Supporting Information

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SI Materials and Methods

Construct. Murine V5–FZD4 was generated by replacing the earlier described V5–FZD5 by that of V5–FZD4 via standard PCR methods.

Pulse-Chase. Cells expressing the indicated constructs were grown in depletion medium (−Met/−Cys) for 30 min at 37 °C. Next, medium was exchanged with labeling medium (100 μCi ^{35}S-Met/^{35}S-Cys per mL, Redivue; GE Healthcare) for 15 min at 37 °C. After the pulse, medium was replaced by chase medium (RPMI with 10% FCS and P/S) at 37 °C for the indicated times. After the chase, cells were washed with PBS containing 10 mM *N*-ethyl maleimide and lysed in lysis buffer (1% Triton X-100, 1 mM EDTA, 10 μM leupeptin, 10 μM aprotinin, and 1 mM PMSF in PBS). Immunoprecipitation was performed by incubation with mouse V5 antibodies (Life Technologies) for 1.5 h and 45 min with protein G beads (Millipore). Samples were washed three times with wash buffer (0.5% SDS, 0.66% Triton X-100, 0.25% deoxycholic acid, 0.5% BSA, and 0.66 mM EDTA, in PBS) and two times with PBS. Samples were resuspended in Laemmli sample buffer and loaded on SDS/PAGE gels. The gels were dried, and a phospho-imager screen (Molecular Dynamics) was used to measure radioactivity with a storm scanner (Amersham BioSciences).

Reagents. A concentration of 0.4 μg/mL SWA (Sigma) and 2 mg/mL BADGP (Merck) was added to the cell culture medium before transfection, 24 h before experimental analysis. BFA (BD Biosciences) was added to the culture medium 4 h before experimental analysis at a concentration of 1 μg/mL.

Isolation of Wnt-Binding Complexes and Proteomics Analysis. For each sample, HEK293T cells were seeded in 15,625-cm^2 tissue culture dishes and grown until confluency. Cells were stimulated with Wnt3a- or Wnt3a–iFlag-conditioned medium for 3 h and extensively washed with PBS, scraped, and pelleted at 500 × *g*. Cells were resuspended in 10 mL of lysis buffer and incubated for 1 h at 4 °C. Cell lysates were cleared by centrifugation at 20,000 × *g* for 30 min and incubated with 200 μL of Flag-M2 beads (Sigma) overnight at 4 °C. Beads were washed three times with lysis buffer and three times with PBS. A total of 100 μL of beads was collected for further analysis by mass spectrometry. Beads were resuspended in a spin filter column (Bio-Rad) and washed three additional times with 200 μL of PBS. Bound proteins were eluted with 100 μL of 0.5% RapiGest (Waters) reagents in 50 mM ammonium bicarbonate, followed by 100 μL of 8 M urea in 50 mM ammonium bicarbonate (pH 8.0). Eluted proteins were reduced with 1 mM DTT and alkylated with 5.5 mM iodoacetamide. For tryptic digestion, proteins were first digested with endoproteinase Lys-C (Wako Chemicals) at room temperature for 4 h, followed by sequencing-grade modified trypsin (Promega) overnight, after fourfold dilution with 50 mM ammonium bicarbonate. Protease digestion was stopped by addition of trifluoroacetic acid, and precipitates were removed after centrifugation. Peptides were desalted by using reversed-phase Sep-Pak C18 cartridges (Waters), then dried and stored at −20 °C. For mass spectrometric analysis, peptides were first separated with a C18 column (Zorbax) and introduced by nanoelectrospray into the LTQ Orbitrap Q Exactive (Thermo Fisher Scientific) and for MS acquisition followed by top 20 MS/MS in the form of collision-induced dissociation. MS raw files were analyzed by using Proteome Discoverer (Version 1.3). Tandem MS spectra, top 6 selected in 100-Da bin, were searched against the IPI Human database (Version 3.37, 69,186 entries). Trypsin/P was chosen as the protease, allowing two missed cleavages. Cysteine carbamidomethylation was set as fixed modification, and oxidation of methionine and acetylation of the N-terminal as variable modifications. Peptide tolerance was set to 15 ppm, while MS/MS tolerance was set to 0.5 Da.

