Structure of Stem Cell Growth Factor R-spondin 1 in Complex with the Ectodomain of Its Receptor LGR5

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SUMMARY

Leucine-rich repeat-containing G protein-coupled receptors 4–6 (LGR4–LGR6) are receptors for R-spondins, potent Wnt agonists that exert profound trophic effects on Wnt-driven stem cells compartments. We present crystal structures of a signaling-competent fragment of R-spondin 1 (Rspo1) at a resolution of 2.0 Å and its complex with the LGR5 ectodomain at a resolution of 3.2 Å. Ecto-LGR5 binds Rspo1 at its concave leucine-rich-repeat (LRR) surface, forming a dimeric 2:2 complex. Fully conserved residues on LGR4–LGR6 explain promiscuous binding of R-spondins. A phenylalanine clamp formed by Rspo1 Phe106 and Phe110 pinches Ala190 of LGR5 and is critical for binding. Mutations related to congenital anonychia reduce signaling, but not binding of Rspo1 to LGR5. Furthermore, antibody binding to the extended loop of the C-terminal LRR cap of LGR5 activates signaling in a ligand-independent manner. Thus, our data reveal binding of R-spondins to conserved sites on LGR4–LGR6 and, in analogy to FSHR and related receptors, suggest a direct signaling role for LGR4–LGR6 in addition to its formation of Wnt receptor and coreceptor complexes.

INTRODUCTION

Vertebrate genomes encode four secreted R-spondin proteins (Rspo1–Rspo4), each defined by two N-terminal Furin (Fu) domains and a thrombospondin (Tsp) domain. Functionally, R-spondin proteins act as potent enhancers of Wnt signals (Kazanskaya et al., 2004). Indeed, Rspo1 strongly promotes proliferation of the Wnt-dependent intestinal-crypt stem cell compartment in vivo (Kim et al., 2005) and in vitro (Sato et al., 2009). This activity can be attributed to the two Fu domains, because a Fu1-Fu2 fragment of Rspo1 retained full signaling activity (Kim et al., 2008; Li et al., 2009).

R-spondin mutations have been found in two hereditary syndromes in humans. Rspo1 is mutated in a recessive syndrome characterized by XX sex reversal, palmoplantar hyperkeratosis, and squamous cell carcinomas (Schuijers and Clevers, 2012). Mutations in the Rspo4 gene result in congenital anonychia, a severe hypoplasia of fingernails and toenails (Blaydon et al., 2006; Brüchle et al., 2008; Wasif and Ahmad, 2013).

The Wnt target gene leucine-rich repeat-containing G protein-coupled receptor 5 (*Lgr5*) encodes a serpentine receptor that is exquisitely specific to Wnt-dependent stem cells of a series of adult tissues, including small intestine and colon (Barker et al., 2007), stomach (Barker et al., 2010), hair follicle (Jaks et al., 2008), liver (Huch et al., 2013), kidney (Barker et al., 2012), and mammary gland (Plaks et al., 2013). The LGRs form a small family of seven-transmembrane (7TM) receptors that include the follicle-stimulating hormone, luteinizing hormone, and thyroid-stimulating hormone receptors (FSHR, LHR, and TSHR, also referred to as LGR1–LGR3, respectively) (Hsu et al., 1998). LGR5 (as well as its homologs LGR4 and LGR6) binds R-spondins with high affinity, thus mediating R-spondin input into the canonical Wnt pathway (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011). Indeed, LGR4 and LGR5 proteins physically reside within Frizzled/LRP receptor complexes (de Lau et al., 2011). Whereas deletion of the *Lgr5* gene in the intestine has little effect, mutation of *Lgr4* (which is expressed by all crypt cells) severely decreases crypt proliferation (Mustata et al., 2011). Double *Lgr4* and *Lgr5* knockout completely abolishes proliferation (de Lau et al., 2011), implying that R-spondins are major drivers of Wnt-dependent crypt self-renewal.

