Supporting Information

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SI Materials and Methods

Plasmids and Recombinant Proteins. Plasmids containing full-length ORFs of LGR4 and LGR5 were purchased from Open Biosystems. The ORF encoding a predicted mature form of mouse LGR4 (amino acids 23–951, GenBank accession no. NP_766259) was fused with sequences encoding an HA tag or a Myc tag at the N terminus, and cloned downstream of a sequence encoding the CD8 signal peptide (MALPVTALLLPLALLLHAA) in the vector pIRESpuro3 (Clontech) using standard, PCR-based molecular cloning procedures. The ORFs encoding the predicted mature forms of human LGR5 (amino acids 21–907, GenBank accession no. NP_003658), the predicted extracellular domain (ECD) of human LGR5 (amino acids 21–563), the predicted ECD of mouse LGR4 (amino acids 23–544), human LRP6 (amino acids 19–1,613, GenBank accession no. NP_002327), and LRP6ECD (amino acids 19–1,408) were fused at the N terminus with sequences encoding a Myc tag (LGR5 and LGR5ECD) or an HA tag (LRP6) and cloned into the same vector as LGR4. All clones were verified by DNA sequencing. The plasmid Super 8× TOPFlash was purchased from Addgene. pRL-SV40 (SV40 promoter-controlled renilla luciferase) was purchased from Promega. All recombinant proteins were purchased from R&D Systems.

Cell Culture, Stable Cell Line Generation, and Production of mRSPO1-Fc. HEK293 cells were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin in a 37 °C incubator with 95% humidity and 5% CO_2. Plasmids of Myc-LGR4 and Myc-LGR5 were transfected into HEK293 cells using Fugene 6 (Roche), and bulk stable cells were selected and maintained with puromycin at 1 μg/mL.

For the production of mRSPO1-Fc, HEK293 cells were transiently transfected with the mRSPO1-Fc plasmid or vector alone using Lipofectamine-2000 (Invitrogen) and incubated for 2 d. The cells were then switched to serum-free Opti-MEM media (Invitrogen), and incubated for 3 d. The media were collected and briefly centrifuged to remove cell debris and then concentrated by ~40-fold using Amicon Ultra-15 10K filter devices which have a molecular weight (MW) cutoff of 10 kDa. The concentration of mRSPO1-Fc in the final concentrate was estimated to be ~0.5 μM using the AlphaScreen HA assay kit (Perkin-Elmer), as the fusion protein has an HA tag at the N terminus.

Immunocytofluorescence Confocal Microscopy. The day before experimentation, cells stably expressing control vector, Myc-LGR4 or Myc-LGR5 were seeded in poly-D-lysine–coated eight-well culture slides (Becton Dickinson). Media were removed and the cells were gently rinsed with ice-cold PBS. Concentrated conditioned media (CM) with and without mRSPO1-Fc were diluted by 1:100 in ice-cold Opti-MEM plus 0.5% BSA and then added to the cells in duplicate slides. One slide was incubated for 2 h at 4 °C, whereas the other one was incubated for 45 min at 37 °C. The cells were washed three times with cold PBS, fixed in 4% paraformaldehyde/PBS for 15 min at room temperature, and then washed three times with PBS with gentle agitation. The cells incubated at 37 °C were permeabilized with 0.1% saponin/PBS followed by three additional washings with PBS. The cells were then costained with Alexa Fluor 488-labeled goat antimouse IgG2a (Invitrogen) at 1:100 dilution in Opti-MEM plus 0.5% BSA) plus Cy3-labeled anti-Myc (Sigma) for 1 h at room temperature. Anti-tag antibodies were used at 1:200 dilution. The cells were then washed three times with PBS. Nuclei were counterstained with TO-PRO-3 iodide (Invitrogen) at 1 μM for 15 min at room temperature. Images were recorded and analyzed using confocal laser scanning microscopy (Leica; TCS SP5 microscope) with LAS AF Lite software.

