RSPO–LGR4 functions via IQGAP1 to potentiate Wnt signaling

Kendra S. Carmon, Xing Gong, Jing Yi, Anthony Thomas, and Qingyun Liu[1]

Brown Foundation Institute of Molecular Medicine and Texas Therapeutics Institute, University of Texas Health Science Center at Houston, Houston, TX 77030

Edited by Roeland Nusse, Stanford University School of Medicine, Stanford, CA, and approved February 20, 2014 (received for review December 11, 2013)

R-spondins (RSPOs) and their receptor leucine-rich repeat-containing G-protein coupled receptor 4 (LGR4) play pleiotropic roles in normal and cancer development as well as the survival of adult stem cells through potentiation of Wnt signaling. Current evidence indicates that RSPO–LGR4 functions to elevate levels of Wnt receptors through direct inhibition of two membrane-bound E3 ligases (RNF43 and ZNRF3), which otherwise ubiquitinate Wnt receptors for degradation. Whether RSPO–LGR4 is coupled to intracellular signaling proteins to regulate Wnt pathways remains unknown. We identified the intracellular scaffold protein IQ motif containing GTPase-activating protein 1 (IQGAP1) as an LGR4-interacting protein that mediates RSPO–LGR4's interaction with the Wnt signalosome. IQGAP1 binds to and modulates the activities of a plethora of signaling molecules, including MAP kinases, Rho GTPases, and components of the Wnt signaling pathways. Interaction of LGR4 with IQGAP1 brings RSPO–LGR4 to the Wnt signaling complex through enhanced IQGAP1–DVL interaction following RSPO stimulation. In this configuration, RSPO–LGR4–IQGAP1 potentiates β-catenin–dependent signaling by promoting MEK1/2-medidated phosphorylation of LRP5/6 as well as β-catenin–independent signaling through regulation of actin dynamics. Overall, these findings reveal that RSPO–LGR4 not only induces the clearance of RNF43/ZNRF3 to increase Wnt receptor levels but also recruits IQGAP1 into the Wnt signaling complex, leading to potent and robust potentiation of both the canonical and noncanonical pathways of Wnt signaling.

cell signaling | receptor activation | adhesion | migration

The four R-spondins (RSPO1–4) and three related leucine-rich repeat-containing G-protein coupled receptors, LGR4, LGR5, and LGR6 (LGR4–6), constitute a ligand–receptor system that plays critical roles in development, stem cell survival, and oncogenesis (1–6). Mutations of RSPO1 and RSPO4 affect sex development and nail formation (7–9), respectively, and haplotype insufficiency of LGR4 in humans is associated with several diseases and other traits (10). In the mouse, knockouts of LGR4 or RSPOs are presented with severe developmental abnormalities, including neonatal/embryonic lethality accompanied by hypoplasia and defects in tubule elongation and branching in the kidney, lung, mammary gland, and testis (11–16). LGR5 and LGR6 are markers of adult stem cells in the intestine and other select solid tissues (2). LGR4 is frequently coexpressed with LGR5 or LGR6 and is required for the survival of crypt stem cells in the gut (4, 17). Recurrent, gain-of-expression gene fusions of RSPO2 (to EIF3E) and RSPO3 (to PTPRK) occur in a subset of colorectal cancers (18). In mouse mammary tumor virus-induced mouse models of breast and colon cancer, RSPO2 and RSPO3 were identified as two of the most commonly activated alleles (19–21). Ectopic expression of RSPO2/3 in mouse mammary epithelial cells led to an increase in invasiveness in vitro and in tumor formation and metastasis in vivo (20, 22). LGR4 is up-regulated in the majority of human colon and lung cancers (23–25). However, the exact roles and mechanisms of RSPO–LGR4 signaling in normal and cancer development remains poorly understood.

LGR4–6 comprise a large extracellular domain (ECD) with 17 leucine-rich repeats and a seven-transmembrane (7TM) domain typical of the rhodopsin family of G-protein–coupled receptors (26). Stimulation of LGRs with RSPO1–4 greatly potentiates the activity of Wnt ligands in both the canonical (β-catenin–dependent) and noncanonical (β-catenin–independent; planar cell polarity) pathways (3–5). This function of the LGRs is independent of heterotrimeric G proteins and β-arrestin (3, 4). Instead, it was shown that the RSPOs function as an extracellular bridge to bring LGR4 and E3 ligases RNF43/ZNRF3 together to form a ternary complex (LGR4–RSPO–RNF43/ZNRF3), which induces clearance of the E3 ligases (27). Because RNF43/ZRNF3 ubiquitinates Wnt receptors for degradation, their removal leads to elevated Wnt receptor levels and increased Wnt signaling (27, 28). The importance of this ternary complex in potentiating Wnt signaling has now been revealed by multiple structural and biochemical studies (29–31), particularly by the finding that higher binding affinity of RSPO2/3 for RNF43/ZNRF3 accounts for their stronger potency in functional assays (32). Conversely, there is strong evidence indicating that RSPO–LGR signaling involves additional mechanisms. Foremost, RSPO treatment leads to an immediate increase in LRP6 phosphorylation, and overexpression of the FZD Wnt receptor does not supersede the effect of RSPO stimulation (33). Upon costimulation with RSPO and Wnt ligands, LGR4 and LGR5 form a supercomplex with the Wnt coreceptors LRP5/6 and FZD to enhance canonical Wnt signaling (4, 34). Here we show that the 7TM domain of LGR4 binds to and functions via the intracellular signaling protein IQGAP1 (IQ motif containing GTPase-activating protein 1) to enhance β-catenin activation through MEK1/2-mediated phosphorylation of LRP6. Furthermore, the interaction of LGR4 with IQGAP1 promotes association with F-actin assembly proteins to regulate focal adhesion (FA) assembly and cell migration. Overall, the maximal function of RSPO–LGR4–IQGAP1 sig-

Significance

R-spondins (RSPOs) and LGR4 emerged as a major ligand–receptor system in the regulation of Wnt signaling as manifested in their pleotropic roles in development and survival of adult stem cells. The mechanism of how RSPO–LGR4 interacts with the Wnt signaling system remains poorly understood. In this work, we describe the identification of IQGAP1 as the first intracellular signaling partner of RSPO–LGR4 and the delineation of IQGAP1's roles and mechanism in mediating RSPO–LGR4-induced potentiation of Wnt signaling. We also elucidate the relationship between the RSPO–LGR4–IQGAP1 pathway and the function of RSPO–LGR4 in inhibiting RNF43/ZNRF3. The findings uncovered a unique mechanism of RSPO–LGR4 signaling and provide a mechanistic basis for the pleiotropic functions of RSPO–LGR4 signaling in normal and pathological processes.

Author contributions: K.S.C., X.G., J.Y., and Q.L. designed research; K.S.C., X.G., J.Y., and A.T. performed research; K.S.C., X.G., J.Y., and Q.L. analyzed data; and K.S.C. and Q.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: qingyun.liu@uth.tmc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1323106111/-/DCSupplemental.

naling depends on the clearance of RNF43/ZNRF3. This unique, dual mechanism of RSPO–LGR4 signaling provides a molecular basis for its robust activity in the potentiation of both the canonical and noncanonical pathways of Wnt signaling.

Results

RSPO–LGR4 Potentiates Wnt/β-Catenin Signaling in the Absence of RNF43/ZNRF3. To determine whether RSPO–LGR4 acts solely through clearance of the E3 ligases, HEK293T cells which only express ZNRF3 (27) were transfected with ZNRF3 siRNA, and their response to Wnt3a and RSPO3 was measured. As expected (27), knockdown (KD) of ZNRF3 led to increases in basal activity and in response to Wnt stimulation (Fig. 1*A*). Surprisingly, cells with KD of ZNRF3 produced a better response to RSPO stimulation (Fig. 1*A*). Ectopic expression of LGR4 in ZNRF3-deficient cells led to much higher basal and RSPO-stimulated β-catenin activity compared with control cells (Fig. 1*A*). These results suggest that RSPO–LGR4 has ZNRF3-independent activity. To corroborate this finding, we generated a mutant (Q71A) of RSPO1 that alters residue Glu-71, which has been shown to be critical for binding to RNF43, but not to LGR5, in the cocrystal structure of LGR5–RSPO1–RNF43 (29). The RSPO1 mutant was expressed and secreted at levels similar to those of WT (Fig. S1*A*). As expected, Q71A failed to bind to ZNRF3 but still bound to LGR4, with slightly lower affinity compared with WT (Fig. S1 *B* and *C*). It should also be noted that the observed binding of WT RSPO1 to ZNRF3 was only slightly above background levels (Fig. S1*B*). In functional assays, Q71A was weaker than WT in HEK293T cells (Fig. 1*B*) but showed the same efficacy as WT with only ∼threefold decrease in potency in cells overexpressing LGR4 (Fig. 1*B*). Furthermore, both mutant and WT RSPO1 elicited a stronger response in cells with KD of ZNRF3 (Fig. 1*C*). The decrease in potency of the Q71A mutant in cells with or without KD of ZNRF3 or with overexpression of LGR4 in potentiating Wnt/β-catenin activity is consistent with its reduced binding affinity for LGR4 (Fig. S1*C*). These results strongly suggest that binding of RSPO to ZNRF3 is only critical for its Wnt-potentiating effect in cells with high levels of ZNRF3, and thus RSPO–LGR4 is likely to function via other mechanisms to enhance Wnt/β-catenin signaling.

