

LGR4 and LGR5 are R-spondin receptors mediating Wnt/ β -catenin and Wnt/PCP signalling

Andrei Glinka^{1*}, Christine Dolde^{1*}, Nadine Kirsch^{1*}, Ya-Lin Huang^{1*}, Olga Kazanskaya¹, Dierk Ingelfinger², Michael Boutros², Cristina-Maria Cruciati¹⁺ & Christof Niehrs^{1,3++}

¹Division of Molecular Embryology, DKFZ-ZMBH Alliance, DKFZ, ²Division of Signalling and Functional Genomics, DKFZ and University of Heidelberg, Heidelberg, Germany, and ³Institute of Molecular Biology, Mainz, Germany

R-spondins are secreted Wnt signalling agonists, which regulate embryonic patterning and stem cell proliferation, but whose mechanism of action is poorly understood. Here we show that R-spondins bind to the orphan G-protein-coupled receptors LGR4 and LGR5 by their Furin domains. Gain- and loss-of-function experiments in mammalian cells and *Xenopus* embryos indicate that LGR4 and LGR5 promote R-spondin-mediated Wnt/ β -catenin and Wnt/PCP signalling. R-spondin-triggered β -catenin signalling requires Clathrin, while Wnt3a-mediated β -catenin signalling requires Caveolin-mediated endocytosis, suggesting that internalization has a mechanistic role in R-spondin signalling.

Keywords: endocytosis; LGR4; LGR5; R-spondin; Wnt
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INTRODUCTION

Wnts have a critical role in development and disease, and understanding their complex signalling mechanisms and biological roles is of wide interest (Nusse, 2005; Grigoryan *et al*, 2008). Besides Wnts, secreted R-spondins (Rspo1–4; roof plate-specific spondin) potently enhance β -catenin signalling (Kazanskaya *et al*, 2004; Kim *et al*, 2005). They are involved in embryonic patterning and differentiation in frogs and mice (Kazanskaya *et al*, 2004, 2008; Aoki *et al*, 2006; Blaydon *et al*, 2006; Parma *et al*, 2006). R-spondins are also implicated in human disease and hold therapeutic promise as potent stem cell growth factors (Kim *et al*, 2005; Blaydon *et al*, 2006; Parma *et al*, 2006; Zhao *et al*, 2009). R-spondins synergize with Wnts and Frizzled (Fz), and require the presence of Wnts to activate β -catenin signalling (Kazanskaya *et al*, 2004; Nam *et al*, 2006; Kim *et al*, 2008).

¹Division of Molecular Embryology, DKFZ-ZMBH Alliance, DKFZ, Im Neuenheimer Feld 280, and

²Division of Signalling and Functional Genomics, DKFZ and University of Heidelberg, Im Neuenheimer Feld 580, Heidelberg D-69120, Germany

³Institute of Molecular Biology, Mainz 55128, Germany

*These authors contributed equally to this work

+Corresponding author. Tel: +49 6221 42 4391; Fax: +49 6221 42 4692; E-mail: c.cruciati@dkfz-heidelberg.de

++Corresponding author. Tel: +49 6221 42 4690; Fax: +49 6221 42 4692; E-mail: niehrs@dkfz-heidelberg.de

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The mechanism of R-spondin signalling is poorly understood and notably the identity of their receptor is controversial. Frizzled, LRP6 and Kremen have been variably proposed and refuted (Wei *et al*, 2007, see also Fig 1A; Nam *et al*, 2006; Binnerts *et al*, 2007). We have recently shown that Rspo3 binds to syndecans, probably as co-receptors, to transduce Wnt/PCP signalling (Ohkawara *et al*, 2011).

In a genome-wide small interfering RNA (siRNA) screen for R-spondin receptors, we have identified leucine-rich repeat containing G-protein-coupled receptor 5 (LGR5). Here we show that LGR4 and LGR5 function as R-spondin receptors in Wnt/ β -catenin and Wnt/PCP signalling. Furthermore, we provide evidence for an important role of endocytosis, where R-spondin-triggered β -catenin signalling requires Clathrin, while Wnt3a-mediated β -catenin signalling requires Caveolin-mediated internalization.