FACS Analysis. HEK293 cells stably expressing Myc-LGR5, HA-LRP6, or vector were detached with enzyme-free cell dissociation buffer and resuspended in binding buffer (PBS + 0.5% BSA). After incubating with mRSPO1-Fc (diluted 1:100) in binding buffer for 2 h at 4 °C, the cells were washed three times with cold binding buffer by centrifugation, fixed in 4% paraformaldehyde/PBS for 15 min at room temperature, and then washed three times with binding buffer. The cells were then incubated with FITC-labeled goat antimouse IgG (Santa Cruz Biotechnology, 1:200 dilution) in binding buffer for 1 h at room temperature, and washed three times with PBS before being analyzed on a guava EasyCyte 8HT flow cytometry system (Millipore/Guava Technologies). For receptor expression verification, the cells were stained with Alexa Fluor 647-labeled anti-HA (Cell Signaling; diluted 1:50) for LRP6 and vector cells and Alexa Fluor 488-labeled anti-Myc (Cell Signaling; diluted 1:50) for Myc-LGR5 and vector cells. A total of 5,000 events were collected for each analysis.

Coprecipitation Analysis. CM from HEK293 cells transiently transfected with mRSPO1-Fc and CM from HEK293 cells stably expressing Myc-LGR5ECD or HA-LGR4ECD were precleared with washed protein A/G plus-agarose beads (Santa Cruz Biotechnology) for 1 h at 4 °C (40 μL of solid beads per milliliter of medium, plus protease inhibitor and 1 mM PMSF). The precleared CM containing mRSPO1-Fc or control mouse IgG was mixed with precleared CM containing Myc-LGR5ECD or HA-LGR4ECD and incubated overnight at 4 °C. Fresh A/G-agarose beads were added, and the mixtures were incubated for 2 h at 4 °C and then washed two times in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM DTT, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). The samples were then boiled for 3 min in 1× Laemmli buffer, resolved by SDS/PAGE, and analyzed using anti-Myc or anti-HA, and antimouse IgG antibodies by standard immunoblotting procedures.

Whole-Cell Competition Binding. HEK293 cells stably expressing Myc-LGR4 or Myc-LGR5 were seeded into poly-D-lysine–coated 96-well black/clear bottom plates (Becton Dickinson) at ~60,000 cells/well. After overnight culturing, the plates were chilled on ice for 5 min, media were removed from each well, and the cells were washed two times with cold PBS. The recombinant RSPOs under investigation were diluted in threefold serial dilutions in cold Opti-MEM + 0.5% BSA and added onto the cells at 50 μL/well, followed immediately by the addition of equal volume of mRSPO1-Fc diluted by 1:100 in the same buffer. The cells were incubated for 3.5 h at 4 °C with gentle agitation, followed by three quick washes with cold PBS. The cells were then fixed in 4% paraformaldehyde/PBS for 15 min at room temperature and washed three times with PBS. The cells were further incubated with Alexa Fluor 647-labeled goat antimouse IgG (H+L) (Invitrogen, diluted by 1:200) in Opti-MEM + 0.5% BSA for 1 h at room temperature. The cells were washed three times with PBS and fluorescence intensity was measured using a Tecan M1000 plate reader with excitation at 630 nm and emission at 670 nm. All experiments were performed at least twice with quadruplicate replicates in each experiment. Data were analyzed using the software GraphPad Prism 5.

β-Catenin Reporter Luciferase Assays. HEK293T cells were cultured in six-well plates, and transient transfections were performed using Fugene HD (Roche) according to the manufacturer's suggested protocol. For transfection, 1 μg of the Super 8× TOPFlash reporter and 100 ng of pRL-SV40 plasmid were used. LGR4 and LGR5 constructs and vector control were transfected at 1 μg/well. LRP6 wild-type and mutant constructs and DKK1 were transfected at 500 ng/well. The total amount of vector transfected per well was normalized using the control vector pIRESpuro3. Twenty-four hours posttransfection, the cells were detached with trypsin and seeded into 384-well plates in Opti-MEM containing 1% FCS. Control CM and Wnt3a CM were prepared by culturing L cells and Wnt3a-L cells, respectively, according to the American Type Culture Collection protocol. Recombinant human RSPO1-4 (R&D Systems) were diluted in control CM or Wnt3a CM in threefold serial dilutions and added to the cells at 6 h postseeding. The final dilution of control CM or Wnt3a CM was 1:5. For experiments performed using recombinant human Wnt3a (R&D Systems), the cells were seeded and the proteins were diluted in DMEM containing 10% FCS. After the cells were stimulated overnight, luciferase assay measurements were carried out using the Dual-Glo luciferase assay kit (Promega) according to the manufacturer's protocol and the plates were read on a PerkinElmer EnVision plate reader. All experiments were performed at least twice with quadruplicate replicates in each experiment and the data were analyzed using GraphPad Prism 5.

