β-Catenin Signaling by Promoting Lrp6 Phosphorylation in Raft Plasma Membrane Domains

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Inventory of Supplementary Material

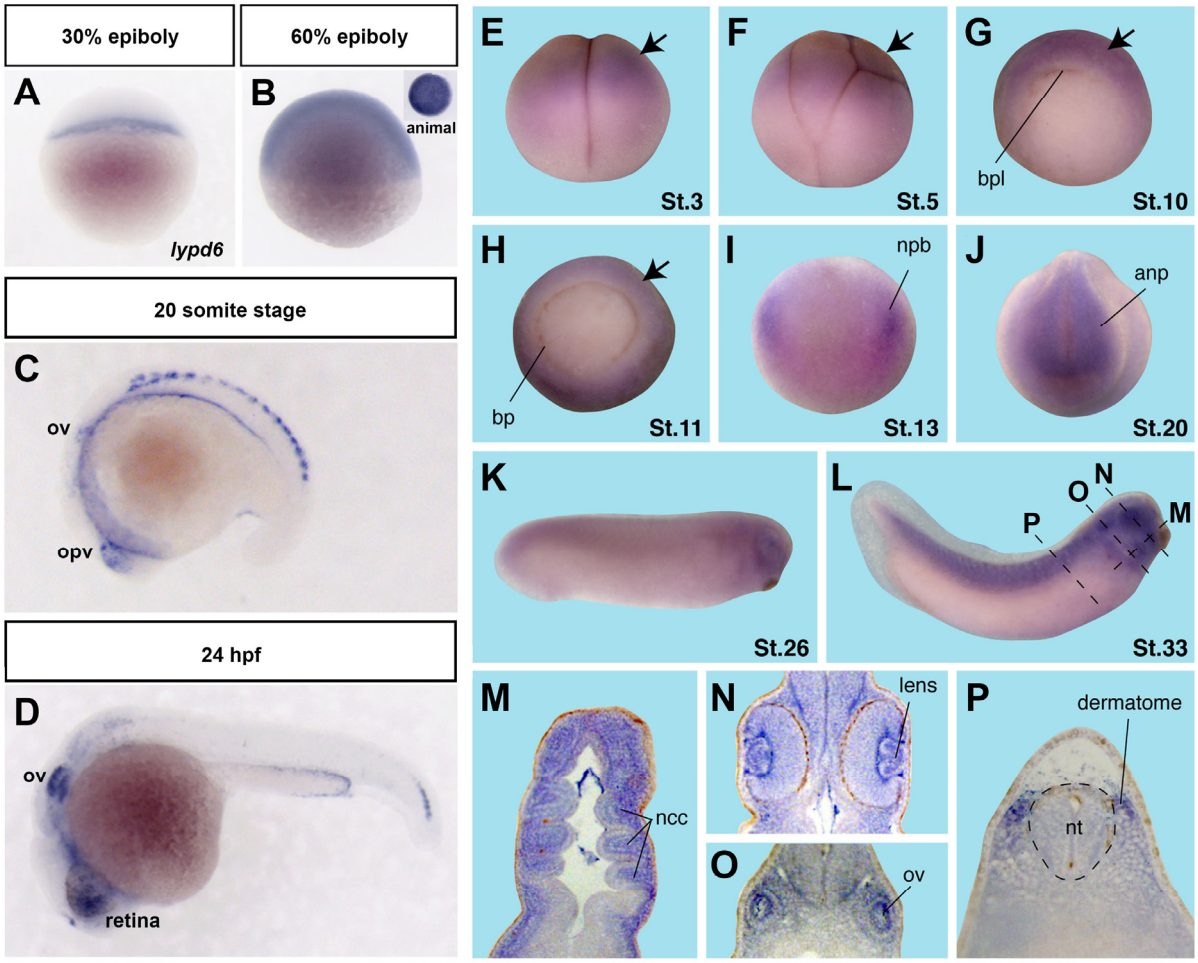
Figure S1: relates to Figure 1 and shows the expression pattern of zebrafish and *Xenopus lypd6* and bioinformatic analysis of the Lypd6 amino acid sequence.

Figure S2: relates to Figure 2 and shows phenotypes caused by *lypd6* knockdown.

Figure S3: relates to Figure 4 and shows effects of *lypd6* knockdown in mammalian cells and transplantation experiments in zebrafish embryos.

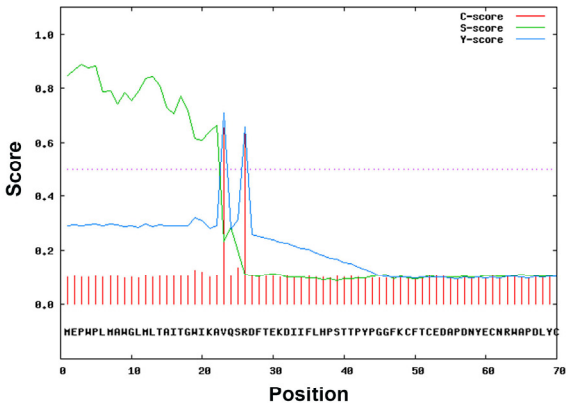
Figure S4: relates to Figure 5 and shows Lypd6-Lrp6 co-IP to test for direct binding and co-IPs of Lypd6-Fz and Lypd6-Wnt.

