Supplementary methods

Cell culture, infection, transfection, and RNA interference

HEK293 cells and its derivatives were grown in DMEM supplemented with 10% FBS. Various constructs were introduced into HEK293 cells through retroviral or lentiviral infection using standard protocols. Plasmid or siRNA transfection was done using Fugene 6 (Roche) or Dharmafect 1 (Dharmacon), respectively.

Surface plasmon resonance assay

Binding affinity between RSPO1 and ZNRF3-ECD recombinant proteins with at least 95% purity was measured on a ProteOn SPR36 protein interaction array system using a GLC sensor chip. Briefly, purified ZNRF3 ECD-Fc was immobilized on the chip surface using standard amine coupling at an optimal density. WT or mutant His-tagged RSPO1 in three-fold serial dilutions was injected over the chip at the same time under constant flow rate. Association and dissociation of protein complex was monitored for 200 s and 480 s, respectively. All data were analyzed using the ProteOn Manager v. 2.0. Double referencing was performed against a flow cell immobilized with unrelated protein (FGF21) control and to the simultaneous buffer blank. For each interacting pair, data from the two independent injections were combined and fit simultaneously, with ka and kd fit globally and Rmax fit locally. To measure interaction between SOMAmers and RSPO1, biotin-labeled SOMAmers were immobilized on the Neutravidin-coated sensor chip. Fc-tagged RSPO1 was injected over the chip. Association/dissociation of SOMAmer/R-spondin was monitored and affinity calculated as mentioned above.

Conditioned media generation and measurement

Construct of WT or mutant RSPO1-GFP was transiently transfected to HEK293 cells. Growth media was changed the second day and supernatant was collected 72 hrs after initial transfection. Concentration of RSPO1-GFP in the solution was measured of fluorescence intensity according to a GFP protein standard (Vector Laboratories) by EnVision Multilabel reader (PerkinElmer).

Pulldown between SOMAmers and R-spondin

Freshly denatured biotin-labeled SOMAmer was mixed with conditioned media containing WT or mutant RSPO1-GFP at the presence of dextran sulfate and protease inhibitors. After two hours incubation at 4°C, prewashed Neutravidin beads (Pierce) were added to the mixture and incubated with rotation overnight at 4°C. Beads were washed three times with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA). The bound proteins were eluted in SDS sample buffer and resolved by SDS PAGE for immunoblotting analysis.

Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitation were done as described previously {Hao, 2012 256 /id}. The sources of primary antibodies are: anti-Myc tag, anti-GFP (Cell Signaling Technology); anti-HA (Roche).

Cell surface protein isolation

Cell surface proteins were isolated by whole cell biotinylation and NeutrAvidin agarose pull-down using Pierce Cell Surface Protein Isolation Kit (#89881) according to manufacturer's instructions.

Flow cytometric analysis

Cells coexpressing LGR4 and Myc-ZNRF3 were treated with wild-type or mutant RSPO1-GFP for twenty four hours. Cell were harvested using Trypsin-free cell dissociation buffer (Invitrogen) and resuspended in FACS buffer (1xPBS with 1% BSA and 0.02% sodium azide). After blocking, cells were incubated with anti-Myc-Alexa fluor 647 (Cell Signaling Technology) for one hour at 4 °C. After extensive washes in FACS buffer, cells were stained with propidium iodide (PI) and subject to multi-channel analysis using BD LSR II flow cytometer. Fluorescence signals from PI negative viable single cells were displayed in histogram plots.

Inhibition of RSPO1/receptor interaction by SOMAmer

GripTite-293 cells (Invitrogen) were transiently transfected with LGR4, ZNRF3 ECD-TM, or empty vector 48 hrs before the binding analysis. 2ug/mL RSPO1-Fc protein was incubated with 50 nM specific SOMAmer, or its scrambled control, in fresh DMEM with 10% FBS for 30 min

at RT. RSPO1/SOMAmer mixture was added to the culture media with transfected cells, and incubated at 37°C for 1 hr. Cells were then washed twice with 1xPBS, fixed in 4% PFA, and stained with goat anti-human IgG Alexa Fluor 488 conjugate (Invitrogen). After final wash, bound RSPO1-Fc was detected by fluorescence microscopy.