For Gα(i/o)-coupling experiments, receptor-transfected HEK293T cells were pretreated with 500 ng/mL of pertussis toxin form *Bordetella pertussis* (Sigma) for 3 h before the addition of RSPO1, and luciferase assays were performed as described above.

The siRNA used in this study were the human LGR4 and LGR5 ON-Targetplus SMARTpool, and Nontargeting pool as a negative control (Dharmacon). HEK293T cells were first transfected with the siRNA in Dharmafect 1 (Dharmacon) and then transfected with Super 8× TOPFlash and pRL-SV40 24 h later. After overnight incubation, the cells were seeded into 384-well plates and stimulated with RSPO1-3 with Wnt3a CM. Luciferase activities were then measured as described above.

Quantitative RT-PCR. Isolation of total RNA from HEK293T and HEK293 cells was performed by lysing the cells with TRIzol (Invitrogen), followed by the successive addition of chloroform and isopropyl alcohol for phase separation and RNA precipitation, respectively. For further purification, the samples were run through RNeasy Mini Kit columns (Qiagen) according to the manufacturer's protocol. RNA was eluted with RNase-free, DEPC-treated water and then treated with DNase. Quantitative RT-PCR of LGR4 and LGR5 and RSPO1–4 was performed by the Quantitative Genomics Core Lab (University of Texas Health Science Center, Houston, TX). Briefly, a total of 100 ng RNA was run in triplicate per assay (along with no-template and nonamplifying controls) using the Taqman primer/probe combinations listed in Table S2. Quantified expression levels of LGR4 and LGR5 and RSPO1–4 were determined from an ssDNA standard curve and expression was normalized to levels of 18S rRNA.

Immunoblot Analysis. For the siRNA knockdown experiment, HEK293T cells were transfected with control siRNA, LGR4-siRNA, LGR5-siRNA, or both LGR4-siRNA and LGR5-siRNA. The next day, the cells were detached and seeded into six-well plates. After overnight culturing in 1% FCS, the cells were treated with RSPO1 or Wnt3a or both for 3 h. The cells were collected and then lysed with RIPA lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM DTT, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors. For the comparison of cells stably expressing vector or LGR5, the cells were seeded into six-well plates and cultured overnight in DMEM + 10% FCS. They were then changed to DMEM with 1% FCS and incubated overnight and treated with Wnt3a CM (1:5) or control CM, with and without RSPO1 at the indicated concentrations for the indicated periods of time.

Phospho-LRP6 was probed with a phospho-Ser1490–specific antibody (Cell Signaling; 2568) and total LRP6 were probed with an anti-LRP6 polyclonal antibody (Cell Signaling; 3395). β-Actin were also probed as protein-loading control. Immunoblotting of cytosolic (nonmembrane bound) β-catenin were carried out using cell lysates that were treated with ConA-sepharose beads overnight followed by centrifugation to remove cadherin-bound β-catenin and probed with the anti–β-catenin antibody that detects total β-catenin (Cell Signaling; 9562). All immunoblotting procedures were carried out using HRP-conjugated secondary antibodies by following the manufacturer's suggested protocols.