Overexpression of the ECD of ZNRF3 anchored to the membrane (ZNRF3–ECDTM), which lacks ubiquitin ligase activity, can act in a dominant negative fashion to inhibit the activities of ZNRF3 and thus increase response to Wnt stimulation (27). Intriguingly, although modestly augmenting Wnt response, ZNRF3–ECDTM completely blocked RSPO's activity, which would otherwise increase Wnt/β-catenin signaling to significantly higher levels (27). We confirmed that overexpression of ZNRF3–ECDTM led to modest increase in response to Wnt stimulation (Fig. 1*D*). Remarkably, ZNRF3–ECDTM was able to completely inhibit the activity of RSPO in an insurmountable fashion in both control cells and cells overexpressing LGR4 (Fig. 1*E*). Because the ECD of ZNRF3 directly interacts with FZD receptors and ZNRF3 (27, 28), these data imply that ZNRF3–ECDTM inhibits RSPO activity, most likely by hindering the interaction of RSPO–LGR4 with the Wnt signalosome. We tested this theory by determining the effect of overexpressing ZNRF3–ECDTM on the interaction between LGR4 and Wnt receptors and found that it blocked the formation of the supercomplex between LGR4 and LRP6 (Fig. 1*F*). Notably, this effect of ZNRF3–ECDTM could not be overcome by Wnt3a and RSPO1 cotreatment, indicating that ZNRF3–ECD has a higher affinity for FZD receptors or

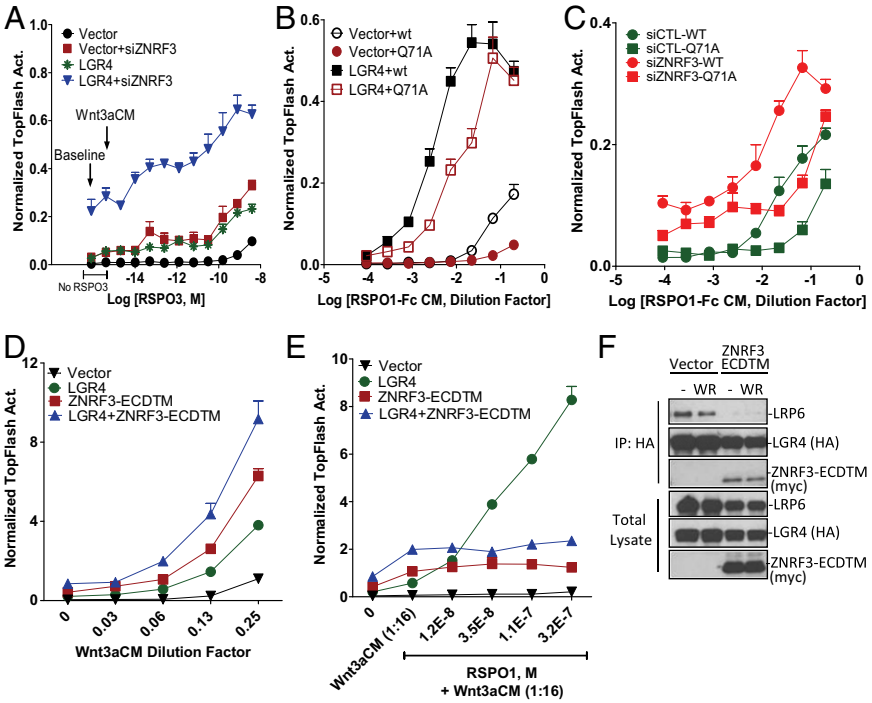

Fig. 1. RSPO–LGR4 has RNF43/ZNRF3-independent function. (*A*) Wnt3a and RSPO3-induced β-catenin activity as measured by the TOPflash assay in HEK293T cells transfected with vector control or LGR4 in the presence of scrambled or ZNRF3 siRNA. The baseline arrow indicates no Wnt3a or RSPO3, and Wnt3a arrow indicates a fixed dilution of Wnt3aCM added to the rest of the samples. (*B*) Dose-dependent response to RSPO1 WT and Q71A mutant in HEK293T cells transfected with vector control or LGR4 in the TOPflash assay. (*C*) Dose-dependent response to RSPO1 WT and Q71A mutant in HEK293T cells transfected with scrambled or ZNRF3 siRNA in the TOPflash assay. (*D* and *E*) HEK293T cells overexpressing ZNRF3–ECDTM in the presence or absence of LGR4 show an enhanced response in the TOPflash assay when treated with serial dilutions of Wnt3aCM (*D*), but failed to respond to RSPO1 (*E*). (*F*) WB analysis of the effect of overexpressing ZNRF3–ECD–TM on interaction of LGR4 with LRP6. HEK293T cells transfected with HA-LGR4 and LRP6 were cotransfected with vector or Myc-ZNRF3-ECD-TM and treated with vehicle or Wnt3a+RSPO1 (WR). IP was performed with anti-HA beads and proteins were detected with Abs as indicated.

possesses an alternative function other than binding to RSPOs. These results, together with the finding that KD of ZNRF3 led to increased response to RSPO stimulation, suggest that RSPO–LGR4 can function by clearing the E3 ligases to liberate the Wnt coreceptor complex and promote its interaction with free RSPO–LGR4 to achieve maximum potentiation of Wnt signaling.

LGR4 Binds to and Requires IQGAP1 to Potentiate Wnt/β-Catenin Signaling. To decipher the mechanism of how RSPO–LGR4 interacts with Wnt receptors to enhance signaling, we searched for cytoplasmic proteins that interact with the 7TM domain of LGR4. Full-length HA-tagged LGR4 expressed in HEK293T cells was completely solubilized and pulled down by immunoprecipitation (IP). Proteins that copurified with LGR4 were identified by mass spectrometry analysis. Of the many putative proteins identified (Table S1), two candidates, IQGAP1 and IQGAP3, were particularly notable for their pleiotropic roles in signal transduction (35, 36). The three highly homologous IQGAPs (IQGAP1–3) contain multiple domains that bind to and modulate the activity of a plethora of signaling molecules, including Rho GTPases, MAP kinase (MAPK), and components of Wnt pathway E-cadherin, β-catenin, and adenomatous polyposis coli (35, 36). We first confirmed that full-length LGR4 interacts with IQGAP1 as well as with IQGAP2 and IQGAP3 by co-IP analysis (Fig. 2*A* and Fig. S2*A*). In contrast, HA-tagged LGR4–ECD fused to the CD4–TM domain (LGR4–ECDTM) did not pull down IQGAP1 (Fig. 2*A*) or IQGAP2–3 (Fig. S2*A*), suggesting that the 7TM domain of LGR4 is required for interaction with IQGAP1–3. Association of LGR4 with IQGAP1 was not altered upon addition of ligand (Fig. 2*A*). To verify that the interaction between LGR4 and IQGAP1 occurs at endogenous receptor levels, co-IP analysis was carried out with HeLa cells, which express high levels of LGR4 and IQGAP1. The two endogenous proteins were found to interact with each other by using reciprocal approaches (Fig. 2*B* and Fig. S2*B*).