Figure S5: relates to Figure 6 and shows effects of Lypd6 mislocalization to non-raft membrane domains on Wnt/β-catenin signaling reporter activity and Lrp6 phosphorylation**.**



Q

Signal peptide prediction: zebrafish Lypd6



SignalP 4.0 Server - Technical University of Denmark

R

GPI anchor modification site prediction: zebrafish Lypd6

```
MEPWPLMAWG LMLTAITGWI KAVQSRDFTE KDIIFLHPST TPYPGGFKCF TCEDAPDNYE
CNRWAPDLYC PRESRYCYTH HKMSWDGNTV SVTKRCVPLE DCLQTGCSDI DHEGNRVCTA
CCEGNICNLP LPRNETDAIF STTSPINRSA QSTQTLPLLL LSVSITSLML HSIN

Best predicted site is shown in red.

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    Prediction of potential C-terminal GPI-Modification Sites
~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

Use of the prediction function for METAZOA


Potential GPI-modification site was found.
Quality of the site ..... :       S
Sequence position of the omega-site :     147
Score of the best site ..... :   -2.70 (PValue = 2.558954e-03)
```

I.M.P. Bioinformatics: big-PI Predictor

Figure S1. *lypd6* expression during zebrafish and *Xenopus* embryonic development and bioinformatic analyses of Lypd6 protein sequence.

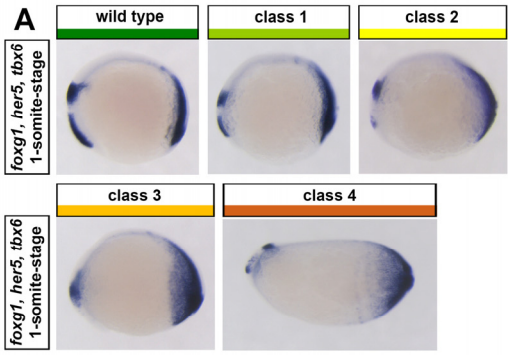
related to Figure 1.

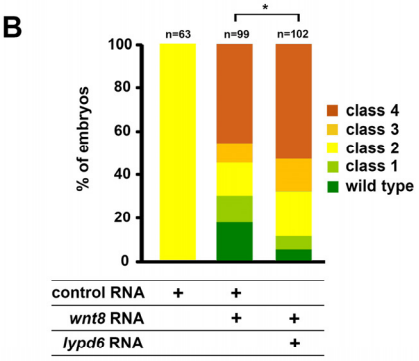
(A-D) Zebrafish *lypd6* expression pattern detected by whole mount in situ hybridization (WMISH) at the indicated stages. ov = otic vesicle, opv = optic vesicle, hpf = hours post fertilization.

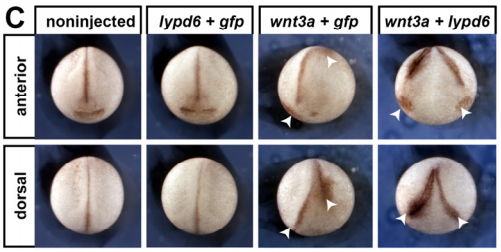
(E-P) *Xenopus lypd6* expression pattern during embryogenesis. bpl = blastoporus lip, bp = blastoporus, npb = neural plate border, anp = anterior neural plate, ncc = neural crest cells, ov = otic vesicle. E, F, K and L lateral views, G and H vegetal views, I and J anterior views. M horizontal section, N-P transversal sections of embryos at stage 33 at the positions shown by dotted lines in L.

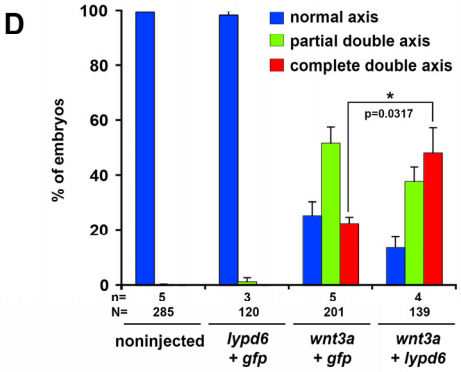
(Q) Signal peptide prediction in zebrafish Lypd6 using Signal P 4.0 Server (University of Denmark). Cleavage site predicted between position 22 and 23: IKA-VQ.

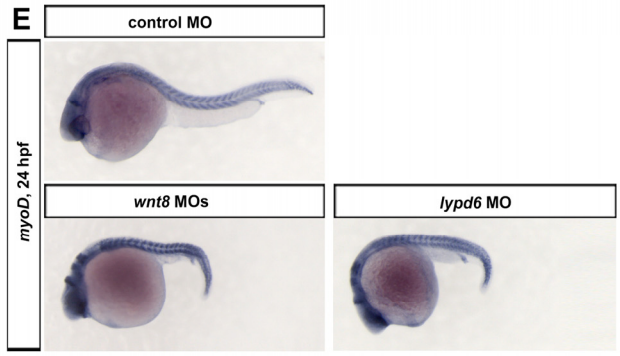
(R) GPI-anchor modification site prediction in zebrafish Lypd6 using big-PI Predictor (I.M.P. Bioinformatics). GPI-anchor attachment site (Omega site) predicted at position 147: N.