GPCR Assays. For cAMP assays, HEK293T cells were transiently transfected with vector, HA-LGR4 and Myc-LGR5 using Fugene 6. The next day, the cells were harvested and cAMP responses were measured with the AlphaScreen cAMP assay kit (PerkinElmer) according to the manufacturer's suggested protocol. RSPO1 and RSPO2 were diluted in 3× serial dilutions in the stimulation buffer. Forskolin was used as a positive control for cAMP production. For Ca^{2+} mobilization assays, HEK293T cells were transfected as above and then seeded into poly-D-lysine–coated black/clear bottom 96-well plates the next day. After overnight culturing, the cells were loaded with Calcium 5 (Molecular Devices) according to the manufacturer's protocol and incubated at 37 °C for 1 h in a CO_2 incubator. RSPO1 and ATP were prepared in Hanks-balanced salt solutions (HBSS) + 0.5% BSA. Fluorescence intensity was measured every 3 s for 6 min using a Tecan M1000 plate reader with excitation at 485 nm and emission at 525 nm. RSPO1 and ATP were injected at 30 s and 3 min, respectively, after the initiation of fluorescence reading. In all cases, receptor expression was verified by immunoblotting analysis.

For the β-arrestin translocation assay, HEK293T cells were transiently transfected with β-arrestin2–GFP plus vector, Myc-LGR4, Myc-LGR5, the β2-adrenergic receptor (β2-ADR) as above, and then seeded into poly-D-lysine–coated eight-well slides (Becton Dickinson). After overnight culturing, the cells were washed once with Opti-MEM and incubated with mRSPO1-Fc diluted by 1:100 in Opti-MEM + 0.2% BSA or buffer alone for 45 min at room temperature. The cells were then washed three times with PBS, fixed for 10 min at room temperature in 4% paraformaldehyde/PBS, washed two times with PBS, and permeabilized with 0.1% saponin in PBS for 10 min. The cells were washed once with PBS and stained with Alexa Fluor 647-labeled antimouse IgG2a plus Cy3–anti-MyC (LGR4 and LGR5 cells) for 1 h at room temperature. The cells were washed three times with PBS and mounted for confocal microscopy.

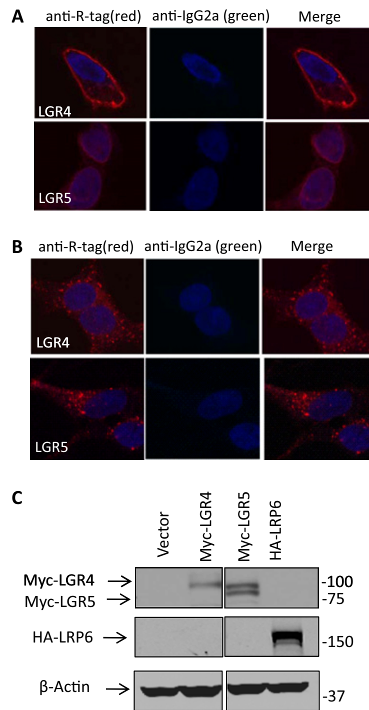

Fig. S1. Immunofluorescence staining of cells without RSPO1-Fc. The binding reactions were carried out side by side with those of Fig. 1, except control CM without mRSPO1-Fc was added to the cells. (*A*) Binding at 4 °C. (*B*) Binding at 37 °C. Receptors were stained with Cy3-labeled anti-Myc (red, LGR4 and LGR5). Alexa Fluor 488-labeled antimouse IgG2a (green) were used to stain mRSPO1-Fc. Nuclei (blue) were counterstained with To-PRO-3. (*C*) Immunblot analysis of the cell lines stably expressing Myc-LGR4, Myc-LGR5, or HA-LRP6. Total cell lysates were probed with anti-Myc (LGR4 and LGR5 cells) and anti-HA (LRP6 cells) antibodies. β-Actin was also probed as loading control.