PNGase F and EndoH Treatment. Immunoprecipitation of V5–FZD was performed as described above. Washed beads were treated with PNGase F (New England Biolabs) or EndoH (Roche) according to the manufacturer's instructions. Note that incubation steps were performed overnight at 37 °C. After enzyme incubation, the beads were taken up in SDS sample buffer.

Sucrose Gradient Sedimentation. Cells were grown to 80% confluency, stimulated for 4 h with Flag–Wnt3a-conditioned medium, washed with 25 mL of PBS, scraped in 6 mL of PBS per plate, and collected. Cell pellets were thoroughly resuspended and lysed for 1 h in cell lysis buffer containing 100 mM NaCL, 50 mM Tris (pH 7.5), 0.5% Triton X-100, 10% glycerol, 50 mM NaF, 10 mM Na3VO4, 10 μM leupeptin, 10 μM aprotinin, and 1 mM PMSF. Cell lysates were cleared by centrifugation for 20 min at 20,000 × *g* at 4 °C, and 450 μL of lysate was layered onto a 10–40% sucrose gradient in 150 mM NaCL, 50 mM Tris (pH 7.5), 0.02% Triton X-100, 10% glycerol, and protease inhibitor mixture tablet (Roche). Ultracentrifugation was done in a Sorvall MLS50 rotor at 100,000 × *g* for 4 h at 4 °C.

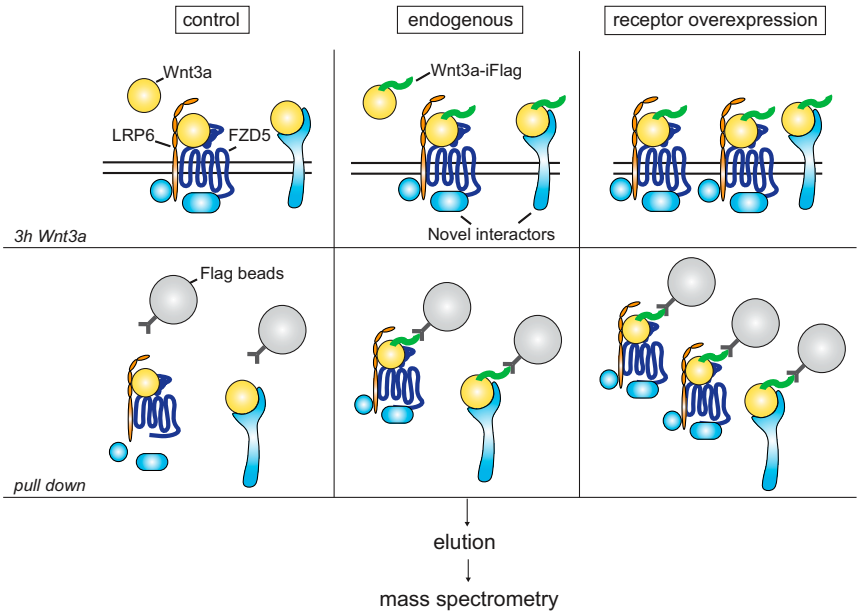

Fig. S1. Schematic representation of Wnt–receptor complex isolation procedure and preparation for proteomic analysis.