RESULTS AND DISCUSSION

Structures of Free and Bound Rspo1

We sought to address the crystal structure of the Fu1-Fu2 fragment of Rspo1 and its complex with the ligand-binding ectodomain of LGR5. A crystal structure of Rspo1-Fu1Fu2 (residues 31–145) was derived at a resolution of 2.0 Å (Figure 1A). Fu domains are rich in cysteine-knotted β-hairpins. In contrast to earlier mass-spectrometry analysis (Li et al., 2009),
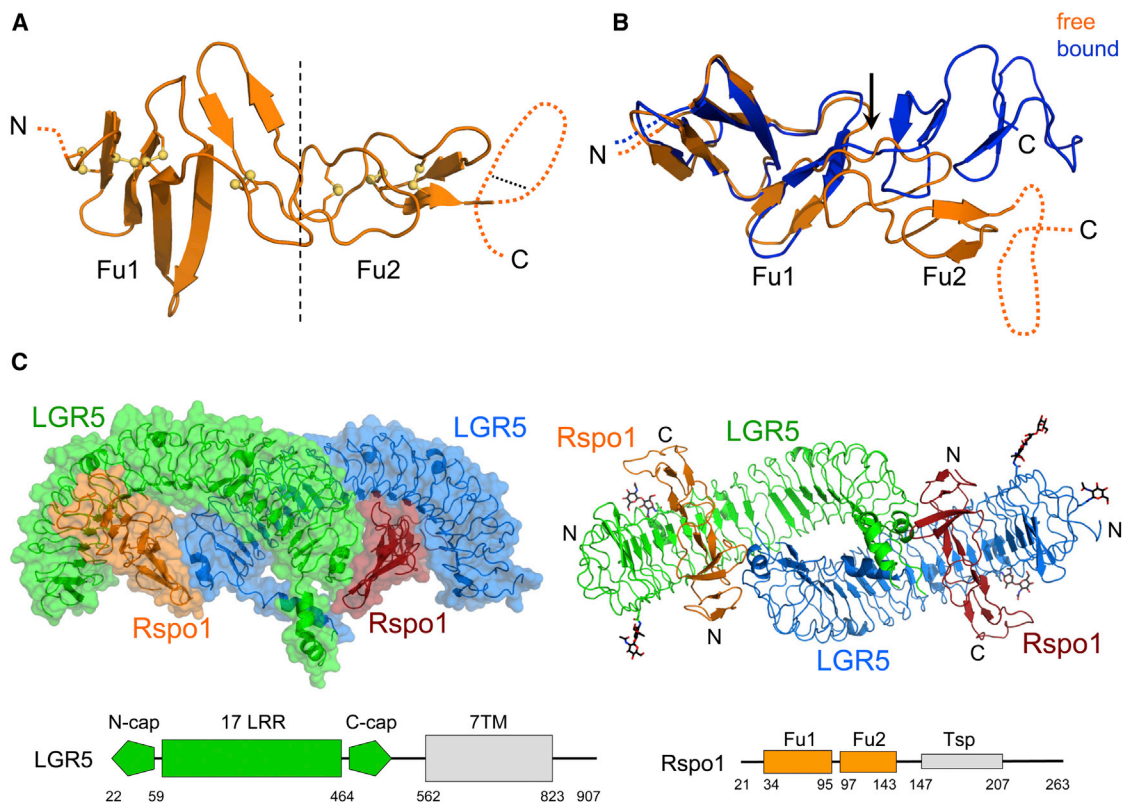
Figure 1. Structure of Rspo1 and the Complex of Rspo1 with Ecto-LGR5

(A) Structure of (unbound) Rspo1-Fu1Fu2 (residues 31–145) at 2 Å resolution. Indicated are disulphide bonds (ball and stick) and disordered residues (dashed line).

(B) Overlay of bound and unbound Rspo1. The arrow indicates a hinge around which the orientation between the Fu1 and Fu2 domains differs by ~90° between the LGR5-bound and unbound structure of Rspo1.

(C) Structure of the LGR5 ectodomain (residues 22–543) in complex with the Rspo1 Fu1-Fu2 domains at 3.2 Å resolution in two views, one without and one with surface representation. Domain compositions of LGR5 and Rspo1 are indicated schematically.

See also Figures S1, S2, S3, S4, S5, S6, and S11.