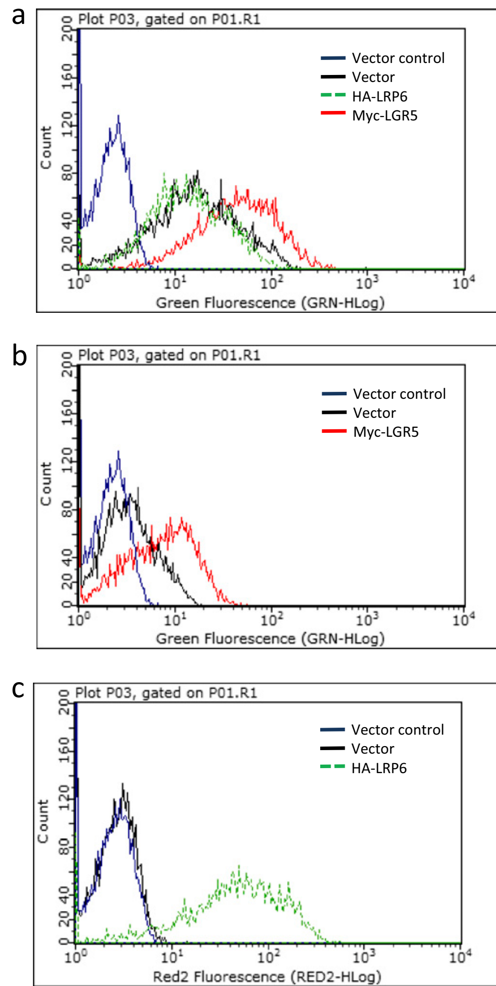

Fig. S2. FACS analysis of mRSPO1-Fc binding and receptor expression. (*A*) Binding of mRSPO1-Fc to Myc-LGR5 cells, but not to vector or HA-LRP6 cells. Vector, Myc-LGR5, or HA-LRP6 cells were incubated with CM containing mRSPO1-Fc, stained with FITC-labeled antimouse IgG, and then sorted for green fluorescence. (*B*) Confirmation of cell surface expression of Myc-LGR5. The cells were stained with Alexa Fluor 488-labeled anti-Myc antibody and then sorted for green fluorescence. (*C*) Confirmation of cell surface expression of HA-LRP6. Cells were stained with Alexa Fluor 647-labeled anti-HA antibody and then sorted for red fluorescence. In all cases, "vector control" represents cells that were not incubated with antibody.

Fig. S3. Quantitative RT-PCR analysis of the constitutive expression levels of RSPO1–4 in HEK293 and HEK293T cells, the effect of LGR4 and LGR5 expression knockdown on RSPO3 response in HEK293T cells, and the effect of LRP6, LRP6ECD, and DKK1 on LGR5-mediated potentitiation. (*A*) Expression level of RSPO1–4 in HEK293 and HEK293T cells normalized to that of 18S RNA. (*B*) Effect of LGR4 and LGR5 expression knockdown on endogenous responses of HEK293T cells to RSPO3 in the β-catenin reporter enzyme. The experiment was carried out along with the one in Fig. 4*B*. (*C*) Effect of LRP6 and LRP6ECD on RSPO1 response in vector and LGR5 cells. (*D*) Effect of DKK1 on RSPO1 response in vector and LGR5 cells.