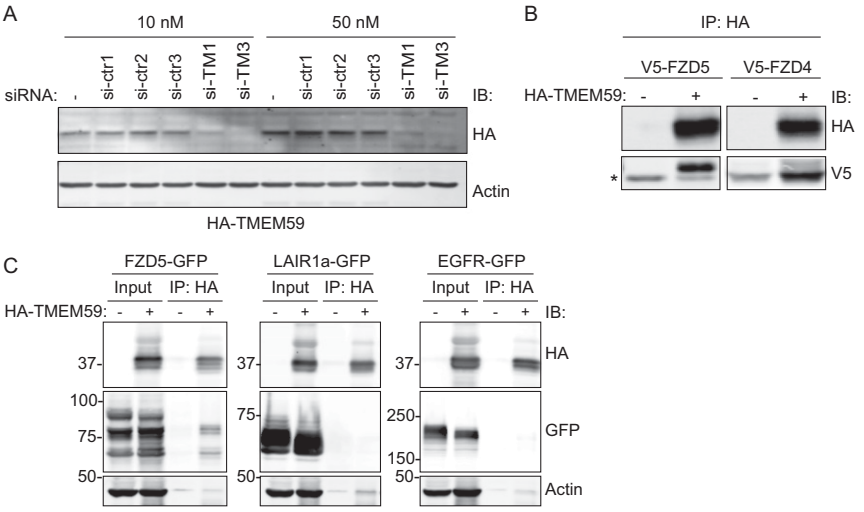

Fig. S2. Validation of TMEM59 siRNAs and determination of specificity of the interaction of TMEM59 with FZD. (*A*) Western blot analysis showing the efficiency of TMEM59-targeting siRNAs (1). (*B*) Coimmunoprecipitation of HA–TMEM59 with V5–FZD4 or–FZD5. Asterisk indicates an aspecific band. (*C*) Coimmunoprecipitation of HA–TMEM59 with FZD5–, LAIR1–, or EGFR–GFP.

1. Boada-Romero E, et al. (2013) TMEM59 defines a novel ATG16L1-binding motif that promotes local activation of LC3. *EMBO J* 32:566–582.

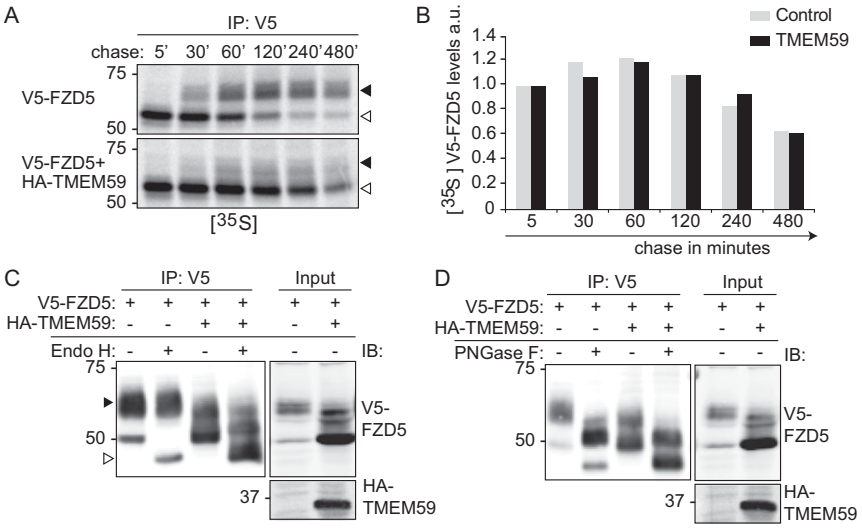

Fig. S3. Analysis of the effect of TMEM59 on FZD5 modifications. (*A*) Pulse-chase experiment of ^{35}S-Met/Cys–labeled V5-FZD5 in the presence or absence of HA–TMEM59. Cells were transfected with the indicated constructs and chased for the indicated times. Mature glycosylated V5-FZD5 (solid arrowhead) and immature V5–FZD5 (open arrowhead) are indicated. (*B*) Quantification of total V5–FZD5 levels of the pulse-chase experiment as shown in *A*. (*C* and *D*) Western blot analysis of Endo H (*C*) or PNGase F (*D*) treated V5–FZD immunoprecipitates.