Rspo1-Fu1Fu2 displayed disulphide-bond patterns common to other Fu domains (Garrett et al., 1998; Ogiso et al., 2002). Each Fu domain formed a leaflet consisting of three β-hairpins connected by disulphide bonds (Figure S1). The sets of Fu1 and Fu2 β-hairpins were oriented at ~90° to each other in unbound Rspo1. Next, we determined the structure of this Rspo1 fragment in complex with the LGR5 ectodomain (residues 22–543) at 3.2 Å resolution (Figures 1B and 1C). In the complex, a twist due to a rotation around the longitudinal axis of Rspo1 aligned the two sets of β-hairpins, thus flattening the shape of Rspo1 when bound to LGR5.

Structure of the LGR5-Rspo1 Complex

Rspo1-Fu1Fu2 binds the ecto-LGR5 domain with a K_D of 2–3 nM (de Lau et al., 2011; Glinka et al., 2011). We observed dimeric, 2:2, arrangements of the complex in four crystal structures, determined up to 3.2 Å resolution (Figure 1C and Figure S2). Size-exclusion chromatography indicated 1:1 complexes (Figure S3). In agreement with a physiological existence of LGR dimers on cells, previous mass-spectrometry analysis has re-

vealed interactions between LGR4 and LGR5 in the cell membrane (de Lau et al., 2011). In the absence of the cell membrane and the 7TM region, the interaction between the LGR ectodomains may be lost in solution. Of note, a similar observation was made for FSHR (Fan and Hendrickson, 2005) and TLR5 (Yoon et al., 2012).

The LGR5 ectodomain adopted a typical horseshoe-shaped structure consisting of the 17 leucine-rich repeat (LRR) units (Figure 1C and Figure S4). The N-terminal and C-terminal caps (N- and C-caps) were similar to those of FSHR (Jiang et al., 2012) (Figures S4C and S4D). A long loop in the C-cap contained an α helix but was largely disordered, possibly adopting a stable structure upon interaction with the extracellular loops of the 7TM region. The LRR curve was kinked and twisted by ~20° between LRR10 and LRR11. This kink coincided with a marked sequence variation in LRR9 and LRR10, wherein two bulky phenylalanines occupied the positions of canonical leucines (Figures S4A and S5). Several LRR-containing receptors show curvatures similar to the N-terminal LRR1–LRR10 or the C-terminal LRR11–LRR17 (Figure S6). Due to the kink and
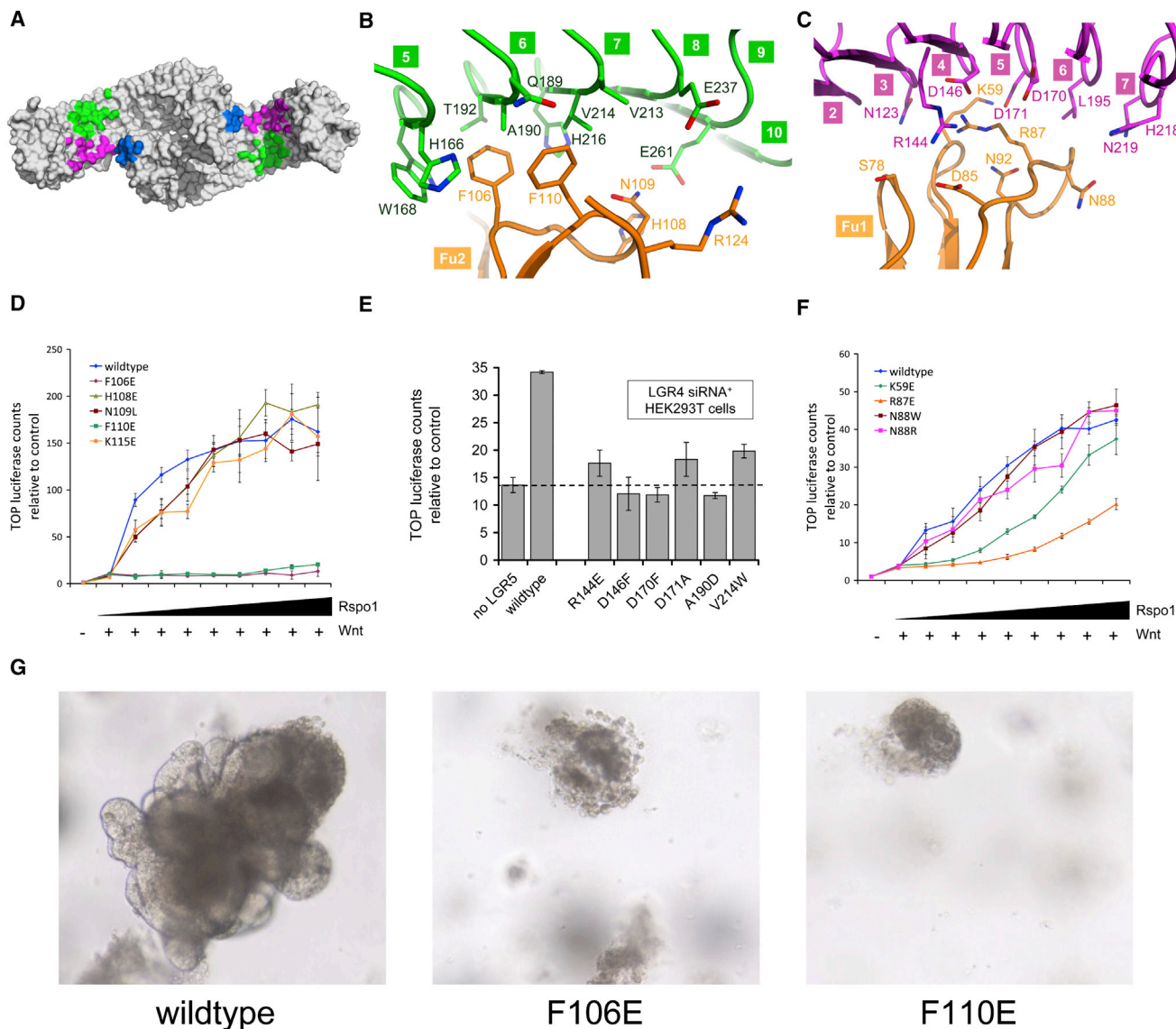
Figure 2. LGR5-Rspo1 Interfaces