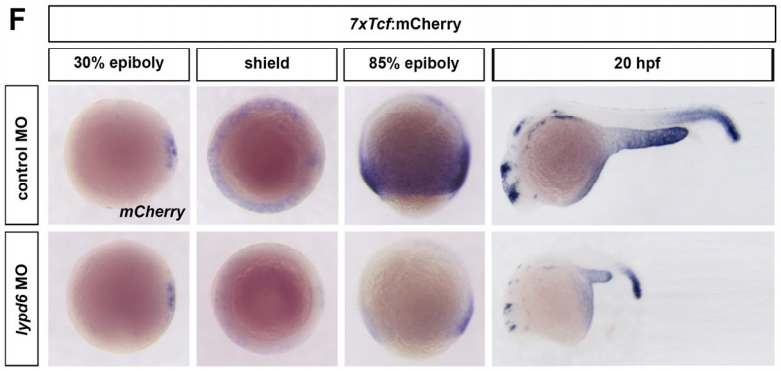

A
wild type
class 1
class 2
foxg1, her5, tbx6
1-somite-stage
class 3
class 4
foxg1, her5, tbx6
1-somite-stage

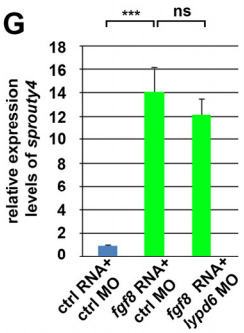

B
n=63
n=99
n=102
*
100
80
60
40
20
0
% of embryos
class 4
class 3
class 2
class 1
wild type
control RNA + +
wnt8 RNA + +
lypd6 RNA +

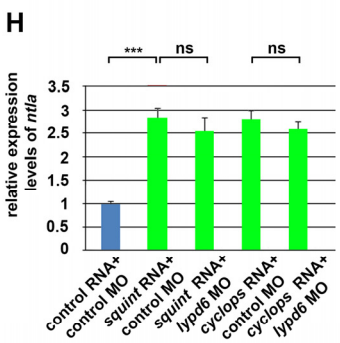

C
noninjected
lypd6 + gfp
wnt3a + gfp
wnt3a + lypd6
anterior
dorsal


D
100
80
60
40
20
0
% of embryos
normal axis
partial double axis
complete double axis
*
p=0.0317
n= 5 3 5 4
N= 285 120 201 139
noninjected
lypd6 + gfp
wnt3a + gfp
wnt3a + lypd6


E
control MO
myoD, 24 hpf
wnt8 MOs
lypd6 MO


F
7xTcf:mCherry
30% epiboly
shield
85% epiboly
20 hpf
control MO
mCherry
lypd6 MO


G
***
ns
relative expression levels of sprouty4
18
16
14
12
10
8
6
4
2
0
ctrl RNA+ ctrl MO
fgf8 RNA+ ctrl MO
fgf8 RNA+ lypd6 MO


H
***
ns
ns
relative expression levels of ntla
3.5
3
2.5
2
1.5
1
0.5
0
control RNA+ control MO
squint RNA+ control MO
squint RNA+ lypd6 MO
cyclops RNA+ control MO
cyclops RNA+ lypd6 MO

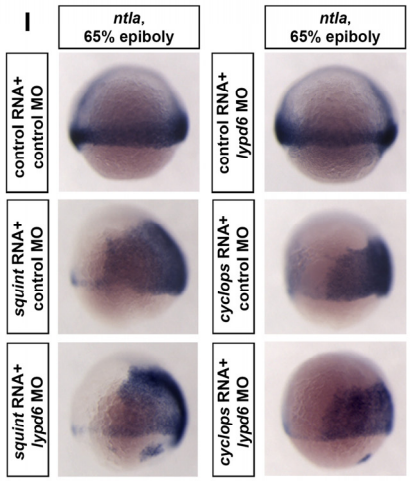

I
ntla, 65% epiboly
ntla, 65% epiboly
control RNA+ control MO
control RNA+ lypd6 MO
squint RNA+ control MO
cyclops RNA+ control MO
squint RNA+ lypd6 MO
cyclops RNA+ lypd6 MO

Figure S2. Lypd6 specifically enhances Wnt/β-catenin signaling in zebrafish and *Xenopus* embryos. *related to Figure 2.*