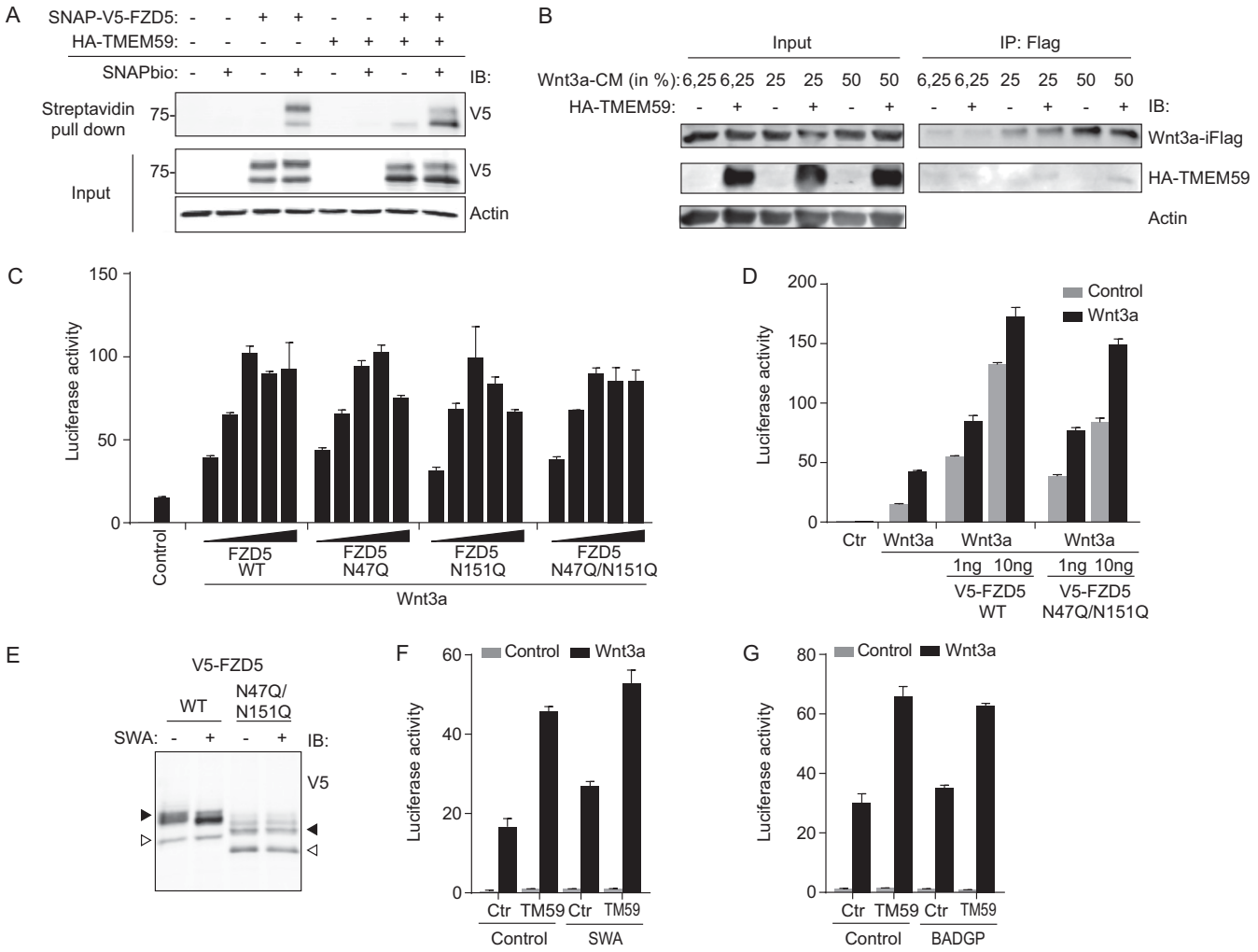

Fig. S4. Glycosylation status of FZD5 does not affect Wnt signaling. (*A*) SNAP–V5–FZD pull-down from HEK293T cells coexpressing HA–TMEM59 or mock plasmid. (*B*) Binding of Wnt3a–iFlag to control cells or cells expressing HA–TMEM59. Cells were transfected with mock plasmid or HA–TMEM59 and stimulated with Wnt3a–iFlag or Wnt3a medium for 3 h. Wnt3a–iFlag bound to cells was immunoprecipitated. (*C*) Wnt luciferase-reporter activity in HEK293T cells expressing increasing amounts of the indicated V5–FZD5 variants. Graph shows average luciferase reporter activities ±SD. (*D*) Wnt luciferase-reporter activity in HEK293T cells coexpressing different amounts of the indicated V5–FZD5 constructs and HA–TMEM59. Graph shows average luciferase reporter activities ±SD. (*E*) Western blot analysis of SWA-treated lysates of HEK293T cells expressing V5–FZD or V5–FZD5 N47Q/N151Q. Cells were treated o/n with 4 μg/mL SWA. Arrow heads indicate the immature (open) and complex glycosylated (solid) forms of each FZD variant. (*F* and *G*) Wnt luciferase-reporter activity in HEK293T cells expressing HA–TMEM59 and treated overnight with SWA (*F*) or BADGP (*G*). Graph shows average luciferase reporter activities ±SD.