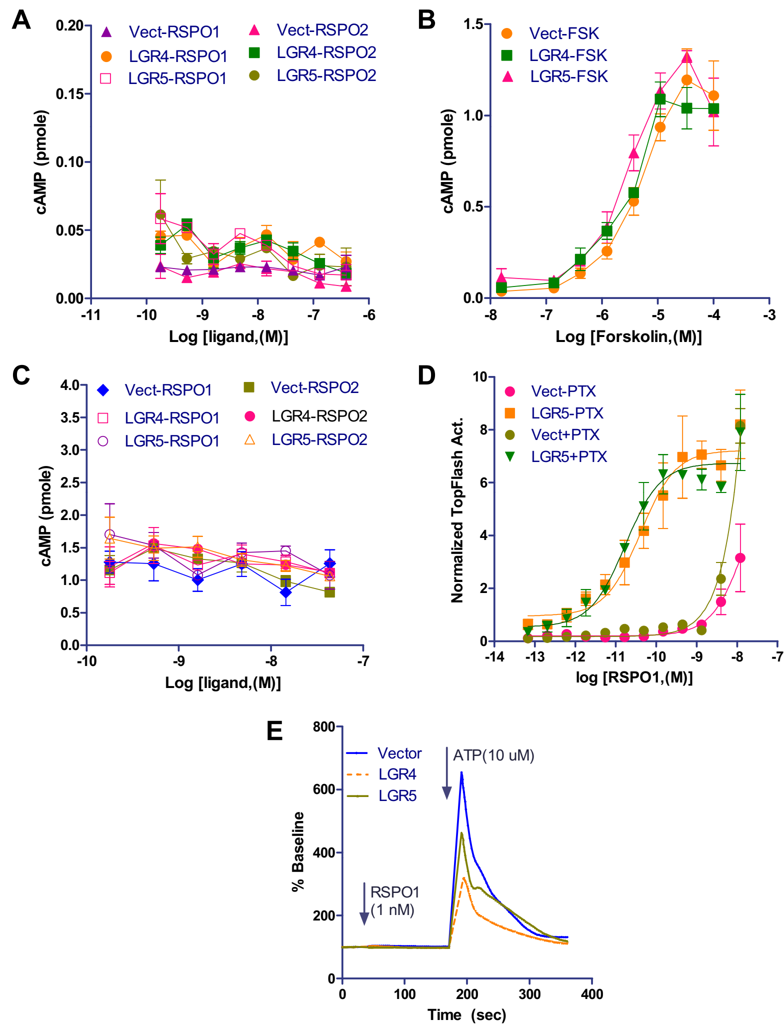

Fig. S4. G protein-coupling assays of LGR4 and LGR5 in response to RSPO1–2. (*A*) cAMP production in vector, LGR4, and LGR5 cells treated with RSPO1 and RSPO2. No cAMP response was observed. (*B*) As positive control, forksolin showed a strong stimulation of cAMP production in these cells. (*C*) RSPO treatments had no effect on forskolin-stimulated cAMP production in vector, LGR4, and LGR5 cells. (*D*) Pertussis toxin (PTX) had no effect on RSPO1-LGR5–mediated Wnt signaling potentiation. (*E*) No Ca^{2+} moblizatoin was induced in vector, LGR4, and LGR5 cells in response to RSPO1, whereas ATP gave a robust response in all cells.

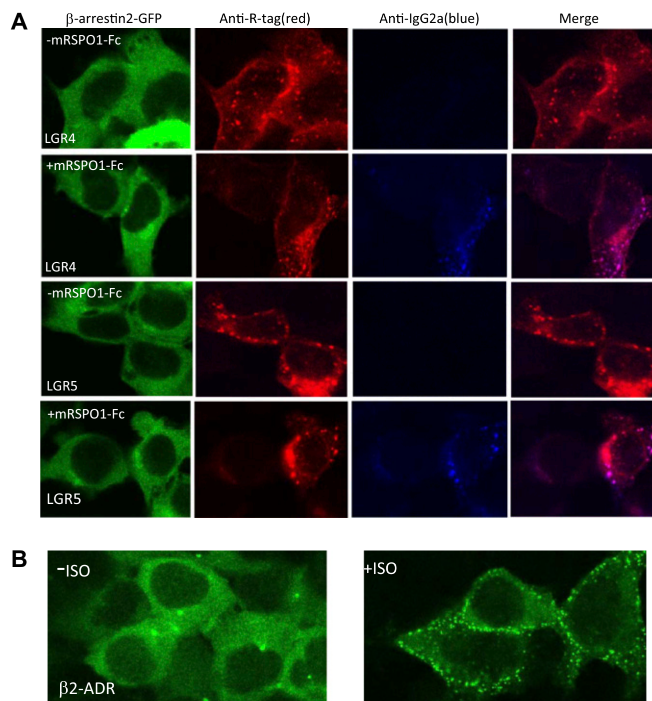

Fig. S5. β-Arrestin translocation assay of LGR4 and LGR5 in response to mRSPO1-Fc. (*A*) HEK293T cells were cotransfected with β-arrestin2–GFP plus Myc-LGR4 or Myc-LGR5, and then stimulated with mRSPO1-Fc (~5 nM) or control CM for 45 min at room temperature. The cells were then fixed, permeabilized, and stained with Alexa Fluor 647-labeled antimouse IgG2a plus Cy3–anti-Myc, and viewed by confocal microscopy. No translocation of β-arrestin was observed in LGR4 and LGR5 cells treated with mRSPO1-Fc, whereas colocalization of mRSPO1-Fc with each receptor was confirmed. (*B*) As positive control, HEK293T cells were transfected with β2-adrenergic receptor (β2-ADR) and stimulated with its agonist isoproterenol (1 μM). Translocation of β-arrestin was clearly visible.