(A-B) *wnt8*-induced anteroposterior patterning defects are enhanced by *lypd6* overexpression during zebrafish gastrulation.
(A) Range of phenotypes at the 1-somite stage caused by *wnt8* RNA (20 pg) overexpression determined by WMISH using the forebrain marker *foxg1a*, the midbrain-hindbrain boundary marker *her5* and the posterior mesoderm marker *tbx6*. Class 1, reduction in *foxg1a* and *her5*; class 2, substantial loss of forebrain marked by *foxg1a*; class 3, complete loss of *foxg1a*, expansion of *tbx6*; class 4, dorsalization indicated by football shape of the embryo.
(B) Co-injection of *lypd6* RNA (80 pg) enhances the severity of phenotypes, defined in A, induced by *wnt8* RNA (20 pg) in comparison to embryos co-injected with equimolar amounts of *spGFP-GPI* control RNA.
(C-D) Secondary axis induction assay in *Xenopus* embryos.
(C) Zebrafish *lypd6* (2 ng) enhances the induction of a secondary body axis by *wnt3a* (5 pg) injection, axes are marked by arrowheads.
(D) Quantification of the experiment shown in (C). n, number of independent injection experiments; N, Total number of analyzed embryos. Error bars, s.e.m.
(E) Injection of 10 ng *lypd6* MO produces a phenotype similar to that generated by injection of 2.5 ng *wnt8* MO1 and MO2 each. Embryos have posterior truncations evident by a reduced number of somites marked by *myoD*. number of somites = 28±1 in control MO [n=20 embryos], 20±3 in *lypd6* MO [n=24 embryos], 17±2 in *wnt8* MOs [n=23 embryos].
(F) Injection of 10 ng *lypd6* MO downregulates *7xTcf*:mCherry reporter activity from shield stage onwards as detected by *mCherry* expression. Note that *lypd6* knockdown has no effect on reporter expression at the 30% epiboly stage.
(G) *lypd6* MO (10 ng) does not affect Fgf signaling as determined by qRT-PCR of the pathway target *sprouty4* at early gastrula stages. Fgf signaling was activated by injection of 20 pg *fgf8* RNA. Error bars, s.e.m.
(H) *lypd6* MO does not affect Nodal signaling determined by qRT-PCR of the pathway target gene *ntla* at early gastrula stages. Nodal signaling was activated by injection of 30 pg *squint (ndr1)* or 30 pg *cyclops (ndr2)* RNA. Error bars, s.e.m.
(I) *lypd6* MO has no effect on ectopic expression of *ntla* induced by *squint* or *cyclops* RNA assayed by WMISH at 65% epiboly.