(A) Overview of three binding interfaces between Rspo1 and LGR5. Shown are the contact footprints of Rspo1 on LGR5; contacts for Fu2 are indicated in green and for Fu1 in magenta (with *cis* LGR5) and blue (with *trans* LGR5).

(B) Interactions between Fu2 of Rspo1 with LGR5.

(C) Interactions between Fu1 of Rspo1 with LGR5 (*cis*).

(D) TOPFlash results for purified Rspo1 mutants in the Fu2 domain from 1.5 to 200 nM (with K115E as additional control). Error bars represent SD (n = 3).

(E) TOPFlash results obtained by transfection-mediated introduction of 0.012 to 15 ng of wild-type and LGR5 variants, mutated on residues interacting with Rspo1. One day prior to introduction of LGR5 and reporter plasmids, cells were transfected with LGR4-specific siRNA. The 15 ng results are shown. Error bars represent SD (n = 3).

(F) TOPFlash results for Rspo1 mutants in the Fu1 domain (concentrations as in D). Error bars represent SD (n = 3).

(G) Small-intestinal organoid growth in the presence of wild-type or Rspo1 mutants.

twisting of the C-terminal LRR11–LRR17 (yielding an overall twist of ~45°), the protomers bent toward each other in the dimer. Contact points in the dimer bridged LRR10 to LRR17 (Figure S7A) with a large "open" area between the dimer partners, except for an H-bonded interaction between Tyr-361 and its dimeric partner at the center.

LGR5-Rspo1 Binding Interfaces

The ecto-LGR5 and Rspo1-Fu1Fu2 complex observed in the crystals revealed three receptor-ligand contact sites (Figures 2A–2C). Two adjoining sites were formed by the concave surface of the LRR3–LRR9 of LGR5 with Fu2 and Fu1 of Rspo1, burying a total surface area of 870 Å². A third site (burying 340 Å²) was

formed "in *trans*" between Rspo1-Fu1 and the second copy of LGR5.