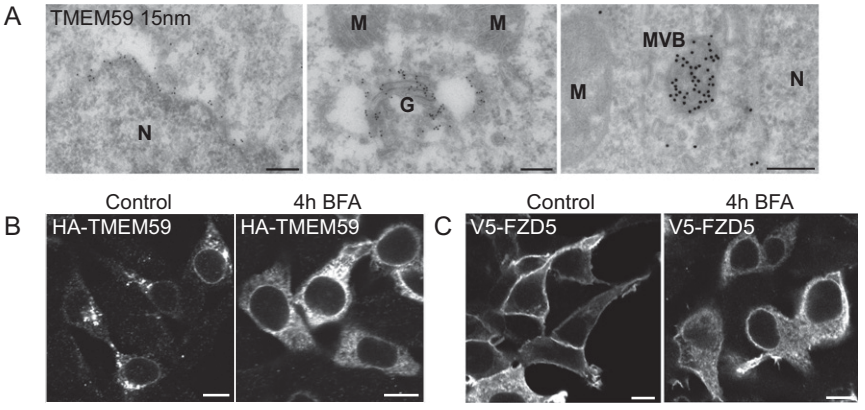

Fig. S5. Subcellular localization of TMEM59 and FZD5. (*A*) HA–TMEM59 localization by EM. Nucleus (N), Golgi (G), mitochondria (M) and multivesicular body (MVB) localization is indicated. (Scale bars, 200 nm.) (*B* and *C*) Confocal microscopy of HEK293T cells expressing HA–TMEM59 (*B*) or V5–FZD5 (*C*). Cells were treated with BFA for 4 h. (Scale bars, 10 μm.)