Next, we tested whether IQGAP1 is involved in RSPO–LGR4-induced potentiation of canonical Wnt signaling. Quantitative RT-PCR (RT-qPCR) analysis indicated that HEK293T cells express the highest levels of IQGAP1 and progressively lower levels of IQGAP3 and IQGAP2 (Fig. S2*C*). KD of IQGAP1 significantly inhibited RSPO1-induced Wnt/β-catenin signaling (~60–70%) (Fig. 2*C*). KD of IQGAP3 alone suppressed signaling to a lesser extent compared with IQGAP1, and no synergistic effect was observed with double KD (Fig. 2*C*). RT-qPCR and Western blot (WB) analysis revealed that KD of IQGAP1 and IQGAP3 was sufficient but incomplete (Fig. S2 *D* and *E*), which could account for the lack of total abolishment of RSPO1 response in IQGAP1/3-KD cells. Ectopic expression of mouse IQGAP1 was able to completely rescue the effect of IQGAP1 KD (Fig. 2*D*). Because phosphorylation of LRP6 is a hallmark of canonical Wnt-signaling activation and is enhanced by RSPO–LGR signaling (3, 33), we tested whether IQGAP1 depletion would affect LRP6 phosphorylation in response to Wnt3a alone or RSPO1+Wnt3a cotreatment. In both instances, loss of IQGAP1 led to decreased LRP6 phosphorylation (p-LRP6) with stronger effect on cells cotreated with RSPO1+Wnt3a (Fig. 2*E*). These data suggest that IQGAP1 transduces RSPO–LGR4-induced potentiation of Wnt/β-catenin signaling through regulation of LRP6 phosphorylation.

IQGAP1-Recruited MAPK MEK1/2 Phosphorylate LRP6 to Enhance Wnt/β-Catenin Signaling. IQGAPs contain several domains that bind to distinct signaling and structural proteins (35, 36). To map the IQGAP1 domain responsible for interacting with LGR4, a series of IQGAP1 truncation and deletion mutants were constructed (Fig. 3*A*). Co-IP studies demonstrated that all IQGAP1 mutants bound to HA-LGR4, except those without the rasGAP-related domain (GRD) (Fig. 3*B*). Furthermore, the GRD alone was able

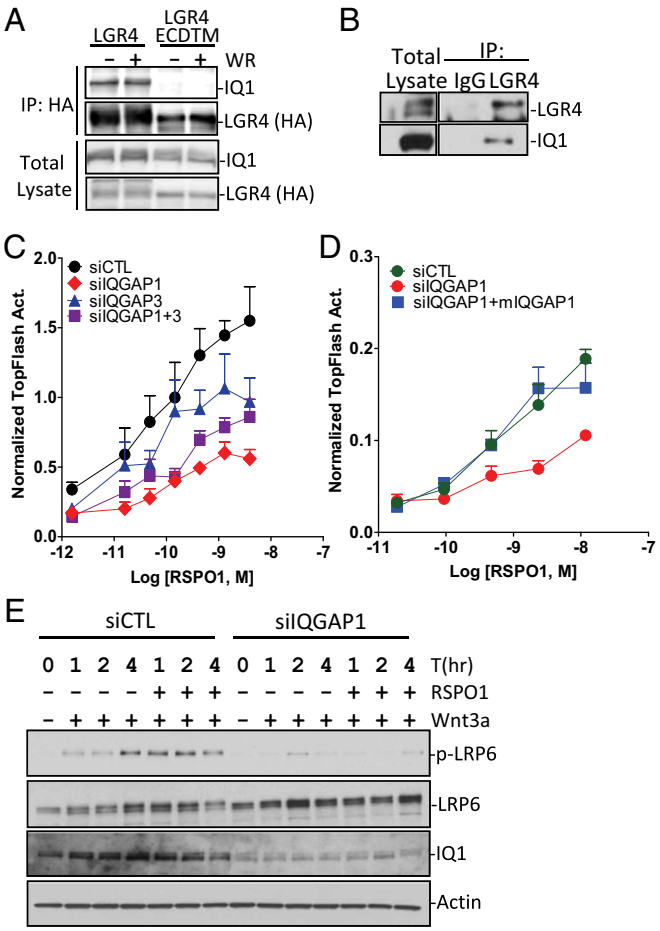

Fig. 2. IQGAP1 binds to LGR4 and mediates Wnt/β-catenin signaling. (*A*) Co-IP analysis of full-length LGR4 or LGR4–ECDTM with IQGAP1. HEK293T cells were transfected with HA-LGR4 or HA-LGR4-ECD-TM, immunoprecipitated with anti-HA beads, and blotted with IQGAP1 or HA Ab. (*B*) Co-IP of endogenously expressed LGR4 and IQGAP1 from HeLa cells. Cell lysates were immunoprecipitated with LGR4 Ab (7E7; described in detail in ref 25) or control IgG and blotted with LGR4 or IQGAP1 Ab. (*C*) Effect of IQGAP1 and IQGAP3 KD on RSPO1-induced Wnt/β-catenin activity in the TOPflash assay. HEK293T cells were transfected with scrambled siRNA or siRNA of IQGAP1 or IQGAP3, or both. (*D*) Overexpression of mouse IQGAP1 rescued IQGAP1 KD effect in HEK293T cells using the TOPflash assay. (*E*) Effect of IQGAP1 KD on RSPO1 and Wnt3a-induced LRP6 phosphorylation. HEK293T cells were transfected with scrambled or IQGAP1 siRNA, treated with RSPO1 +Wnt3a for the indicated periods of time as indicated, and probed for LRP6 phosphorylation (S1490) and levels of IQGAP1. All error bars are SEM ($n \geq 3$ for all samples).

to bind to LGR4 (Fig. 3 *B*, *Right*), indicating that it is both necessary and sufficient for interacting with LGR4. We then tested the functional activity of the mutants and found that overexpression of IQGAP1–ΔNT and –ΔIQ as well as the GRD inhibited RSPO1 response, presumably by acting in dominant-negative fashion and blocking LGR4 binding to endogenous IQGAPs (Fig. 3*C* and Fig. S2*F*). In contrast, IQGAP1 mutants without the WW or the C-terminal half had no effect (Fig. 3*C*). Moreover, overexpression of the ΔIQ mutant suppressed both baseline and peak p-LRP6 levels induced by Wnt3a+ RSPO1 cotreatment in HEK293T and HeLa cells (Fig. S3 *A* and *B*). These results indicate that IQGAP1 interacts with LGR4 through the GRD and requires the IQ domain to mediate the effect of RSPO–LGR4 function on Wnt/β-catenin signaling.

The IQ and WW domains of IQGAP1 bind the MEK1/2 and ERK1/2 class of MAPKs, respectively (37, 38). Both MEK and