In the first contact site, phenylalanine residues 106 and 110 of the Rspo1-Fu2 domain formed a clamp-like arrangement around Ala190 of LGR5 (Figure 2B). Ala190 was surrounded by a hydrophobic rim formed by Cβ atoms and/or side chains of His166, Trp168, Gln189, Val213, Val214, and His216. On the side of this hydrophobic patch, glutamates 237 and 261 of LGR5 formed H-bonds and salt bridges with His108 and Asn109 from the ^{106}FSHNF^{110} loop and with Arg-124 from a neighboring loop. All these contact residues of LGR5 are strictly conserved among LGR4–LGR6 (Figure S5). Between the R-spondins, Phe106 and Phe110 are fully conserved, the intervening residues are all hydrophilic or charged, and position 124 is either an arginine or a lysine (Figure S1B). We generated the Rspo1-Fu1Fu2 mutants F106E and F110E and found that these had no activity in the TOPFlash Wnt reporter assay (Figure 2D). Mutations A190D and V214W of LGR5, disrupting the shallow hydrophobic bowl, showed reduced signaling activity (Figure 2E).

Residues from Rspo1-Fu1 and LGR5 residues from the "lower" part of the concave surface of LRR3–LRR7 formed the second contact site (Figure 2C). This site was predominantly of charged character. Again, the LGR5 residues at the interface were strictly conserved between LGR4–LGR6. These residues were Asn123, Arg144, Asp146, Asp170, Asp171, Leu195, His218, and Asn219. The corresponding Rspo1 residues were Lys59, Ser78, Asp85, Arg87, Asn88, and Asn92. Lys59 and Arg87, at the center of this site, were conserved as lysines or arginines between all four R-spondins. Charge-reversal mutations R87E and K59E showed reduced activity, confirming their role in the interaction (Figure 2F). Mutation of the nonconserved Asn88 (on the side of the interface) had no effect on Rspo1 activity. LGR5 mutants D146F and D170F had lost all signaling activity, whereas mutants R144E and D171A showed reduced activity (Figure 2E). We concluded that Rspo1 utilizes both its Fu1 and Fu2 domains to bind the ectodomain of LGR5 at sites that are fully conserved between LGR4–LGR6. This was in agreement with the observed lack of specificity of each of the R-spondins for LGR4–LGR6 (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011). In vitro "minigut" culture, which depends entirely on functional R-spondin (Sato et al., 2009), confirmed that Rspo1-FuFu2 stimulated organoid growth and two binding-defective Rspo1-Fu1Fu2 F106E and F110E mutants failed to support growth (Figure 2G).

The *trans* Site and Dimer Interface

In the third site, Rspo1-Fu1 contacted the dimeric partner LGR5 at the last LRR and short α helix of the C-cap (Figure 3A). The interface was formed by LGR5 residues from the C-cap, Gln457, Ser458, Leu459, and Tyr477 and Rspo1 residues Asn51, Leu54, Leu64, Gln71, Asn88, and Met91. However, in several protein copies in the crystal structures, we observed disorder at this interface (Figure S8). The side chain of Rspo1 Gln71 made a H-bond to backbone carbonyl oxygen of the Tyr477 of LGR5 (Figure 3A); both residues are strictly conserved between Rspo1–Rspo4 and LGR4–LGR6, respectively. Gln71 of Rspo1 coincides with the position of one of the four Rspo4 mu-

tations described in patients with congenital anonychia (Blaydon et al., 2006; de Lau et al., 2012). The four related residues are Arg66, Arg70, Gln71, and Gly73, which were located in the second β-hairpin loop of Rspo1-Fu1. Anonychia mutants R66W, Q71R, R70C, and G73R and related mutations in Rspo1-Fu1Fu2 did not affect binding to ecto-LGR5, but showed reduced signaling activity (Figures 3B and 3C). Q71R and G73R point toward the "*trans*" interface and may disrupt interactions. LGR5 mutations in the *trans* site showed both reduced (S458R) and enhanced (L459R) TOPFlash activity (Figure S7); these findings could not be corroborated further due to lack of expression on the cell surface of the tested mutants. Next, we mutated LGR5 residues in the dimer interface (Figure S7). Residues Tyr289, Asp290, and His454 were observed at the LGR5-LGR5 dimer interface (Figure S7A). Mutation Y289A/D290A, Y289W/D290A, or H454A did not significantly reduce TOPFlash activity (Figure S7B), indicating that the receptor-receptor interface observed in the crystal structure is not critical and that dimer formation may depend on receptor-receptor interactions in the membrane (de Lau et al., 2011).