Statistical analysis

Student's *t* tests were used to determine statistical significance. It is considered significant when *P* is smaller than 0.01.

Legends of Supplementary Figures

Figure S1 Alignment of human RSPO1, RSPO2, RSPO3 and RSPO4

Alignment of furin-like domains of human RSPO1, RSPO2, RSPO3, and RSPO4. The asterisks indicate identical amino acid residues, the dots indicate homologous amino acid residues, the filled triangles indicate two residues critical for ZNRF3 binding in the FU1 domain, and the non-filled triangles indicate two residues critical for LGR4 binding in the FU2 domain.

Figure S2 Interaction between RSPO1 FU1 and FU2 double mutants and LGR4 or ZNRF3 ECD-TM in the cell-based binding assay

HEK293 cells transiently transfected with empty vector (EV) or LGR4, ZNRF3 ECD-TM expression plasmid were incubated with RSPO1-GFP conditioned medium (CM), and binding of RSPO1-GFP was analyzed by fluorescence microscopy.

Figure S3 Interaction between R-spondin recombinant proteins and RSPO1 SOMAmer or scrambled control in the SPR assay

To measure interaction between SOMAmers and RSPO1 or RSPO3, biotin-labeled SOMAmers were immobilized on the Neutravidin-coated sensor chip. Fc-tagged RSPO1 or RSPO3 at indicated concentrations was injected over the chip. Association/dissociation of SOMAmer/R-spondin was monitored and affinity calculated.