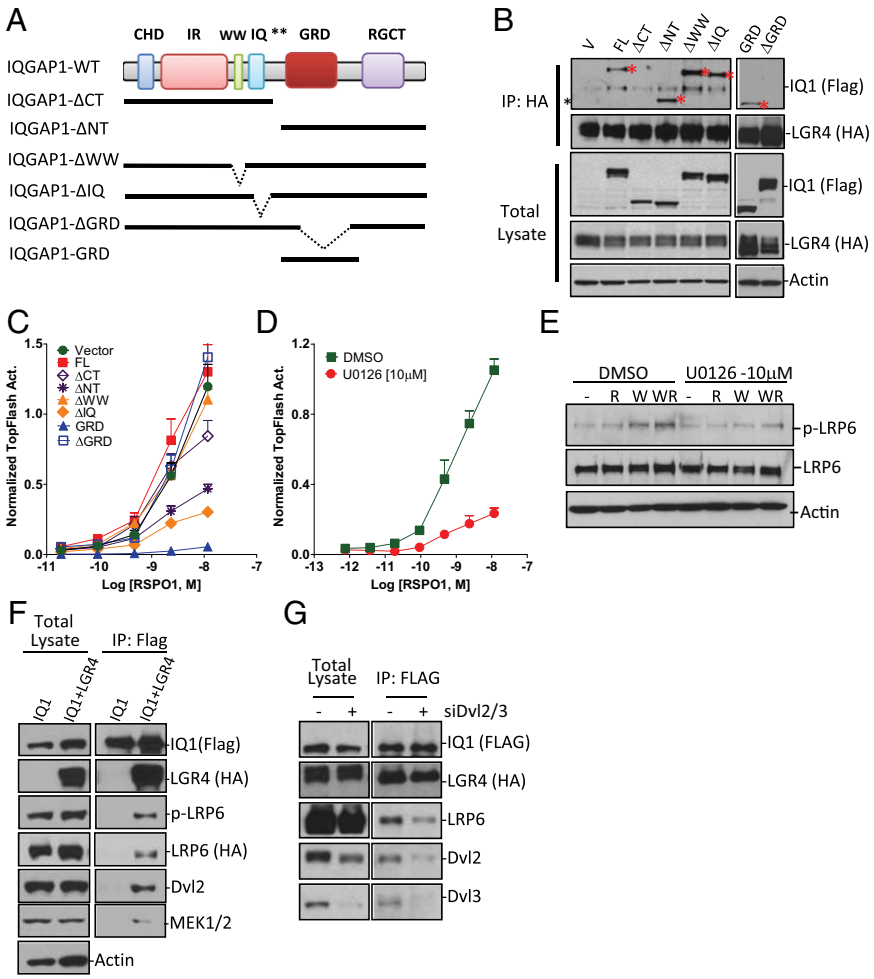

Fig. 3. IQGAP1 bridges RSPO–LGR4 to the Wnt signalosome via DVL and promotes MEK1/2-mediated LRP6 phosphorylation. (*A*) A schematic diagram of IQGAP1 domain structure and deletion/truncation mutants. CHD, calponin homology domain; IR, IQ repeats; GRD, rasGAP-related domain; RGCT, C-terminal domain of RasGAP-related proteins. **Putative DVL binding region. (*B*) Co-IP analysis of various IQGAP1 mutants with LGR4. HEK293 cells were cotransfected with FLAG-tagged IQGAP1 WT or mutants and HA-LGR4, immunoprecipitated with anti-HA beads, and probed with FLAG or HA Abs. *Specific bands that coimmunoprecipitated with LGR4. (*C*) Effect of overexpressing IQGAP1 WT or mutants on RSPO1-potentiated Wnt/β-catenin signaling in the TOPflash assay. (*D*) Effect of MEK1/2 inhibitor U0126 on RSPO1 potentiation of Wnt/β-catenin signaling in the TOPflash assay. (*E*) WB analysis of the effect of U0126 on Wnt3a (W), RSPO1 (R), or both (WR)-induced LRP6 phosphorylation. (*F*) Co-IP analysis of IQGAP1 with p-LRP6, LRP6, DVL2, and MEK1/2 with or without LGR4 overexpression. (*G*) KD of DVL2 and DVL3 reduced complex formation of LGR4 with Wnt signalosome. HEK293T cells were cotransfected with siRNAs targeting DVL1–3, FLAG-IQGAP1, HA-LGR4, and LRP6. Cell lysates were immunoprecipitated with anti-FLAG beads and probed with anti-HA (LGR4), -LRP6, -DVL2, and -DVL3 Abs.

ERK are capable of phosphorylating LRP6 to promote canonical Wnt signaling (39). Because loss of IQGAP1 led to failure of LRP6 phosphorylation following Wnt3a+RSPO stimulation, we reasoned that RSPO–LGR4 may interact with IQGAP1 to recruit MAPKs to phosphorylate LRP6 and therefore enhance Wnt signaling. U0126, a specific inhibitor of MEK1/2 (40), was able to abolish RSPO-induced Wnt/β-catenin signaling and LRP6 phosphorylation (Fig. 3 *D* and *E* and Fig. S4 *A* and *B*), whereas inhibitors of p38, ERK and JNK had no effect (Fig. S4 *C* and *D*). Furthermore, co-IP analysis showed that IQGAP1 was able to pull down p-LRP6 and MEK1/2 when coexpressed with LGR4 (Fig. 3*F*). Loss of MEK1/2 binding would account for the dominant-negative activity of the ΔIQ mutant in the functional assays. Just recently, DVL was shown to modulate Wnt signaling by directly binding to the region of IQGAP1 that lies between the IQ domain and GRD (41). We examined whether IQGAP1–DVL interaction would function as a bridge to promote complex formation between RSPO–LGR4 and the Wnt signalosome because DVL binds to FZDs and is essential for both the canonical and noncanonical pathways. Indeed, endogenous DVL was found to coimmunoprecipitate with IQGAP1 when coexpressed with LGR4 (Fig. 3*F*). Partial KD of DVLs resulted in the decreased association of LGR4–IQGAP1 with LRP6 of the Wnt signalosome (Fig. 3*G*). Costimulation of cells with Wnt3a and RSPO3 led to a further increase in the amount of DVL and MEK1/2, which coimmunoprecipitated with IQGAP1 in the presence of LGR4 overexpression (Fig. 4*A*). Overall, these results suggest that the interaction of IQGAP1 with DVL mediates the supercomplex formation between RSPO–LGR4 and the Wnt signalosome and that IQGAP1 recruits MEK1/2 to phosphorylate LRP5/6, leading to the potentiation of the canonical Wnt-signaling pathway.

RSPO–LGR4–IQGAP1 Is Associated with F-Actin Assembly Machinery. RSPO–LGR4 signaling was also shown to potentiate the β-catenin–independent, noncanonical pathway of Wnt signaling (5). This pathway regulates cell polarity and migration through modulating activities of the Rho family of GTPases (Rho, Rac, and CDC42), which all interact with IQGAPs directly (35, 36, 42). Importantly, IQGAP1 can recruit the actin assembly proteins

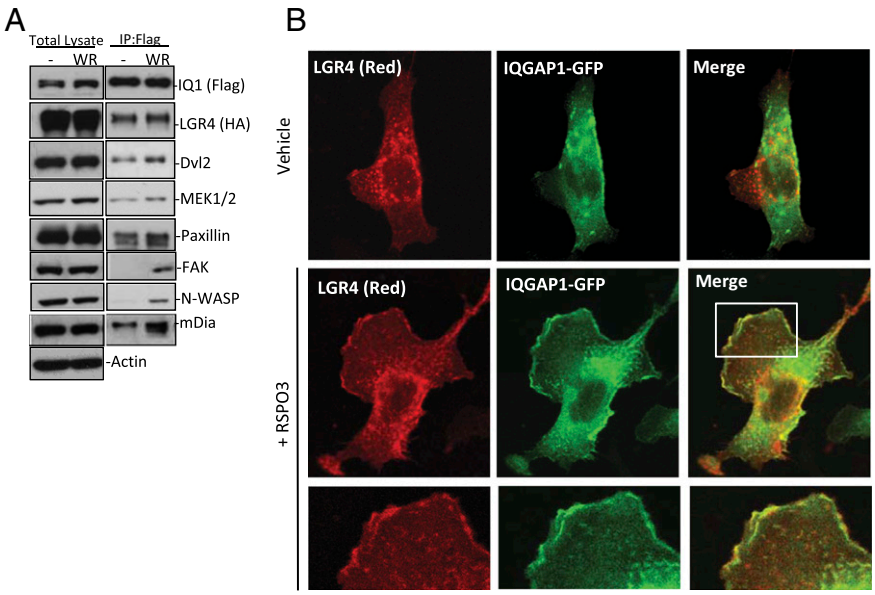

Fig. 4. RSPO–LGR4-recruited IQGAP1 interacts with Wnt signalosome and F-actin assembly components. (*A*) Co-IP analysis of LGR4–IQGAP1 with Wnt and FA complex molecules with or without Wnt3a+ RSPO1 (WR) costimulation. (*B*) Confocal microscopy analysis of RSPO3-induced colocalization of IQGAP1 with LGR4 at the leading edge of MDCK cells. MDCK cells transfected with IQGAP1–EGFP (green) and HA-LGR4 were starved and treated with vehicle or RSPO3 for 30 min. Cells were stained with Alexa 594-labeled anti-HA (red) and imaged. The leading edge is highlighted by the white box and in *Bottom*.

neural Wiskott–Aldrich syndrome protein (N-WASP) and mDia1 to coordinate receptor activation with FA assembly, which is critical to the control of cell adhesion and migration (43–45). We reasoned that RSPO–LGR4 may potentiate the noncanonical pathway by recruiting IQGAP1, which interacts with N-WASP and mDia1 into the Wnt signalosome to promote actin assembly. First, we examined whether RSPO3-induced LGR4 activation could induce translocation of IQGAP1 to the cell membrane by using confocal immunofluorescence analysis of Madin–Darby canine kidney (MDCK) cells coexpressing HA-LGR4 and IQGAP1–EGFP. In serum-starved cells without RSPO3, LGR4 was mostly located in vesicles due its strong constitutive internalization (3), whereas IQGAP1 was diffusely expressed throughout the cytoplasm (Fig. 4 *B*, *Upper*). In cells treated with RSPO3, both LGR4 and IQGAP1 were found at the front of the leading edges with near-complete colocalization (Fig. 4 *B*, *Lower*). We then examined whether LGR4–IQGAP1 was located in FA complexes following ligand stimulation. In cells overexpressing LGR4 and IQGAP1, cotreatment with Wnt3a, which promotes RhoA-dependent cell motility (46), and RSPO3 led to dramatic increases in the association of IQGAP1 with N-WASP and mDia1 (Fig. 4*A*). FA kinase (FAK) and paxillin, two key players in FA assembly, were found in the LGR4–IQGAP1 complex following cotreatment with both ligands (Fig. 4*A*). These data support the notion that RSPO-induced activation of LGR4 enhances the recruitment of IQGAP1 into the Wnt signalosome through a DVL bridge. In turn, this recruitment provides a mechanism for RSPO–LGR4 to regulate actin dynamics and FA assembly through IQGAP1's modulation of N-WASP and mDia1 activity.