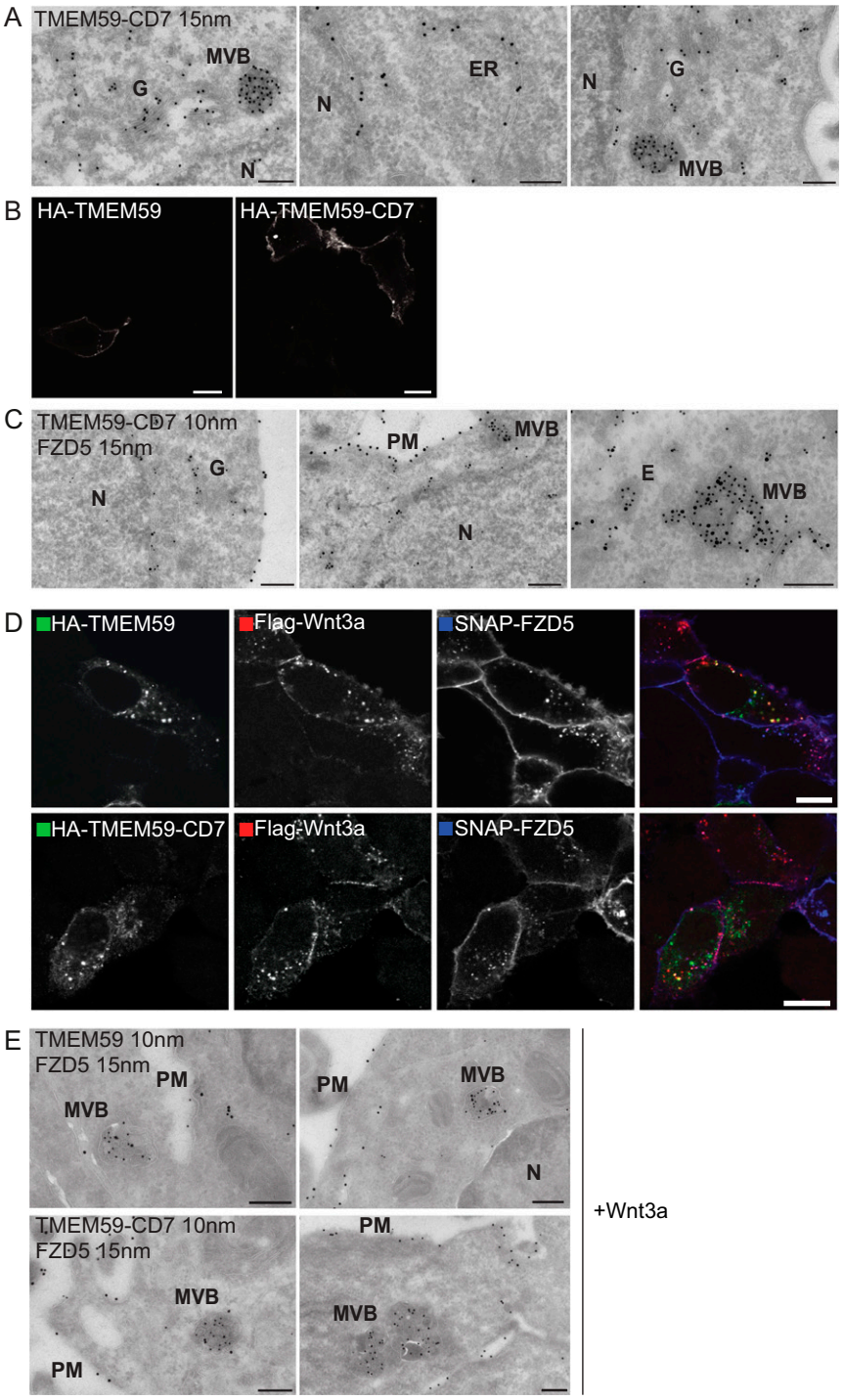

Fig. S6. Subcellular localization of TMEM59–CD7 and FZD5. (*A*) EM images of cells expressing HA–TMEM59–CD7 labeled with 10-nm gold particles. Endosomes (E), Golgi (G), mitochondria (M), and multivesicular bodies (MVB) are indicated. (Scale bars, 200 nm.) (*B*) Confocal microscopy of HEK293T cells expressing HA–TMEM59 or HA–TMEM59–CD7 in unpermeabilized cells. (Scale bars, 10 μm.) (*C*) EM images of cells coexpressing HA–TMEM59–CD7 and V5–FZD5, labeled with 15- and 10-nm gold particles, respectively. Endosomes (E), nucleus (N), PM, mitochondria (M), and multivesicular bodies (MVB) are indicated. (Scale bars, 200 nm.) (*D*) Confocal microscopy of HEK293T cells expressing HA–TMEM59 (*Upper*) or HA–TMEM59–CD7 (*Lower*) and SNAP-FZD5. Cell surface SNAP–FZD5 was labeled with surface $SNAP^{Alexa649}$ and cells were chased for 3 h in the presence of Flag–Wnt3a. (Scale bars, 10 μm.) (*E*) EM images of cells coexpressing HA–TMEM59 (*Upper*) or HA–TMEm59–CD7 (*Lower*) and V5–FZD5, labeled with 15- and 10-nm gold particles, respectively. Nucleus (N), PM, and multivesicular bodies (MVB) are indicated. (Scale bars, 200 nm.)