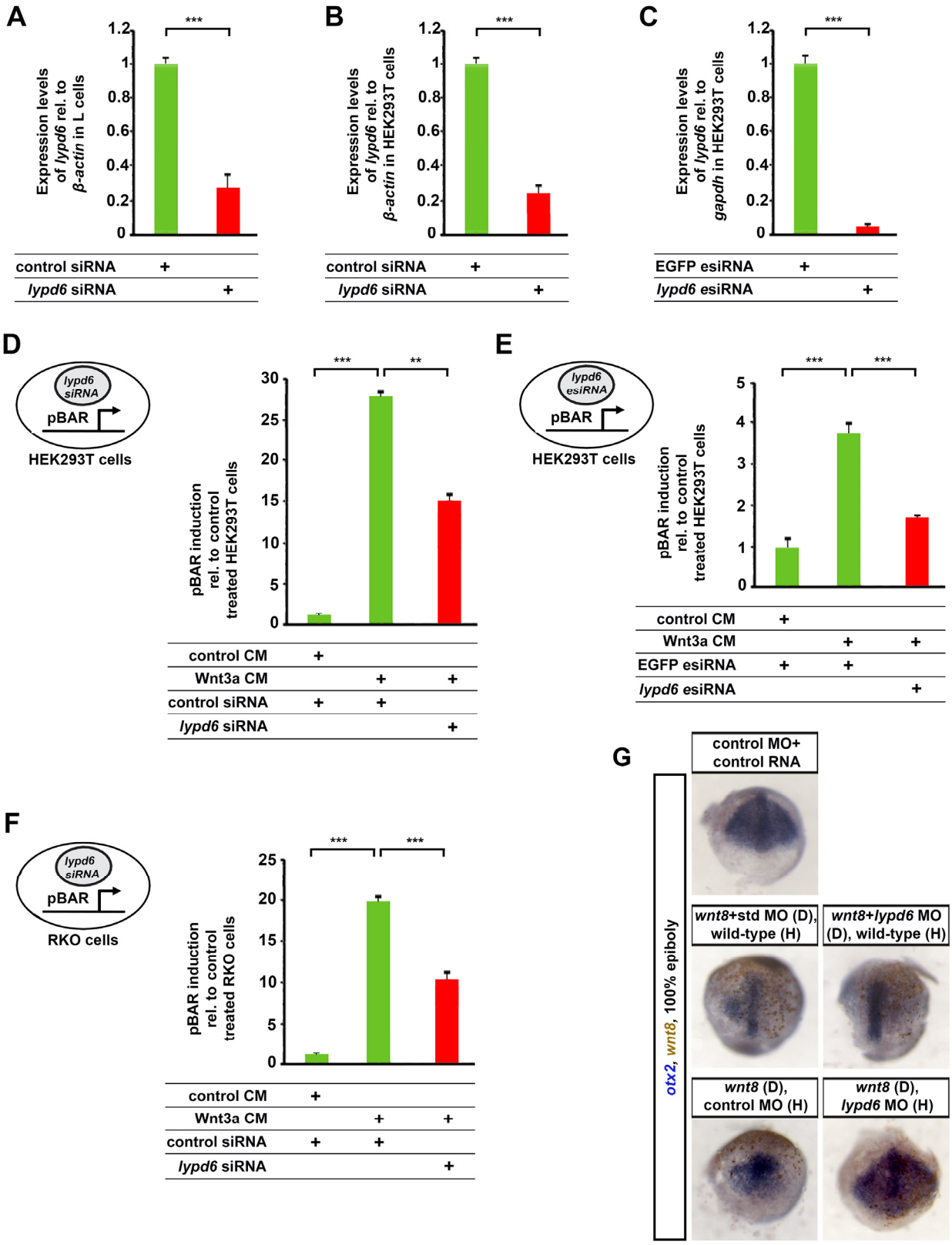

A
Expression levels of lypd6 rel. to β-actin in L cells
1.2
1
0.8
0.6
0.4
0.2
0
***
control siRNA +
lypd6 siRNA +
B
Expression levels of lypd6 rel. to β-actin in HEK293T cells
1.2
1
0.8
0.6
0.4
0.2
0
***
control siRNA +
lypd6 siRNA +
C
Expression levels of lypd6 rel. to gapdh in HEK293T cells
1.2
1
0.8
0.6
0.4
0.2
0
***
EGFP esiRNA +
lypd6 esiRNA +
D
lypd6 siRNA
pBAR
HEK293T cells
pBAR induction rel. to control treated HEK293T cells
30
25
20
15
10
5
0
***
**
control CM +
Wnt3a CM + +
control siRNA + +
lypd6 siRNA +
E
lypd6 esiRNA
pBAR
HEK293T cells
pBAR induction rel. to control treated HEK293T cells
5
4
3
2
1
0
***
***
control CM +
Wnt3a CM + +
EGFP esiRNA + +
lypd6 esiRNA +
F
lypd6 siRNA
pBAR
RKO cells
pBAR induction rel. to control treated RKO cells
25
20
15
10
5
0
***
***
control CM +
Wnt3a CM + +
control siRNA + +
lypd6 siRNA +
G
otx2, wnt8, 100% epiboly
control MO+ control RNA
wnt8+std MO (D), wild-type (H)
wnt8+lypd6 MO (D), wild-type (H)
wnt8 (D), control MO (H)
wnt8 (D), lypd6 MO (H)

Figure S3. Lypd6 is required for Wnt/β-catenin signaling in Wnt-receiving cells.
related to Figure 4.

(A) Mouse *lypd6* transcripts are knocked down to about 27% in murine L cells transfected with *lypd6* siRNA as determined by qRT-PCR at 48 hours after transfection.
(B) Human *lypd6* transcripts are knocked down to about 23% in HEK293T cells transfected with *lypd6* siRNA as determined by qRT-PCR at 48 hours after transfection..
(C) Human *lypd6* transcripts are knocked down to about 5% in HEK293T cells transfected with *lypd6* esiRNA as determined by qRT-PCR at 48 hours after transfection.
(D) siRNA mediated knockdown of human *lypd6* in HEK293T cells reduces induction of pBAR by Wnt3a conditioned medium (CM). pBAR activity is shown relative to that in cells transfected with negative control siRNA and treated with control CM.
(E) esiRNA mediated knockdown of human *lypd6* in HEK293T cells reduces induction of pBAR by Wnt3a CM.
(F) siRNA mediated knockdown of human *lypd6* in RKO cells reduces induction of pBAR by Wnt3a CM.
(A-F) Error bars, s.e.m.
(G) *otx2* expression (blue) in zebrafish host embryos transplanted with *wnt8* expressing cells, marked by fluorescein-tagged dextran detected in brown at 100% epiboly. D: donor, H: host, anterior views.
Upper row: Control non-transplanted embryo injected with control MO and control RNA (n=15/15).
Middle row: *wnt8* expressing cells, transplanted into a wild type (uninjected) embryo at dome stage, can repress *otx2* (blue) no matter whether the donor cells have been co-injected with *lypd6* MO (n=8/11) or control MO (n=12/13).
Bottom row: *wnt8* expressing cells inhibit *otx2* expression in control MO injected hosts (n=8/9), but much less so if the host was injected with *lypd6* MO (n=10/13).