RSPO3–LGR4–IQGAP1 Signaling Regulates the Cytoskeletal Organization and Migration of Cancer Cells. LGR4 was also shown to be one of the few 7TM receptors that were highly up-regulated in the majority of non-small-cell lung cancer cases (24). However, whether LGR4 plays any roles in lung carcinogenesis remains unknown. Mining of the Cancer Cell Line Encyclopedia database identified that the lung cancer cell lines A549 and H460 express high levels of RSPO3 as well as LGR4 and IQGAP1, with lower levels of IQGAP3 (47). These cell lines also express various Wnt ligands and coreceptors, but no LGR5, LGR6, or the other three RSPOs. RT-qPCR confirmed that A549 and H460 cells express high levels of LGR4 with low levels of LGR6, but no other RSPOs or LGR5 (Fig. S5*A*). A series of LGR4 shRNA constructs were screened, and one (no. 40) was able to knock down LGR4 mRNA and protein levels by ∼80% in A549 cells (Fig. 5*A* and Fig. S5*B*). Importantly, only cells with shRNA-40 displayed reduced levels of p-LRP6 and non-membrane-bound β-catenin as well as DVL2 phosphorylation (Fig. 5*A*). Similar results were obtained with H460 cells (Fig. S5*C*). A549 cells stably expressing a panel of RSPO3 shRNA were also generated. Two independent shRNAs were able to knock down RSPO3 expression by 99.9% KD effect (Fig. S5*D*). Analysis of Wnt signaling pathways also revealed significant reduction in Wnt signaling (Fig. 5*B*). One IQGAP1–shRNA (#85) was identified with potent KD effect on IQGAP1 expression (Fig. S6*A*). A549 cells with loss of IQGAP1 showed a decrease in p-LRP6, especially in Wnt3a+RSPO-stimulated cells (Fig. S6*B*). These results clearly indicate that RSPO–LGR4–IQGAP1 signaling plays a major role in Wnt/β-catenin signaling in lung cancer cells with high endogenous expression of RSPO3.

Given that RSPO–LGR4–IQGAP1 is associated with the machinery of F-actin assembly, we examined whether their deficiency would affect F-actin–mediated cytoskeletal organization. KD of RSPO3, LGR4, or IQGAP1 in A549 cells led to nearly identical phenotypes: loss of actin stress fibers, increased levels of cortical F-actin, and reduced cell size (Fig. 5*C* and Fig. S7). Staining of paxillin (FA marker) and F-actin clearly revealed that A549 cells with KD of IQGAP1 or RSPO3 contained no FAs, whereas control shRNA cells displayed prominent FAs at the ends of stress fibers (Fig. 5*C*). WB analysis showed that A549 cells with KD of either RSPO3 or IQGAP1 had lower levels of pJNK, pFAK, paxillin, and pERK1/2 (Fig. 5*D*), consistent with defects in noncanonical Wnt signaling and FA assembly. We then tested whether RSPO–LGR4–IQGAP1 controls cell migration and invasion, given the critical function of F-actin assembly in these processes. KD of LGR4 or IQGAP1 in A549 cells led to ∼50% reduction in cell migration and invasion (Fig. 5 *E* and *F*). Similar results were obtained with KD of LGR4 in H460 cells (Fig. S8*A*). Remarkably, A549 cells with complete KD of RSPO3 displayed

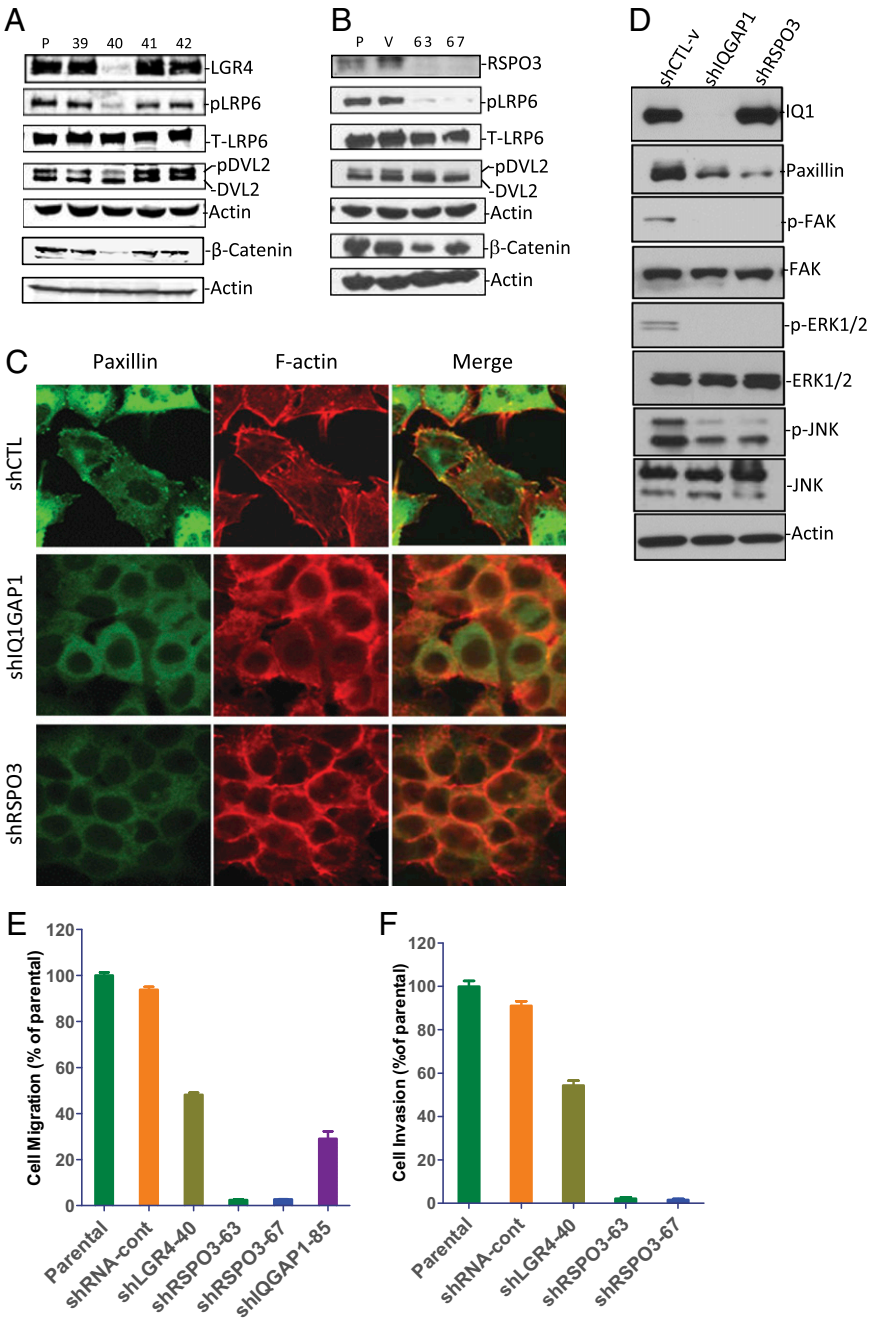

Fig. 5. RSPO3–LGR4–IQGAP1 signaling regulates the formation of FA and migration in lung cancer cells. (*A*) Effect of LGR4 KD on Wnt signaling. A549 cells stably expressing four distinct shRNA constructs (nos. 39–42) were generated and probed for levels of LGR4 and Wnt signaling markers. P, parental cells. (*B*) Effect of RSPO3 KD on Wnt signaling. A549 cells stably expressing two distinct shRNA constructs of RSPO3 (nos. 63 and 67) were generated and probed for levels of RSPO3 and Wnt signaling markers. V, vector control. (*C*) Effect of KD of RSPO3 or IQGAP1 on FA and cytoskeletal structures. A549 cells stably expressing RSPO3 (no. 63), IQGAP1 (no. 85), or control shRNA were costained with anti-paxillin (green) and rhodamine-labeled phalloidin (red) and viewed by confocal microscopy. (*D*) WB analysis of Wnt signaling and FA assembly markers in A549 cells stably expressing RSPO3 (no. 63), IQGAP1 (no. 85), or control shRNA. (*E*) Migration results of A549 cells with KD of LGR4, RSPO3, or IQGAP1. (*F*) Invasion results of A549 cells with KD of LGR4 or RSPO3.