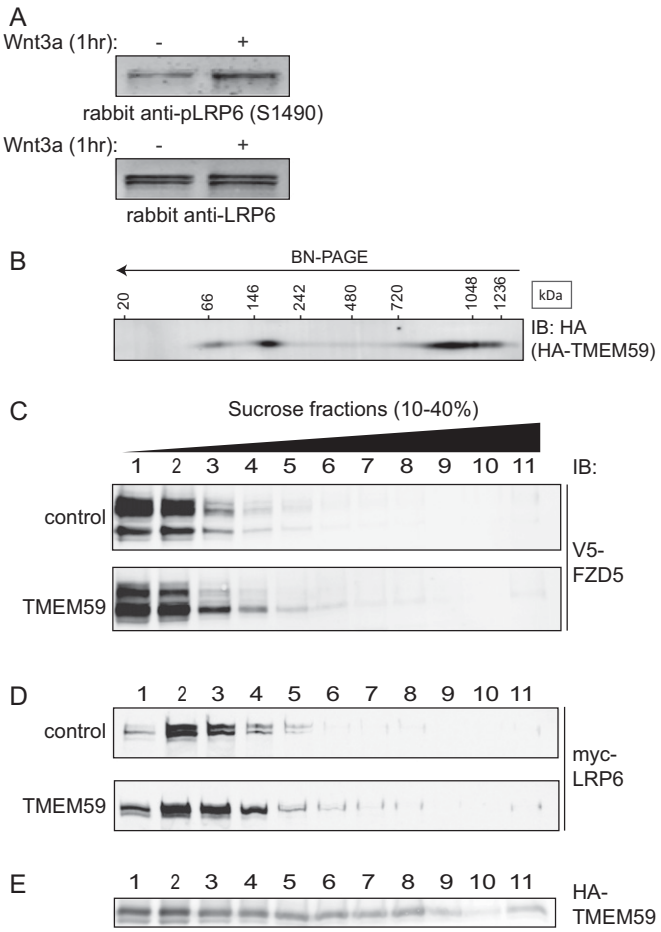

Fig. S7. Wnt receptor analysis in sucrose gradient sedimentation. (*A*) Western blot analysis of total and phospho-LRP6 (S1490) in HEK293T lysates treated with Wnt3a-conditioned medium for 1 h. (*B*) Western blot showing HA–TMEM59 detection corresponding to Fig. 5*E*. (*C*–*E*) Sucrose gradient sedimentation analysis of Triton X-100 solubilized HEK293T cells. Cells were transfected with V5–FZD5, myc–LRP6, and either empty vector or HA–TMEM59 and stimulated for 4 h with Wnt3a–iFlag medium. Lysates were separated on sucrose gradients of 10–40%, and fractions were analyzed by Western blot.

Table S1. Results of the single siRNA screen to identify Wnt pathway regulators

Gene name	Average	SD
Smartpool siRNA screen		
RPN2	2.337368488	1.468024889
OPRS1	2.17201201	0.364105405
DAD1	1.686740775	0.355373955
SEC61A1	1.635094858	0.178183293
GPSN2	1.542464246	0.275494309
NUP210	1.522693663	0.227732157
ITM2B	1.378823408	0.158407275
CANX	1.358721339	1.017811672
LRP5	0.614280514	0.069071578
TMEM161A	0.600062942	0.072301908
SMBP	0.560517047	0.063912758
ATP2A2	0.538866179	0.097269858
TMEM33	0.487169945	0.129598898
TMEM59	0.480706671	0.071317994
HSPC163	0.475629259	0.112644614
FZD8	0.404163829	0.094325995
LRP6	0.075969343	0.035404835
Controls Smartpool siRNA screen		
APC_B1	5.884755877	1.993384221
APC_B2	5.283998969	0.825753679
APC_A2	5.142846798	0.615530813
APC_A1	5.080122439	1.520597287
b-catenin_A2	0.041731469	0.026297221
b-catenin_B2	0.039031718	0.024078737
b-catenin_B1	0.032011218	0.012394444
b-catenin_A1	0.029486911	0.025564608
Controls single siRNA deconvolution		
APC_P1_A	4.127224135	0.628925367
APC_P1_B	3.657764292	0.950024897
APC_P2_A	4.408365405	1.021531432
APC_P2_B	4.424452483	1.021383363
APC_P3_A	3.977787014	1.479400283
APC_P3_B	3.190871308	1.072152495
b-catenin_P1_A	0.005476981	0.001641369
b-catenin_P1_B	0.004349972	0.001608506
b-catenin_P2_A	0.006156614	0.003022435
b-catenin_P2_B	0.004026905	0.001353844
b-catenin_P3_A	0.016841367	0.020818128
b-catenin_P3_B	0.013128122	0.01250512

Wnt luciferase-reporter activity in HEK293T cells transfected with the indicated smartpool siRNAs. Shown are the averages and SD of triplicate measurements.