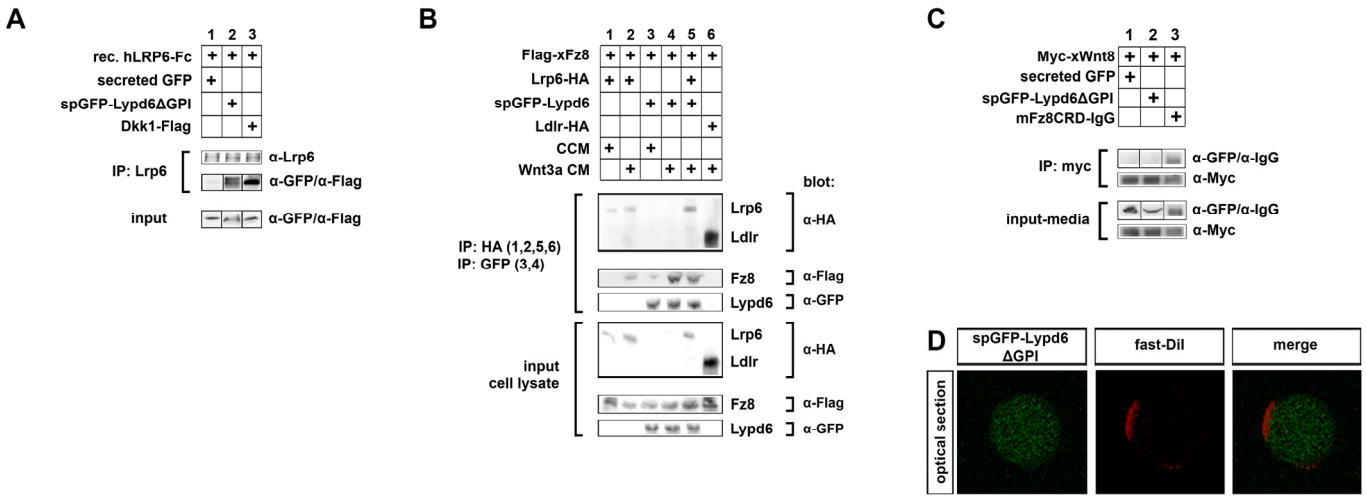


Figure S4. Lypd6 directly interacts with Lrp6, while it binds to Fz8 in a Wnt3a-dependent manner but does not interact with Wnt8.

related to Figure 5.

(A) Purified secreted Lypd6 (spGFP-Lypd6ΔGPI) co-IPs with secreted recombinant hLrp6-Fc chimeric protein (lane 2), while secreted GFP does not (lane 1). Dkk1-Flag can also be co-IPed with hLrp6-Fc (lane 3).

(B) Flag-tagged *Xenopus* Frizzled8 (xFz8) co-IPs with spGFP-Lypd6 in HEK293T cells and this interaction is strongly enhanced by Wnt3a (compare lanes 3 & 4). Flag-xFz8 can also be IPed with Lrp6-HA in the presence of Wnt3a, but not with Ldlr-HA. Lypd6 and Lrp6 still bind to Fz8 when expressed together.

(C) Immunoprecipitation experiments of conditioned media show that Myc-tagged *Xenopus* Wnt8 (Myc-xWnt8) does not bind to spGFP-Lypd6ΔGPI or the negative control secreted GFP, while it does bind to the positive control, the CRD domain of mouse Fz8 fused to IgG (mFz8CRD-IgG).

(D) spGFP-Lypd6ΔGPI does not localize to the membrane of GPMVs derived from CHO cells.