almost no migration or invasion (Fig. 5 *E* and *F*). The more modest effect in cells with KD of LGR4 or IQGAP1 on migration and invasion is most likely due to incomplete KD (only ~80% for LGR4) or the abundance of IQGAP1 and IQGAP3, respectively. To further validate the results of RSPO3 KD on cell migration, we tested an RSPO3-neutralizing antibody (Ab), which was validated to inhibit RSPO3-induced potentiation of β-catenin signaling (Fig. S8*B*). A549 cells incubated with the Ab showed ~50% reduction in cell migration compared with control IgG-treated cells (Fig. S8*C*). The lack of total inhibition of migration may reflect inadequate neutralization by the antisera due to an autocrine-like nature of RSPO3–LGR4 signaling. Overall, these results strongly suggest that endogenous RSPO3–LGR4–IQGAP1 signaling regulates FA assembly in lung cancer cells with high RSPO3 expression, likely by directly recruiting N-WASP and mDia1 and coordinating actin dynamics.

Discussion

The newly discovered RSPO–LGR4 ligand receptor system has emerged as a major axis in the regulation of Wnt signaling as manifested in its pleotropic roles in development and survival of adult stem cells. The mechanism of how RSPO–LGR4 functions to potentiate Wnt signaling, however, remains enigmatic. Despite containing a 7TM domain with strong homology to those of the rhodopsin family of G-protein–coupled receptors, stimulation of LGR4 or its related receptor LGR5 and LGR6 by RSPOs does not lead to the activation of heterotrimeric G proteins or to β-arrestin translocation (3, 4, 48). Hao et al. discovered that RSPO–LGR4 induces the clearance of RNF43/ZNRF3 to increase Wnt receptor level and thus augment Wnt signaling activity (27). This mechanism has now been confirmed by a series of papers with structural information detailing the interactions among RSPO, LGR4, and RNF43/ZNRF3 (29, 30, 32). These studies unequivocally concluded that binding of RSPOs to RNF43/ZNRF3 is essential for RSPO–LGR4-induced potentiation of Wnt signaling in cells expressing RNF43/ZNRF3.

However, we and others have presented direct evidence that RSPO–LGR forms a supercomplex with the Wnt receptor system to potentiate Wnt signaling (4, 34). There is also indirect evidence that RSPOs–LGR must act via additional mechanisms to potentiate Wnt signaling, including the immediate increase in LRP6 phosphorylation following RSPO treatment and the lack of a superseding effect of FZD overexpression (33). From the proteins that coimmunoprecipitated with LGR4, we identified IQGAP1 and IQGAP3 as potential candidates that could mediate the intracellular signaling of RSPO–LGR4 to the Wnt signalosome. Through a series of biochemical, genetic, and cell biology experiments, we demonstrated that IQGAP1, the most abundant IQGAP in HEK293T cells, is essential for RSPO–LGR4-induced potentiation of Wnt/β-catenin signaling. Interaction between LGR4 and IQGAP1 occurs between the 7TM domain of LGR4 and the GRD of IQGAP1. Stimulation of LGR4 by RSPOs increases the affinity of IQGAP1 to DVL, leading to the formation of a supercomplex between RSPO–LGR4 and the Wnt signalosome. Potentiation of Wnt signaling requires the MEK1/2-binding domain of IQGAP1, which provided us with the hint that LGR4-bound IQGAP1 brings in MEK1/2 to phosphorylate LRP6. Although the supercomplex had been consistently detected with overexpression systems, further studies are needed to confirm the interactions with endogenously expressed receptors using more sensitive methods. Interestingly, the growth factor FGF was also shown to potentiate Wnt/β-catenin signaling through a MEK1/2-dependent pathway (39). Because IQGAP1 also interacts directly with the FGF receptor, it is tempting to speculate that FGF and RSPOs signal in a similar intracellular mechanism to augment the Wnt/β-catenin pathway.

The question is how the RSPO–LGR4–IQGAP1 mechanism relates to the clearance of RNF43/ZNRF induced by RSPO–LGR4. It has been reported that the potencies of the four RSPOs in potentiating Wnt/β-catenin signaling in HEK293T cells are largely governed by their affinities for RNF43/ZNRF3 (32), indicating that inhibition of the E3 ligases is critical to the function of RSPO–LGR4. However, RSPO1 and RSPO4 have affinity for ZNRF3 in the micromolar range and do not promote the formation of ZNRF3–RSPO–LGR4 ternary complex (30); however, both are able to potentiate Wnt/β-catenin signaling through LGR4 (3). Here we found that RSPO1 had a much more potent effect in HEK293T cells when the level of ZNRF3 was reduced and a ZNRF3-binding–defective RSPO1 mutant (Q71A) was fully functional in ZNRF3–KD cells (Fig. 1*C*). These findings suggest that the low-affinity binding of RSPO1, and probably RSPO4, for RNF43/ZNRF3 is not physiologically relevant. Instead, RSPO1/4 may exert their Wnt signaling function primarily on cells with low expression of ZNRF3/RNF43. Conversely, the dominant effect of ZNRF3–ECD over RSPO1–LGR4 activity (Fig. 1 *D* and *E*) indicates that FZD receptors bound by ZNRF3–ECD were prevented from interacting with RSPO–LGR4. Based on these observations, we propose a model (Fig. 6) in which RSPO–LGR4 interacts with IQGAP1 to potentiate both the canonical and noncanonical pathways of Wnt signaling. Binding of RSPO2/3 to LGR4 induces the clearance of RNF43/ZNRF3, leading to the release of Wnt receptors from ubiquitination-mediated degradation and a moderate level of Wnt-signaling activation. The removal of RNF43/ZNRF3 frees up and raises the levels of Wnt receptors, which can then interact with a pool of LGR4 that is bound to IQGAP1 and free of RNF43/ZNRF3. RSPO stimulation of LGR4–IQGAP1 increases in the affinity of IQGAP1 for DVL in the Wnt signalosome, leading to the eventual formation of a supercomplex. In this configuration, IQGAP1 not only engages MEK1/2 to phosphorylate LRP5/6 and significantly enhance canonical Wnt signaling but also recruits actin-polymerization complexes through binding to N-WASP and mDia1 to coordinate actin dynamics (Fig. 6). This dual-mechanism model provides an explanation for the roles of RNF43/ZNRF3 and IQGAP1 in RSPO–LGR4 action and the pleiotropic functions of RSPO–LGR4 signaling in normal and cancer development, particularly for the crucial role of LGR4 in tubule elongation and branching in multiple organs.