RESULTS AND DISCUSSION

LGR4 and LGR5 promote R-spondin signalling

In a search for an R-spondin receptor we carried out a genome-wide siRNA screen (Cruciati *et al*, 2010). In brief, human embryonic kidney (HEK293T) cells were transfected with siRNA pools targeting about 18,500 human genes and analysed for transcription of a Wnt-responsive luciferase reporter. Cells were stimulated with six different Wnt activators, two of which were recombinant Rspo3, and transfected *Wnt1 + Rspo3*. When the hits were sorted for genes with high score in both R-spondin regimes, and low in the other four Wnt stimulations, the orphan leucine-rich repeat containing G-protein-coupled receptor 5 was the number one hit (supplementary Table S1 online). LGR4 and LGR5 are related orphan receptors characterized by the presence of seven transmembrane domains and an extracellular domain containing leucine-rich repeat motifs, implicated in stem cell biology (Barker & Clevers, 2010; Mustata *et al*, 2011). Knock down of LGR4 and LGR5 by siRNA inhibited Wnt/ β -catenin signalling in TOPFLASH reporter assays when stimulated by Wnt3a, Rspo3 or their combination (Fig 1A). In contrast, siLGR4/5 did not inhibit TOPFLASH reporter stimulated intracellularly by constitutively active LRP6 (LRP6 Δ E1–4), Dvl1 or β -catenin (Fig 1B). The siLGR4 and -5 effects were rescued by co-transfection with LGR4 cDNA, attesting specificity (supplementary Fig S1A,B online). Limiting doses of Wnt3a and Rspo1 strongly synergized in Wnt signalling