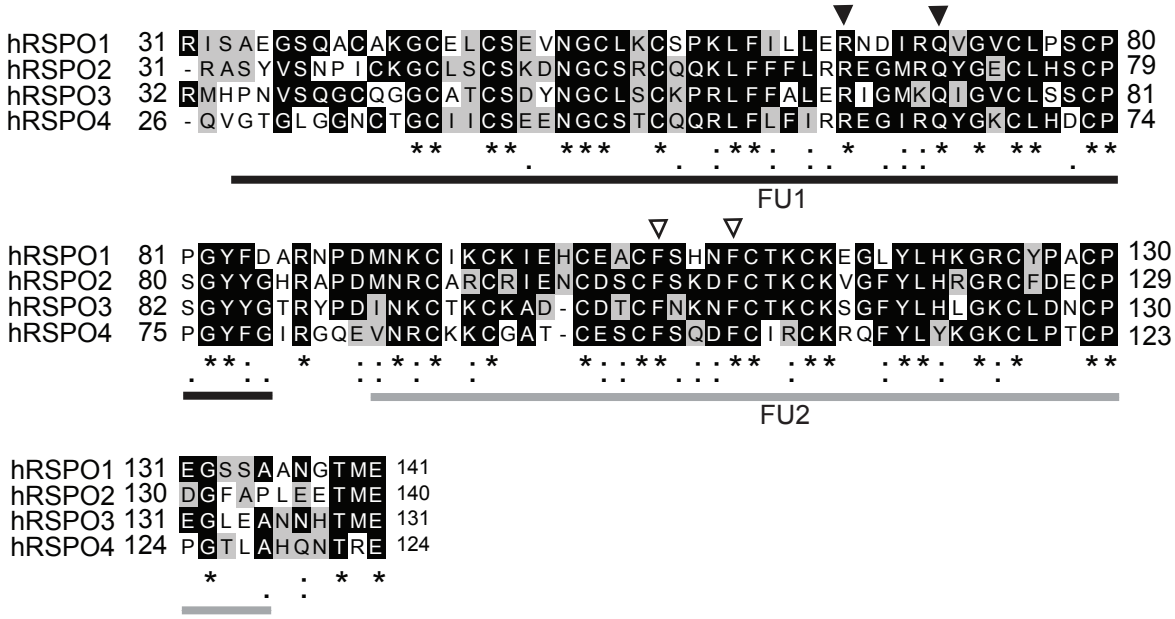

hRSPO1 31 RISAEGSQACAKGCELCSEVNGCLKCSPKLFILLERNDIRQVGVCLPSCP 80
hRSPO2 31 -RASYVSNPICKGCLSCSKDNGCSRCQQKLFFFLRREGMRQYGECLHSCP 79
hRSPO3 32 RMHPNVSQGCQGGCATCSDYNGCLSCKPRLFFALERIGMKQIGVCLSSCP 81
hRSPO4 26 -QVGTGLGGNCTGCIICSEENGCSTCQQRLFLFIRREGIRQYGKCLHDCP 74
FU1
hRSPO1 81 PGYFDARNPDMNKCIKCKIEHCEACFSHNFCTKCKEGLYLHKGRCYPACP 130
hRSPO2 80 SGYYGHRAPDMNRCARCRIENCDSCFSKDFCTKCKVGFYLHRGRCFDECP 129
hRSPO3 82 SGYYGTRYPDINKCTKCKADCDTCFNKNFCTKCKSGFYLHLGKCLDNCP 130
hRSPO4 75 PGYFGIRGQEVNRCKKCGATCESCFSQDFCIRCKRQFYLYKGKCLPTCP 123
FU2
hRSPO1 131 EGSSAANGTME 141
hRSPO2 130 DGFAPLEETME 140
hRSPO3 131 EGLEANNHTME 131
hRSPO4 124 PGTLAHQNTRE 124

Figure S1.

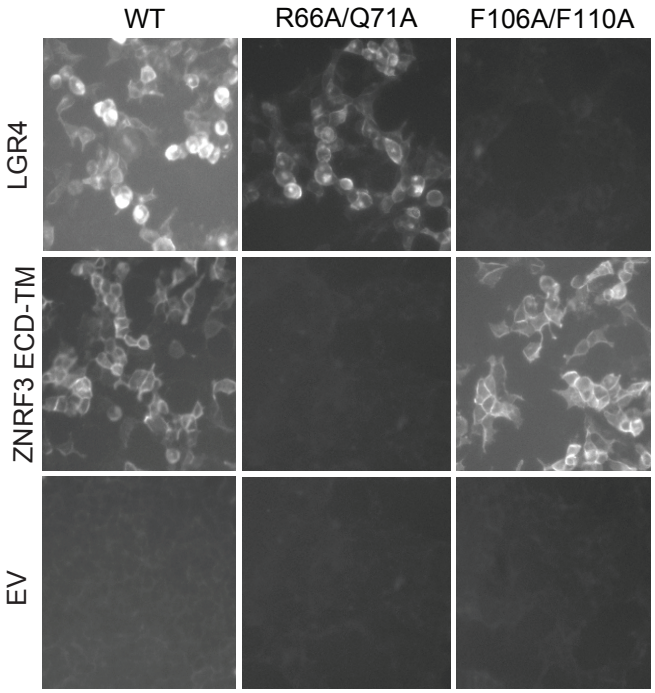
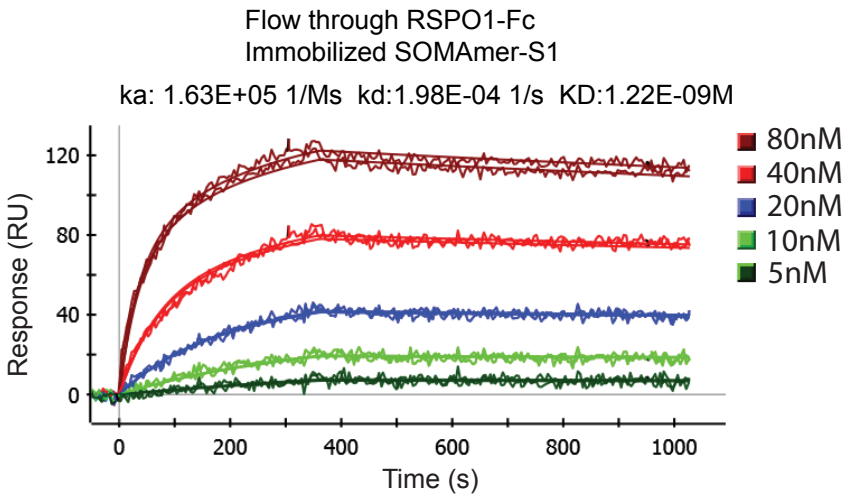
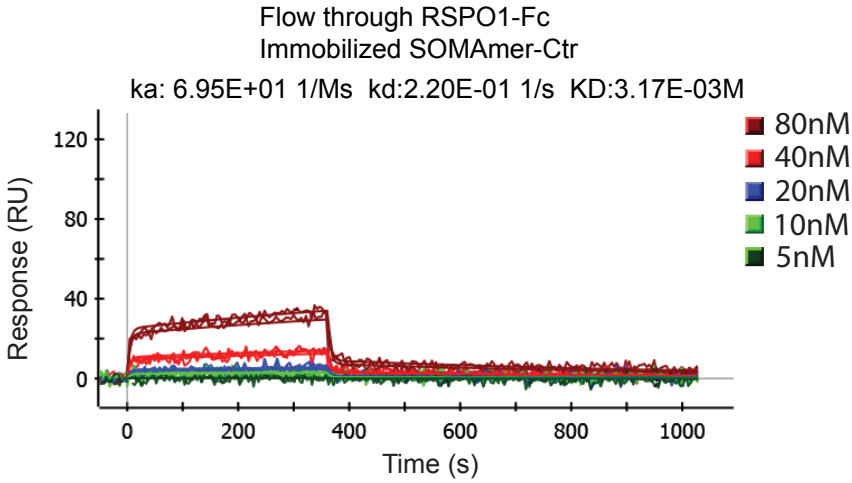