Activation by Antibody Binding to Disordered C-Cap Loop in the Absence of Rspo1

Ligand-activated signaling is well established for the glycohormone receptors FSHR, TSHR, and LHR (Jeoung et al., 2007; Ryu et al., 1998; Simoni et al., 1997). However, a downstream G protein has not been identified for LGR5. It is unknown whether LGR5, or its homologs LGR4 and LGR6, is directly involved in transmembrane signaling, or whether the receptor serves to capture its ligand and affect Wnt signaling solely through ternary-complex formation, with LRP5/LRP6 (Wei et al., 2007), Frizzled (Nam et al., 2006), and RNF43/ZNRF3 (Hao et al., 2012), for example. We tested a series of LGR5-specific monoclonal antibodies and observed that three of them (1D9, RD20, and RD42) induced TOPFlash activity in human embryonic kidney 293 cells (HEK293 cells) stably expressing LGR5 in the absence of R-spondins (Figures 4A and 4B). The epitopes of these antibodies were mapped onto a flexible region in the ecto-LGR5 C-cap loop (notably, the C-cap was not required for ligand binding as shown for a shortened construct in Figure S3). In contrast, antibody 4D11, which binds to LRR9–LRR11, showed no activity in the TOPFlash assay (data not shown). For the glycohormone receptors, this region (referred to as the hinge region) has been implied to have an autoinhibitory, reverse-agonistic role (Agrawal and Dighe, 2009; Majumdar et al., 2012; Mueller et al., 2010; Vlaeminck-Guillem et al., 2002; Zeng et al., 2001; Zhang et al., 2000). Similarly, antibodies against FSHR (Majumdar et al., 2012) and TSHR (Majumdar and Dighe, 2012) bind between the LRR and 7TM regions and activate signaling, putatively through inducing a conformational change that alleviates the autoinhibitory activity. The TOPFlash activity upon antibody binding may indicate a similar process in LGR5. Moreover, the positions of the anonychia-related mutations provide further indication of interactions beyond the contact sites formed by the ecto-LGR5, with residues Arg60 and Arg77 pointing away from the *trans* site to where the extended C-cap loop or 7TM is expected (see Figure S9 for a presentation of a hypothetical ectodomain and 7TM region arrangement). To
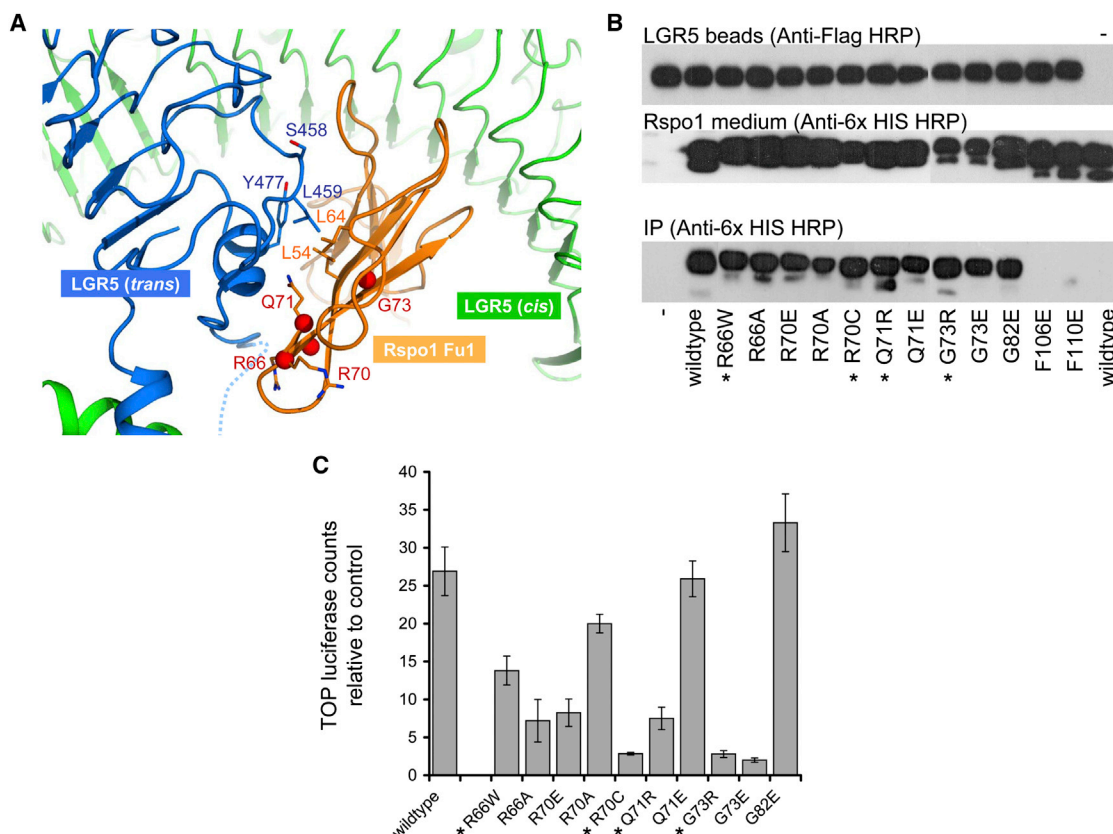
Figure 3. trans and Dimer Interactions