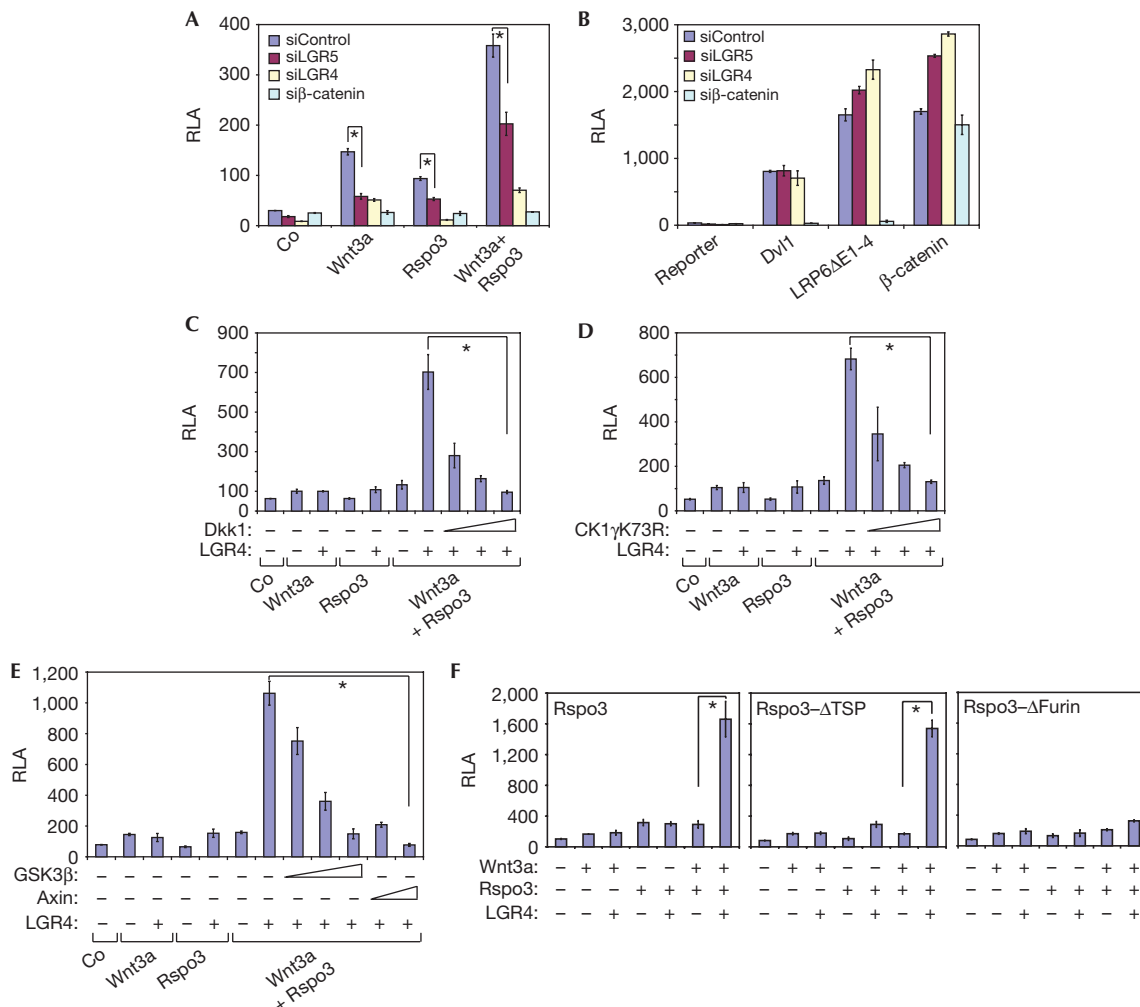


Fig 1 | LGR4 and LGR5 are new regulators of Wnt and R-spondin signalling. (A–F) Wnt luciferase reporter assays in HEK293T cells stimulated with the indicated constructs or Wnt3a and/or Rspo3-ΔC, or Rspo3-ΔC + ΔTSP, or Rspo3-ΔC + ΔFurin-conditioned medium, in the presence of the indicated small interfering RNAs. Co, control medium. RLA, relative luciferase activity. TSP, thrombospondin. Error bars indicate s.d. values, $n = 3$; * indicates $P < 0.001$ by Student's t -test.

when co-transfected with *LGR4* or *LGR5* (supplementary Fig S2A,B online). R-spondin–LGR4 signalling was inhibited by the LRP6 antagonist *Dkk1*, dominant-negative *Casein kinase 1γ*, *GSK3β* and *Axin* (Fig 1C–E). This indicates that the R-spondin–LGR4 complex functions upstream or at the level of LRP6 signalling. Consistent with this, R-spondin–LGR4/5 signalling was inhibited by siLRP6 (supplementary Fig S2C,D online).

R-spondins contain two Furin type domains, which are essential for Wnt/β-catenin signalling (Kazanskaya et al, 2004), and a thrombospondin type 1 domain (TSP1), which in the case of Rspo3 mediates PCP signalling and binds to syndecans (Ohkawara et al, 2011). Rspo3 deletion mutants lacking the TSP1 domain signalled with LGR4, while ΔFurin mutants failed to activate the reporter (Fig 1F). We conclude that Rspo3 signalling by the β-catenin pathway requires LGR4 and LGR5, and is mediated by the Furin domains.

LGR4 and LGR5 bind to R-spondins

As LGR4 and LGR5 are transmembrane proteins and are required for Rspo3 signalling epistatically at the Wnt receptor level, we

tested for direct interaction. In cell surface binding assays, alkaline-phosphatase (AP) fusion proteins of Rspo1,2,3,4 bound to cells transfected with *LGR4* and -5 (Fig 2A). Weaker binding was found with *LGR5*-transfected cells, but this might reflect its low expression (not shown) rather than a major difference in affinity (see below). Rspo2 and Rspo3 bound to syndecan 4 (SDC4), while no R-spondin binding was detected with *LRP6*-transfected cells, which however bound recombinant *Dkk1*. Rspo3 deletion mutants lacking the TSP1 domain also bound to *LGR4/5*-transfected cells, while ΔFurin mutants failed to bind, consistent with the requirement of the Furin domains in Wnt reporter assays. The reverse was true for binding of Rspo3 deletion mutants to *SDC4*-transfected cells. Using immunopurified LGR4 and -5 and purified recombinant hRspo3–AP, we determined their apparent K_d as 2.2 and 3.0 nM, respectively (Fig 2B,C). Taken together with the functional data, these results indicate that LGR4 and LGR5 function as R-spondin receptors.

Clathrin endocytosis is required for Rspo3 signalling

There is a good evidence that Wnt signalling proceeds by an endocytic compartment and that Wnt–receptor complex