Figure S2.


Flow through RSPO1-Fc
Immobilized SOMAmer-S1
ka: 1.63E+05 1/Ms kd:1.98E-04 1/s KD:1.22E-09M
Response (RU)
120
80
40
0
0
200
400
600
800
1000
Time (s)
80nM
40nM
20nM
10nM
5nM


Flow through RSPO1-Fc
Immobilized SOMAmer-Ctr
ka: 6.95E+01 1/Ms kd:2.20E-01 1/s KD:3.17E-03M
Response (RU)
120
80
40
0
0
200
400
600
800
1000
Time (s)
80nM
40nM
20nM
10nM
5nM

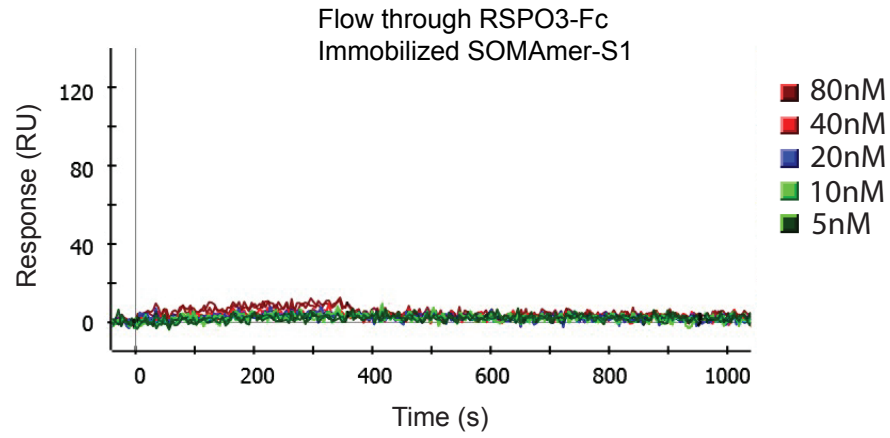

Flow through RSPO3-Fc
Immobilized SOMAmer-S1
Response (RU)
120
80
40
0
0
200
400
600
800
1000
Time (s)
80nM
40nM
20nM
10nM
5nM

Figure S3.

Supplementary Table

Binding of RSPO1-GFP mutants to LGR4 and ZNRF3 ECD-TM in cell-based binding assay

RSPO1-GFP	LGR4	ZNRF3 ECD-TM
WT	++	++
G43A	++	+
S48A	++	+
N51A	++	++
G52A	++	+
L60A	++	++
F61A	++	+
R66A	++	-
Q71A	++	-
G73A	++	++
L76A	++	++
P80A	-	-
G82A	++	++
Y83A	-	-
R87A	++	++
N92A	++	++
F106A	-	++
F110A	-	++
Y119A	++	++
L120A	++	++
G123A	++	++
G132A	++	++
T139A	++	++
E141A	++	++