Materials and Methods

Plasmids, siRNA, shRNA, Recombinant Proteins, Abs, and Chemical Inhibitors. Plasmids encoding human HA-LGR4, HA-LGR4-ECD-TM, Myc-LGR4, LRP6, and HA-LRP6 were generated as described (3, 34). FLAG-tagged mouse IQGAP1 and truncation mutants were constructed by amplifying the following fragments from mouse IQGAP1 pCMV-sport6 (Open Biosystems): IQGAP1-ΔCT (expressing amino acids 2–865), IQGAP1-ΔNT (expressing amino acids 893–1,657), IQGAP1-ΔWW (deletion of 643–743), IQGAP1-ΔIQ (deletion of 704–905), IQGAP1-ΔGRD (deletion of amino acids 893–1,432), and IQGAP1-GRD (expression of 893–1,432). The PCR products were then subcloned into the pcDNA3.1 vector modified to incorporate an N-terminal FLAG-tag. The Q71A mutant of RSPO1 was generated in the plasmid RSPO1-FC (49) with standard site-directed mutagenesis methodology. Super 8×TopFlash and peGFP-IQGAP1 (50) were purchased from Addgene. pRL-SV40 was purchased from Promega. siRNA targeting human IQGAP1 (J-004694-07) and Smartpool

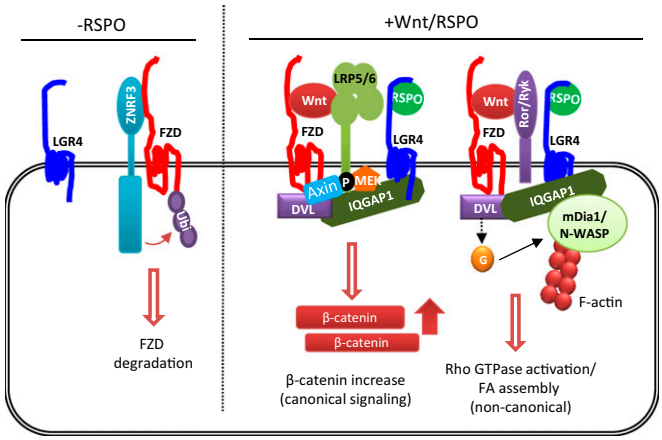

Fig. 6. A schematic diagram illustrating the dual mechanism of RSPO3–LGR4 signaling. In the absence of RSPO, RNF43/ZNRF3 ubiquitinates the FZD receptors for degradation, resulting in low Wnt signaling activity. In the presence of RSPOs, LGR4 recruits IQGAP1 and increases its affinity toward DVL, leading to the formation of a supercomplex with the Wnt signalosome through IQGAP1–DVL interaction. IQGAP1-bound MEK1/2 then phosphorylates LRP5/6, which binds Axin and inhibits its activity in organizing β-catenin phosphorylation. RSPO–LGR4-bound IQGAP1 can also interact with noncanonical Wnt signalosome to coordinate actin dynamics due to IQGAP1's direct binding to actin polymerization machinery, leading to enhanced FA assembly and cell migration.

control was obtained from Dharmacon. The sequence for human IQGAP3 siRNA was acquired from the literature (51). The siRNA targeting human ZNRF3 was purchased from Qiagen. siRNAs targeting DVL1–3 were synthesized based on the sequences described (52).

Recombinant human RSPOs and Wnt3a proteins were purchased from R&D Systems. Wnt3a conditioned media (Wnt3aCM) was produced from L cells as described (3). All commercial Abs were used in accordance to manufacturer's guidelines: anti-LRP6, anti–p-LRP6 (Ser-1490), anti-HA, anti-myc, anti-p42/44, anti–p-p42/44, anti-Dvl2, anti–β-catenin, anti-MEK1/2, anti-FAK, anti–p-FAK, anti-JNK1/2, anti–p-JNK1/2, anti-actin (Cell Signaling); anti-RSPO3 (ProteinTech Group); anti-FLAG (Sigma); anti–N-Wasp (Pierce); anti-mDia1, anti-IQGAP, anti-paxillin, and anti–e-cadherin (BD Biosciences). RSPO3-neutralizing Ab was from R&D Systems. SB203580, U0126, SP600125, and FR180204 were purchased from Tocris.

Mass Spectrometry Analysis. HEK293T cells stably expressing HA-tagged LGR4 were harvested, and their membranes were isolated and completely solubilized by using 1% n-dodecyl β-D-maltoside as described (53). HA-LGR4 was pulled down from total lysate by using anti-HA Ab-labeled agarose beads. The samples were subjected to SDS/PAGE followed by Coomassie blue staining. The gel lane was sliced into lower- and higher-molecular-weight sections and digested. Peptide extraction was performed, and extracted peptides were subjected to mass spectrometry analysis by using the Applied Biosystems QStar Elite tandem LC MS/MS in the proteomics center at the Brown Foundation Institute of Molecular Medicine at University of Texas Health Science Center at Houston.

Cell Culture, Transfection, Stable Cell Line Generation, and Luciferase Assays. HEK293T and HeLa cells were purchased from ATCC. MDCK cells were obtained from Wenliang Li (University of Texas Health Science Center at Houston). A549 and H460 cells were from Bingliang Fang (M. D. Anderson Cancer Center, Houston). Cells were cultured in high-glucose DMEM supplemented with 10% (vol/vol) FBS and penicillin/streptomycin at 37 °C under 95% (vol/vol) humidity/5% (vol/vol) CO_2. Transient transfections were performed by using Fugene HD (Promega) or Dharmafect Duo (Dharmacon). For transfection of siRNA or cotransfection of siRNA with plasmids, Dharmafect Duo was used, and cells were incubated 72 h before lysis. Luciferase assays were performed as described (34). All experiments were performed at least three times with quadruplicates in each experiment.

RT-qPCR, WB, IP, Immunofluorescence, and Confocal Microscopy. Total RNA isolation, RT-qPCR of LGR4–6 and RSPO1–4, and immunofluorescence analysis were carried out as we described (3). All RT-qPCR probes were purchased from Life Technologies. For WB analysis, cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris·Cl, pH 7.4, 150 mM NaCl, 1 mM DTT, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors. HRP-labeled secondary Abs were used for detection along with the standard ECL protocol. For co-IP experiments, cell lysates were incubated overnight at 4 °C with anti-HA–labeled agarose (Pierce), anti-FLAG magnetic beads (Sigma), or primary Ab and protein A/G agarose beads (Santa Cruz). Precipitates were washed with lysis buffer followed by PBS and boiled with 2× SDS sample buffer before loading for SDS/PAGE and WB analysis. All ligand treatments were performed at 50 ng/mL Wnt3a and 10 ng/mL RSPO, unless otherwise stated. For immunofluorescence, HEK293T and MDCK cells transfected with the indicated plasmids or A549 KD cells were reseeded into poly-D-lysine–coated eight-well chamber slides (BD Biosciences) and allowed to adhere overnight. Cells were then washed, fixed with 4% (vol/vol) paraformaldehyde for 15 min, and permeabilized with 0.1% saponin for 10 min. Cells were incubated with anti–HA-Alexa 594 (Invitrogen) or anti–Myc-Cy3 (Sigma) for detection of LGR4 and anti–HA-488 for labeling of HA-ZNRF3-ECD-TM. Confocal microscopy images were collected by using the Leica TSC SP5 system.

Cell Migration and Invasion Assays. For the migration assays, cells (1×10^5) in serum-free DMEM were seeded into the top of a Transwell migration chamber (six-well, 8-μm pore) and allowed to migrate for 16 h with DMEM plus 10% (vol/vol) FBS in the bottom chamber. Migrated cells were counted according the manufacturer's protocol. Invasion assays were carried out by seeding 2,000 cells in BD BioCoat Matrigel invasion chambers (BD Biosciences) with overnight incubation. The number of invaded cells was determined according to the manufacturer's protocol.

ACKNOWLEDGMENTS. We thank Dr. Wenliang Li (University of Texas Health Science Center at Houston) for the MDCK cells, Dr. Bingliang Fang (M. D. Anderson Cancer Center) for A549 and H460 cells, Dr. Calvin Kuo (Stanford University) for the RPSO1-FC plasmid, and Dr. Feng Cong (Novartis) for the ZNRF3-ECDTM plasmid. This work was supported in part by Cancer Prevention and Research Institute of Texas Grant RP100678; National Institutes of Health Grant R01GM102485; the Texas Emerging Technology Fund; and the Janice D. Gordon Endowment for Bowel Cancer Research (to Q.L.).