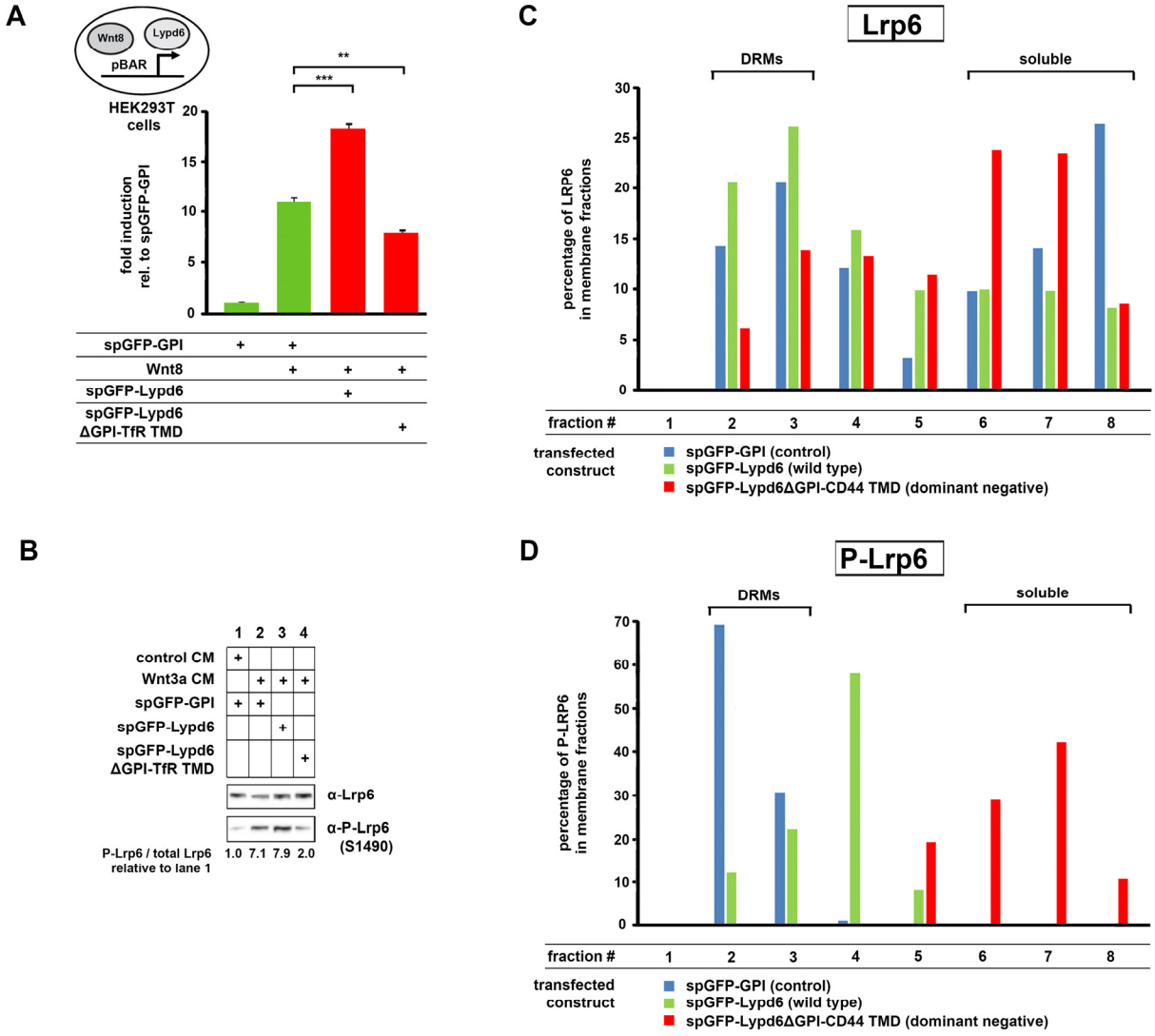


Figure S5. Mislocalization of Lypd6 to non-raft membrane domains inhibits Wnt/β-catenin signaling via shifting Lrp6 phosphorylation to these domains.

related to Figure 6.

(A) pBAR luciferase reporter activity in HEK293T cells transfected with Wnt8 (20 ng) plus spGFP-Lypd6 (100 ng) or equimolar amounts of spGFP-Lypd6ΔGPI-TfR TMD (95 ng) or spGFP-GPI control. Note that Wnt8-induced reporter activity is significantly reduced by the spGFP-Lypd6ΔGPI-TfR TMD construct. Error bars, s.e.m.

(B) spGFP-Lypd6ΔGPI-TfR TMD reduces Wnt3a-induced phosphorylation of Lrp6 at S1490 in HEK293T cells assayed at 6 h post stimulation with Wnt3a CM.

(C) Quantification of Lrp6 distribution in membrane fractions shown in Figure 6E, fourth panel.

(D) Quantification of P-Lrp6 (S1490) distribution in membrane fractions shown in Figure 6E, fifth panel.

Supplemental Experimental Procedures

Constructs

The following modifications of the zebrafish *lypd6* (genbank BC081426) coding sequence were created in the pCS2P+ expression vector:

pCS2P+Lypd6: full length coding sequence.

pCS2P+spGFP-Lypd6: full length Lypd6 fused in frame with mmGFP5 between amino acids (aa) 22 and 23.

pCS2P+spmCherry-Lypd6: full length Lypd6 fused in frame with mCherry between aa 22 and 23.

pCS2P+spGFP-Lypd6ΔGPI: Lypd6 lacking the carboxy terminus after aa 146, fused in frame with mmGFP5 between aa 22 and 23.

pCS2P+spGFP-Lypd6ΔGPI-TfR TMD: carboxy terminus after aa 146 replaced by the first 97 aa of the human Transferrin Receptor (TfR) including the TMD, fused in frame with mmGFP5 between aa 22 and 23.

pCS2P+spGFP-Lypd6ΔGPI-CD44 TMD: carboxy terminus after aa 146 replaced by the TMD of CD44, fused in frame with mmGFP5 between aa 22 and 23.

pCS2P+spmCherry-Lypd6ΔGPI-CD44 TMD: carboxy terminus after aa 146 replaced by the TMD of CD44, fused in frame with mCherry between aa 22 and 23.

pCS2P+Lypd6 rescue: full length coding sequence with 8 silent mutations introduced in the first 9 aa to block MO binding.

To generate an antisense probe for WMISH in *Xenopus*, a partial *Xenopus laevis lypd6* cDNA (genbank KF042353) was cloned into pSC-B (Stratagene) using primers designed against *Xenopus tropicalis lypd6* (genbank NM_001113032).

Quantitative reverse transcription PCR (qRT-PCR)

RNA was isolated using Trizol (Life Technologies) and cDNA was synthesized with Thermoscript RT (Life Technologies) using a 1:1 mixture of oligodT and random primers. (-RT) controls were generated by replacing the Thermoscript RT with water. qPCR was performed in triplicates with 1:10 diluted cDNA using a Stratagene MX 3000 QPCR machine. Relative expression levels were determined after normalization to *ß-actin* or *gapdh.*

Primers used:
Zebrafish *lypd6*: 5'-ATGCTGACCGCCATTACCGG-3' (Weidinger lab ID 1880) and 5'-GCGGCACACAACGCTTCGT-3' (1881).
Zebrafish *ß-actin:* 5'-GAAGGAGATCACCTCTCTTGCTC-3' and 5'-GTTCTGTTTAGAAGCACTTCCTGTG-3'.
Mouse *lypd6*: 5'-CTGGTGGCTGATTGTCTGAA-3' (2680) and 5'-GGGTTGCTGTGATTGTGTTG-3' (2681).
Mouse *ß-actin*: 5'-GGGAATGGGTCAGAAGGACT-3' (2692) and 5'-CCATCACAATGCCTGTGGTA-3' (2693).
Human *lypd6*: 5'-CCTGAGCCTGCTGGCGGATTG-3' (1706) and 5'-GCATCAGTTTCATTTCGGGGCAGTG-3' (1707)
Human *ß-actin:* 5'-GCAGAAGGAGATCACATCCCTGGC-3' (2217) and 5'-CATTGCCGTCACCTTCACCGTTC-3' (2218)
Human *gapdh*: 5'-TCAGACACCATGGGGAAGGTGAAGG-3' (1540) and 5'-GAGGGATCTCGCTCCTGGAAGATGG-3' (1541)