(A) trans contact site between Rspo1-Fu1 and the neighboring C-cap of LGR5; positions of anonychia-related (Rspo4) residues are indicated by red spheres.

(B) Binding assay of Rspo1 anonychia location-specific mutations, present in conditioned media (middle panel), to immobilized LGR5 ectodomain (top panel). The bottom panel shows the amount of protein captured. Actual patient mutations are indicated (*). G82E is used as positive control, and F106E and F110E are used as negative controls. HRP, horseradish peroxidase; HIS, histidine; IP, immunoprecipitation.

(C) TOPFlash assay using conditioned media derived from wild-type and Rspo1 mutants. Error bars represent SD (n = 3).

See also Figures S7 and S8.

verify a role of the 7TM in signaling, we rigorously substituted residues 562–907 of LGR5 with the corresponding region of an unrelated, single-pass membrane protein, GPA33. Fluorescence-activated cell sorting (FACS) analysis showed that the chimeric receptor is expressed on the cell surface (Figure S10A), and Rspo1-alkaline phosphate staining on the cell surface indicates that the Rspo1 is able to bind to the chimeric receptor (Figure S10B). However, TOPFlash activity reduced to basal levels (Figure S10C), supporting the notion that binding of Rspo1 to the LGR5 ectodomain is not sufficient for signaling.

Concluding Remarks

The current structural analysis of Rspo1 in complex with the ectodomain of LGR5 provides a framework from which to build insights into the molecular mechanism by which these two molecules support a wide range of Wnt-dependent stem cell types. R-spondins have been reported to interact with additional membrane receptors such as LRP5/LRP6 (Wei et al., 2007), Frizzled (Nam et al., 2006), RNF43/ZNRF3 (Hao et al., 2012), and Syndecan-4 (Ohkawara et al., 2011). The Wnt agonist Norrin binds LGR4–LGR6 in addition to its interaction with Frizzled4 (Deng

et al., 2013). These proteins either compete for binding or, potentially, form complexes. The short R-spondin Fu1Fu2 fragment suffices for full activity in assays in vitro (Kim et al., 2008; Li et al., 2009). Though most of the Fu1-Fu2 surfaces of R-spondins will be in contact with LGR4–LGR6, additional interactions with Fu1 and Fu2 domains may allow the formation of ternary complexes with LRP5/LRP6, Frizzled, or RNF43/ZNRF3, with or without changing the overall arrangement of the R-spondin-LGR4–LGR6 complexes. Furthermore, in full-length R-spondins, the Tsp and C-terminal tail, not studied here, probably point outward from the complex, providing additional potential contact sites close to the membrane surface—for Syndecan-4, for instance (Ohkawara et al., 2011) (Figure S9). Ligand-independent signaling through antibody binding and residues in the C-cap region that are critical for signaling are features reminiscent of signaling in FSHR, TSHR, and LHR (Mizutori et al., 2008; Mueller et al., 2010). Critical interactions beyond ligand binding to the LRR of LGR5 are further supported by the location of the anonychia-related (Rspo4) mutations (Blaydon et al., 2006; de Lau et al., 2012). Taken together, our data indicate that R-spondins bind tightly to the strictly conserved binding sites formed by
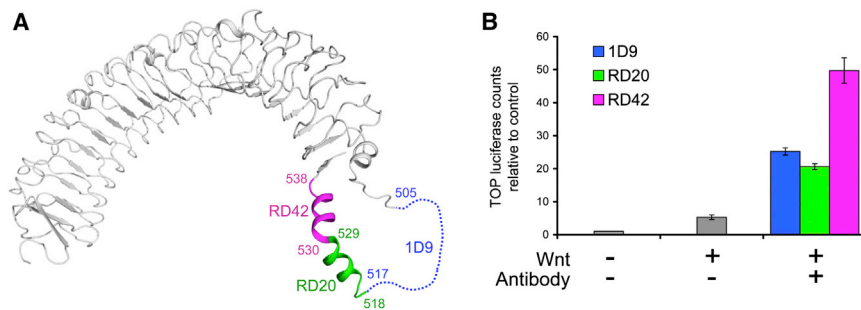
Figure 4. Ligand-Independent Antibody Activation of LGR5