1. de Lau WB, Snel B, Clevers HC (2012) The R-spondin protein family. *Genome Biol* 13(3):242.
2. Barker N, Clevers H (2010) Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. *Gastroenterology* 138(5):1681–1696.
3. Carmon KS, Gong X, Lin Q, Thomas A, Liu Q (2011) R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. *Proc Natl Acad Sci USA* 108(28):11452–11457.
4. de Lau W, et al. (2011) Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* 476(7360):293–297.
5. Glinka A, et al. (2011) LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signalling. *EMBO Rep* 12(10):1055–1061.
6. Yoon JK, Lee JS (2012) Cellular signaling and biological functions of R-spondins. *Cell Signal* 24(2):369–377.
7. Parma P, et al. (2006) R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat Genet* 38(11):1304–1309.
8. Bergmann C, et al. (2006) Mutations in the gene encoding the Wnt-signaling component R-spondin 4 (RSPO4) cause autosomal recessive anonychia. *Am J Hum Genet* 79(6):1105–1109.
9. Blaydon DC, et al. (2006) The gene encoding R-spondin 4 (RSPO4), a secreted protein implicated in Wnt signaling, is mutated in inherited anonychia. *Nat Genet* 38(11): 1245–1247.
10. Styrkarsdottir U, et al. (2013) Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits. *Nature* 497(7450):517–520.
11. Mazerbourg S, et al. (2004) Leucine-rich repeat-containing, G protein-coupled receptor 4 null mice exhibit intrauterine growth retardation associated with embryonic and perinatal lethality. *Mol Endocrinol* 18(9):2241–2254.
12. Kato S, et al. (2006) Leucine-rich repeat-containing G protein-coupled receptor-4 (LGR4, Gpr48) is essential for renal development in mice. *Nephron, Exp Nephrol* 104 (2):e63–e75.
13. Mendive F, et al. (2006) Defective postnatal development of the male reproductive tract in LGR4 knockout mice. *Dev Biol* 290(2):421–434.
14. Wang Y, et al. (2013) Lgr4 regulates mammary gland development and stem cell activity through the pluripotency transcription factor Sox2. *Stem Cells* 31(9):1921–1931.
15. Bell SM, et al. (2008) R-spondin 2 is required for normal laryngeal-tracheal, lung and limb morphogenesis. *Development* 135(6):1049–1058.
16. Chadi S, et al. (2009) R-spondin1 is required for normal epithelial morphogenesis during mammary gland development. *Biochem Biophys Res Commun* 390(3):1040–1043.
17. Mustata RC, et al. (2011) Lgr4 is required for Paneth cell differentiation and maintenance of intestinal stem cells ex vivo. *EMBO Rep* 12(6):558–564.
18. Seshagiri S, et al. (2012) Recurrent R-spondin fusions in colon cancer. *Nature* 488 (7413):660–664.
19. Lowther W, Wiley K, Smith GH, Callahan R (2005) A new common integration site, Int7, for the mouse mammary tumor virus in mouse mammary tumors identifies a gene whose product has furin-like and thrombospondin-like sequences. *J Virol* 79 (15):10093–10096.
20. Theodorou V, et al. (2007) MMTV insertional mutagenesis identifies genes, gene families and pathways involved in mammary cancer. *Nat Genet* 39(6):759–769.
21. Starr TK, et al. (2009) A transposon-based genetic screen in mice identifies genes altered in colorectal cancer. *Science* 323(5922):1747–1750.
22. Klauzinska M, et al. (2012) Rspo2/Int7 regulates invasiveness and tumorigenic properties of mammary epithelial cells. *J Cell Physiol* 227(5):1960–1971.
23. Gao Y, et al. (2006) Up-regulation of GPR48 induced by down-regulation of p27Kip1 enhances carcinoma cell invasiveness and metastasis. *Cancer Res* 66(24):11623–11631.
24. Gugger M, et al. (2008) GPR87 is an overexpressed G-protein coupled receptor in squamous cell carcinoma of the lung. *Dis Markers* 24(1):41–50.
25. Yi J, et al. (2013) Analysis of LGR4 receptor distribution in human and mouse tissues. *PLoS ONE* 8(10):e78144.
26. Hsu SY, et al. (2000) The three subfamilies of leucine-rich repeat-containing G protein-coupled receptors (LGR): Identification of LGR6 and LGR7 and the signaling mechanism for LGR7. *Mol Endocrinol* 14(8):1257–1271.
27. Hao HX, et al. (2012) ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature* 485(7397):195–200.
28. Koo BK, et al. (2012) Controlled gene expression in primary Lgr5 organoid cultures. *Nat Methods* 9(1):81–83.
29. Chen PH, Chen X, Lin Z, Fang D, He X (2013) The structural basis of R-spondin recognition by LGR5 and RNF43. *Genes Dev* 27(12):1345–1350.
30. Moad HE, Pioszak AA (2013) Reconstitution of R-spondin:LGR4:ZNRF3 adult stem cell growth factor signaling complexes with recombinant proteins produced in Escherichia coli. *Biochemistry* 52(41):7295–7304.
31. Peng WC, et al. (2013) Structures of Wnt-antagonist ZNRF3 and its complex with R-Spondin 1 and implications for signaling. *PLoS ONE* 8(12):e83110.
32. Zebisch M, et al. (2013) Structural and molecular basis of ZNRF3/RNF43 transmembrane ubiquitin ligase inhibition by the Wnt agonist R-spondin. *Nat Commun* 4:2787.
33. Wei Q, et al. (2007) R-spondin1 is a high affinity ligand for LRP6 and induces LRP6 phosphorylation and beta-catenin signaling. *J Biol Chem* 282(21):15903–15911.

34. Carmon KS, Lin Q, Gong X, Thomas A, Liu Q (2012) LGR5 interacts and cointernalizes with Wnt receptors to modulate Wnt/β-catenin signaling. *Mol Cell Biol* 32(11): 2054–2064.
35. Noritake J, Watanabe T, Sato K, Wang S, Kaibuchi K (2005) IQGAP1: A key regulator of adhesion and migration. *J Cell Sci* 118(Pt 10):2085–2092.
36. White CD, Erdemir HH, Sacks DB (2012) IQGAP1 and its binding proteins control diverse biological functions. *Cell Signal* 24(4):826–834.
37. Roy M, Li Z, Sacks DB (2005) IQGAP1 is a scaffold for mitogen-activated protein kinase signaling. *Mol Cell Biol* 25(18):7940–7952.
38. Sacks DB (2006) The role of scaffold proteins in MEK/ERK signalling. *Biochem Soc Trans* 34(Pt 5):833–836.
39. Červenka I, et al. (2011) Mitogen-activated protein kinases promote WNT/beta-catenin signaling via phosphorylation of LRP6. *Mol Cell Biol* 31(1):179–189.
40. Favata MF, et al. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 273(29):18623–18632.
41. Goto T, et al. (2013) IQGAP1 functions as a modulator of dishevelled nuclear localization in Wnt signaling. *PLoS ONE* 8(4):e60865.
42. Schlessinger K, Hall A, Tolwinski N (2009) Wnt signaling pathways meet Rho GTPases. *Genes Dev* 23(3):265–277.
43. Brandt DT, Grosse R (2007) Get to grips: Steering local actin dynamics with IQGAPs. *EMBO Rep* 8(11):1019–1023.
44. Parsons JT, Horwitz AR, Schwartz MA (2010) Cell adhesion: Integrating cytoskeletal dynamics and cellular tension. *Nat Rev Mol Cell Biol* 11(9):633–643.
45. Benseñor LB, et al. (2007) IQGAP1 regulates cell motility by linking growth factor signaling to actin assembly. *J Cell Sci* 120(Pt 4):658–669.
46. Endo Y, et al. (2005) Wnt-3a-dependent cell motility involves RhoA activation and is specifically regulated by dishevelled-2. *J Biol Chem* 280(1):777–786.
47. Barretina J, et al. (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 483(7391):603–607.
48. Ruffner H, et al. (2012) R-Spondin potentiates Wnt/β-catenin signaling through orphan receptors LGR4 and LGR5. *PLoS ONE* 7(7):e40976.
49. Ootani A, et al. (2009) Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nat Med* 15(6):701–706.
50. Ren JG, Li Z, Crimmins DL, Sacks DB (2005) Self-association of IQGAP1: Characterization and functional sequelae. *J Biol Chem* 280(41):34548–34557.
51. Liu Z, et al. (2010) IQGAP1 plays an important role in the invasiveness of thyroid cancer. *Clin Cancer Res* 16(24):6009–6018.
52. Li L, Mao J, Sun L, Liu W, Wu D (2002) Second cysteine-rich domain of Dickkopf-2 activates canonical Wnt signaling pathway via LRP-6 independently of dishevelled. *J Biol Chem* 277(8):5977–5981.
53. Daulat AM, Maurice P, Jockers R (2011) Tandem affinity purification and identification of GPCR-associated protein complexes. *Methods Mol Biol* 746:399–409.