Table S2. Results of the single siRNA screen to identify Wnt pathway regulators: Single siRNA deconvolution averages

	Average			
Gene name	siRNA1	siRNA2	siRNA3	siRNA4
RPN2	0.53768086	0.722143016	0.540919762	0.509147687
OPRS1	0.579841388	0.410538947	3.151137392	0.477744114
DAD1	0.489641863	0.738383156	0.454801603	0.987589107
SEC61A1	0.777156254	0.931509404	0.52465231	0.615411411
GPSN2	0.738713133	0.665824231	0.716662805	2.144469475
NUP210	0.495901934	0.746921914	0.615606664	0.72450877
ITM2B	0.542281685	0.889657383	0.554717814	0.645009742
CANX	0.468228225	0.330478821	0.596462912	0.680635692
LRP5	0.407572092	0.473788214	0.468645528	0.354653745
TMEM161A	0.503896974	0.768030811	0.975494396	0.736876158
SMBP	0.561523456	0.609488204	0.456636878	0.447900937
ATP2A2	0.359323076	0.341938438	0.456757905	0.287571139
TMEM33	0.48353857	0.444220692	0.522213369	0.549919411
TMEM59	0.490585822	0.541617722	0.373386992	0.507998236
HSPC163	0.509551912	0.286362034	0.514512346	0.456286189
FZD8	0.448673228	0.362249307	0.318612615	0.268006144
LRP6	0.754807354	0.019111884	0.224635784	0.136610841

Wnt luciferase-reporter activity in HEK293T cells transfected with the indicated single siRNAs. Shown are the averages of triplicate measurements.

Table S3. Results of the single siRNA screen to identify Wnt pathway regulators: Single siRNA deconvolution SDs

	SD			
Gene name	siRNA1	siRNA2	siRNA3	siRNA4
RPN2	0.113641971	0.173813823	0.142633139	0.080528103
OPRS1	0.07198508	0.080926042	0.915615073	0.099487587
DAD1	0.2065013	0.250619281	0.109561432	0.316139249
SEC61A1	0.143496822	0.328852549	0.137715248	0.156031267
GPSN2	0.188090963	0.120847469	0.301111635	1.029098147
NUP210	0.101900513	0.145914941	0.319718416	0.161759611
ITM2B	0.142793587	0.469847738	0.12195158	0.215630929
CANX	0.126640609	0.185473297	0.441284667	0.220430964
LRP5	0.082026712	0.290943731	0.281479277	0.084367588
TMEM161A	0.354960183	0.237635873	0.531890901	0.332486348
SMBP	0.101441723	0.11991236	0.240431747	0.099236492
ATP2A2	0.089148224	0.08371503	0.09699205	0.035885502
TMEM33	0.086583311	0.108142407	0.088530629	0.080002345
TMEM59	0.091125619	0.095513677	0.121233869	0.079522411
HSPC163	0.142527374	0.098677408	0.149184834	0.079436145
FZD8	0.095557842	0.049966163	0.100712564	0.057974104
LRP6	0.220547864	0.003211963	0.057344214	0.040428241

Wnt luciferase-reporter activity in HEK293T cells transfected with the indicated single siRNAs. Shown are the SDs of triplicate measurements.

Dataset S1. List of Wnt receptor complex-associated proteins identified by mass spectrometry-based proteomics analysis, leading to the identification of TMEM59 as a member of the Wnt receptor complex

Dataset S1

Cells were either left untreated or transfected with V5–FZD5, myc–LRP6, and MESD, as indicated. Cells were stimulated with Wnt3a or Wnt3a–iFlag for 3 h before lysis and subsequently subjected to Flag-immunoprecipitations. Isolated complexes were analyzed with liquid chromatography-tandem mass spectrometry. The number of identified unique peptides for each protein hit is indicated. Known Wnt pathway modifiers are highlighted in green; TMEM59 is highlighted in orange.