(A) Epitopes of antibodies RD20, RD42, and 1D9 mapped onto ecto-LGR5 (the precise location of this protruding region varies among crystal structures; see Figure S2B).

(B) TOPFlash assay for LGR5-specific antibodies RD20, RD42, and 1D9 in the presence of Wnt3a, but in the absence of R-spondins. Error bars represent SD (n = 3).

See also Figures S2, S9, and S10.

LRR3–LRR9 of LGR4–LGR6 and that monomeric 1:1 interactions are sufficient for this binding to occur. Full-length LGR4–LGR6 molecules, however, dimerize to establish the *trans* contact sites that are implied in signaling. Signaling may also occur upon antibody binding to the C-cap loop region independent of binding R-spondins. In analogy to FSHR, TSHR, and LHR (Mueller et al., 2010; Vassart et al., 2004), this binding may induce conformational changes alleviating the putative autoinhibitory, reverse-agonistic activity. Thus, these data suggest a direct role of signaling by the LGR4–LGR6 in addition to ternary-complex formations. Hence, a bewildering complexity exists at the level of the initiation of Wnt signals at the cell surface because of the existence of 19 Wnts, 10 Frizzleds, and 2 LRP Wnt coreceptors. A variety of secreted and membrane-bound Wnt agonists and antagonists add a further level of complexity. The facultative R-spondin-LGR receptor module appears to represent a vertebrate invention for magnifying Wnt signal strength and thus enlarging stem cell compartments. Recent structures have been resolved for Wnt-Frizzled (Janda et al., 2012) and Dkk1-LRP (Ahn et al., 2011; Bourhis et al., 2011; Chen et al., 2011; Cheng et al., 2011). Many additional studies will be required for understanding the inner workings of what must be among the most complex receptor-ligand systems in animal biology.

EXPERIMENTAL PROCEDURES

Ecto-LGR5 (residues 22–543) and Rspo1-Fu1Fu2 (residues 31–145) proteins were produced recombinantly in HEK293 N-acetylglucoaminyltransferase I-deficient (GnTI⁻) Epstein-Barr virus nuclear antigen I (EBNA) cells, purified to homogeneity, and crystallized with the hanging-drop vapor-diffusion method. Diffraction data were collected at the Swiss Light Source (SLS) in Villigen, Switzerland and at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The structures of Rspo-Fu1Fu2 and the ecto-LGR5-Rspo-Fu1Fu2 complex were determined by experimental phasing and molecular replacement, respectively. Diffraction-data and refinement statistics are provided in Table S1; examples of the electron densities are shown in Figure S11. Monitoring the potential of wild-type and mutated variants of Rspo1-Fu1Fu2 to enhance Wnt signals was done by employing the TCF-dependent TOP luciferase assay in HEK293T cells (Staal et al., 1999). Measuring the ability of mutated versions of the LGR5 to transmit Rspo1-Fu1fu2-driven signaling was performed using the same luciferase assay but with simultaneous small interfering RNA (siRNA)-driven knockdown of endogenous LGR4. The LGR5-binding ability of Rspo1-Fu1Fu2 proteins mutated in the LGR5-interacting domain and Rspo1-Fu1Fu2 mutants representing congenital anonychia mutations were tested in an immunoprecipitation experiment with the ectodomain of LGR5 coated on agarose beads. Additional details are available in the Extended Experimental Procedures.

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