Wnt3a conditioned media (CM) production

Wnt3a-producing murine L cells (American Type Culture Collection CRL-2647) were grown in 10 cm culture dishes to a confluence of 90-95% in DMEM containing 10% FBS and 1% Pen-Strep. Cells were diluted 1:10 and seeded into new dishes. Media were exchanged 1 day after transfection, conditioned media were collected at 2 days, 4 days and 6 days and stored at 4°C.

Luciferase assays

HEK 293T cells were transiently transfected with the Tcf/Lef firefly luciferase reporter pGL3 BAR (pBAR, 20 ng) (Biechele and Moon, 2008) and normalization control pGL4.73 hRLuc/SV40 (RLuc, 5 ng) (Promega, Madison) in triplicates. At 24 hours post transfection, cells were stimulated with Wnt3a-conditioned media (CM) or control CM for 6 hours at 37°C. Following stimulation, firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and firefly activity was normalized to renilla luciferase levels.

***Xenopus* secondary axis induction assay**

5 pg GFP, 5 pg mouse *wnt3a*, or 2 ng zebrafish *lypd6* RNA were injected bilaterally into the marginal zone of both ventral blastomeres of 4-cell stage *Xenopus* embryos. Embryos were cultivated until stage 20 for evaluation.

Zebrafish cell transplantation assay

Host embryos were either injected at the 1-cell stage with 10 ng *lypd6* MO or control MO or left uninjected. To test the effect of *lypd6* on Wnt production, donor embryos were injected with 200 pg *wnt8* RNA and 10 ng *lypd6* MO or control MO plus fluorescein-tagged *dextran* (MW 40000*,* anionic, Life Technologies). To test the effect on Wnt reception, donor embryos were injected with 200 pg *wnt8* RNA plus fluorescein-tagged dextran. Transplantations were performed at dome stage using trimmed borosilicate capillaries and embryos were fixed for WMISH at the 100% epiboly stage. Transplanted cells were detected by incubation with alkaline phosphatase conjugated anti-Fluorescein-Ab (1:4000) followed by INT/BCIP staining (brown).

Purification of secreted proteins and immunoprecipitation (IP) to assay for direct interaction

HEK293T cells were seeded in 10 cm dishes and, after replacing the media with serum-free media on the next day, transfected with spGFP-Lypd6ΔGPI (9 µg) or with equimolar amounts of spGFP as negative control. At 24 hours post transfection, conditioned media of cells were collected and mixed at a ratio of 30% (v/v) with IP buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton-X100). For Dkk1 collection, cells were similarly transfected in serum-free media with Dkk1-Flag (9 µg) in 10 cm dishes. At 24 hours post transfection, conditioned media of cells were collected and concentrated using centrifugal filter units (Amicon Ultra-15, Millipore) and the buffer was exchanged with PBS. All samples were pre-cleared with Protein A/G PLUS Agarose beads (Santa Cruz Biotechnology, Inc.) and the beads were removed. Next, samples were incubated with goat anti-GFP antibody (MPI-CBG Dresden Antibody Facility, 1:1000) and Protein A/G PLUS Agarose beads by rotating at 4°C o/n. Next day, the beads were washed five times with NET low-salt buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton-X100), eluted with 0.1 M Glycine (pH 2.5) three times and immediately neutralized with Tris pH 8. Eluates with the highest protein content, confirmed by Coomassie staining, were used for IP. Eluates containing spGFP-Lypd6ΔGPI, spGFP or Dkk1-Flag were incubated with recombinant hLrp6-Fc chimera (Ala20 – Pro 1368, R&D Systems). Next, samples were again pre-cleared with pre-blocked Protein A/G PLUS Agarose beads by rotating at 4°C for 30 min rotation, followed by removal of beads. IP experiments were performed as described previously (Kagermeier-Schenk et al., 2011) using rabbit anti-Lrp6 (Cell Signaling, 1:1000). Rabbit anti-Lrp6, goat anti-GFP and rabbit anti-Flag were used for Western blotting.

Supplemental References

Biechele, T.L., and Moon, R.T. (2008). Assaying beta-catenin/TCF transcription with beta-catenin/TCF transcription-based reporter constructs. Methods Mol Biol *468*, 99-110.

Kagermeier-Schenk, B., Wehner, D., Ozhan-Kizil, G., Yamamoto, H., Li, J., Kirchner, K., Hoffmann, C., Stern, P., Kikuchi, A., Schambony, A.*, et al.* (2011). Waif1/5T4 inhibits Wnt/beta-catenin signaling and activates noncanonical Wnt pathways by modifying LRP6 subcellular localization. Dev Cell *21*, 1129-1143.