Table S1. List of candidate ligands tested on LGR4 and LGR5 in GPCR assays

Candidate	Known function	Rationale
BMP7	BMP receptor ligand.	KO phenotype in the kidney is strikingly similar to that of LGR4.
BMPER	BMP receptor antagonist as well as agonist. Binds to BMP 2 and BMP7.	KO phenotype in the kidney is also similar to that of LGR4.
DAND5	BMP antagonist.	Member of the DAN/bursicon family.
Grem1	BMP antagonist. Binds to BMP2, BMP4, etc.	Expressed in stromal cells of the crypts in the intestine; homologous to the bursicons, which are ligands of *Drosophila* LGR2; KO is embryonic lethal.
Grem2	BMP antagonist. Binds to BMP2, BMP4, etc.	Closely related to Grem1, also expressed in the stromal cells surrounding the crypts in the intestine.
Norrin	Binds to FZD4 and activates the Wnt/β-catenin pathway.	KO phenotype in the eye is similar to that of LGR4. Also a member of the bursicon family.
R-spondins	Wnt coligand and potentiate Wnt/β-catenin signaling activity. Stimulates the growth of intestinal stem cells.	Strongly stimulates the growth of LGR5-positive intestinal stem cells. No receptor was clearly identified.
TDGF1	Nodal coreceptor; independently functions as ligand. Binds to Nodal, GRP78, TMEFF1, glypican-1, activin.	Stem cell marker, GPI-anchored protein; coexpressed with LGR4 and LGR5 in stem cells; extracellular domain functions as ligand with unknown receptor; KO are embryonic lethal.

Table S2. List of primers used for qPCR analysis of LGR4, LGR5, and RSPO1–4

Gene nomenclature	GenBank accession no.	Amplicon length	Amplicon location	Forward primer sequence	Reverse primer sequence	Probe sequence	PCR efficiency, %
LGR4	NM_003667	80	Exon 18	2,559(+) CTTTGTTTGCCATTTCCTA	2,634(–) CTAGTGAGTTTAATAGCACTAA	2,586(+) FAM-ACGCCATCATTAGGATTCACTGTAAC-BHQ1	99
LGR5	NM_018490	86	Exon 11/12 boundary	1,066(+) ATCTCATCTCTTCCTCAAA	1,144(–) CTTCTAATAGGTTGTAAGACA	1,092(+) FAM-CAATCAGTTACCTAATCTCCAAGTGCT-BHQ1	95
RSPO1	NM_001038633	81	Exon 3/4 boundary	487(+) TACTCAGTATTAAGGTTGG	562(–) CCTCGGAATATCATATGAG	515(+) FAM-TAGTCCCTGCTGACGTGAC-BHQ1	99
RSPO2	NM_178565	72	Exon 4/5 boundary	1,076(+) GAATGTGTGGAAGGATG	1,143(–) GTGCGATTATTTCTGCTA	1,115(+) FAM-ATTCGCTCCAATGACCAACTTC-BHQ1	93
RSPO3	NM_032784	87	Exon 5	1,007(+) ATCCAGCAAAGAAATCC	1,090(–) GATACCGATTTCTGTTTATC	1,061(–) FAM-TCGCTTCTTCTGCTGCTGT-BHQ1	94
RSPO4	NM_001029871	77	Exon 5	895(+) CTCTGTCTTCTCCATTTG	966(–) AAAGGGAAGGTAGACTG	915(+) FAM-CTCTCTTTCTTTCCACCCTTCTATCA-BHQ1	98