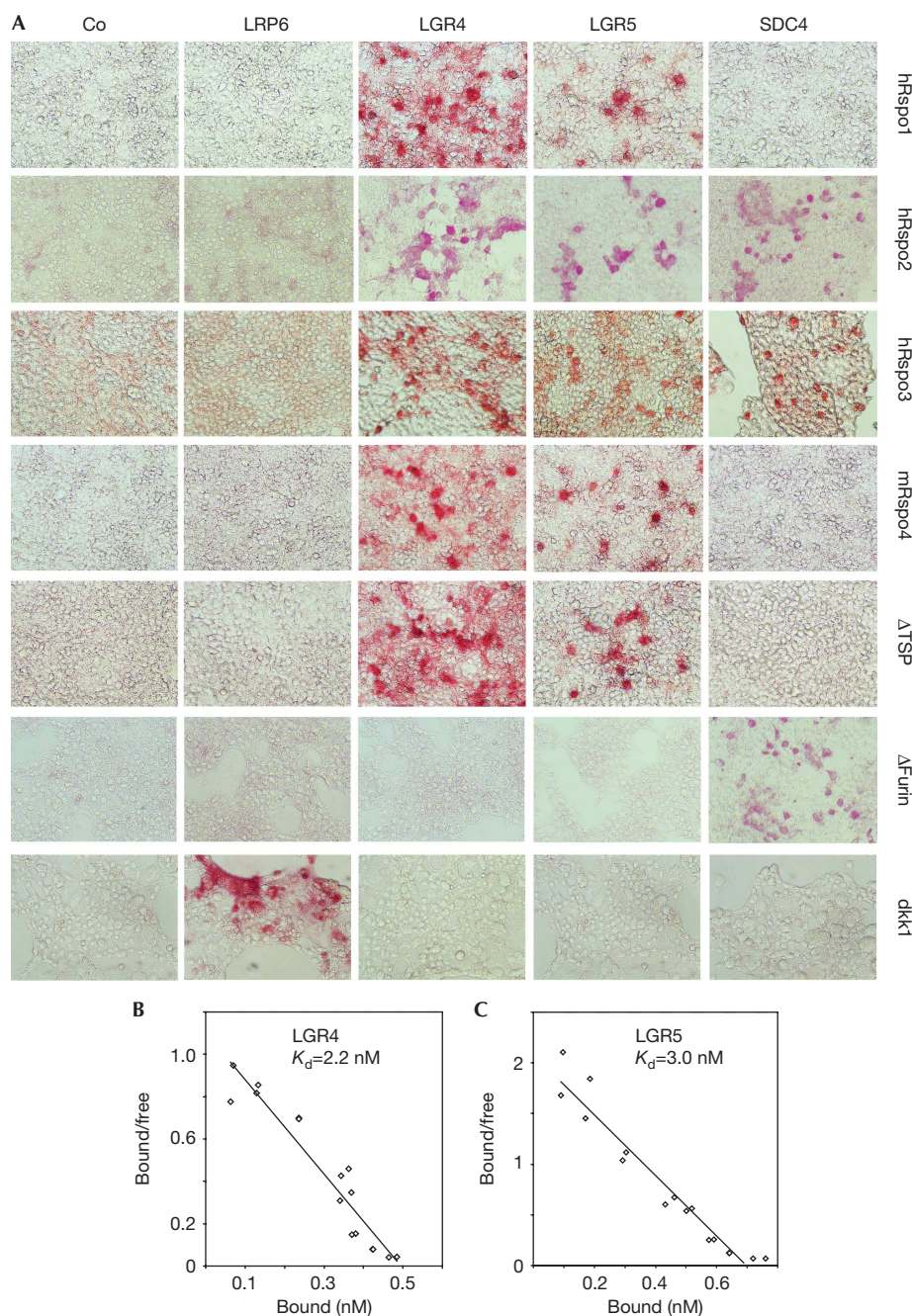


Fig 2 | R-spondins bind to LGR4 and LGR5. (A) Cell surface binding assay. Cells were transfected with the indicated plasmids and subjected to binding assays with conditioned medium containing alkaline-phosphatase (AP) fusion proteins of R-spondins- Δ C, Dkk1 or hRspo3- Δ C deletion constructs (Δ TSP and Δ Furin). (B,C) Scatchard plot analysis of *in vitro* binding assay using LGR4, LGR5 and hRspo3- Δ C-AP. Co, control medium; SDC4, syndecan 4; TSP, thrombospondin.

internalization is an essential step in both canonical and non-canonical Wnt signalling (reviewed in Kikuchi & Yamamoto (2007)). Furthermore, Rspo3 PCP signalling requires Clathrin-mediated endocytosis (Ohkawara *et al*, 2011). We therefore asked whether Rspo3 and LGR4 are co-internalized and whether endocytosis is also essential for R-spondin signalling by the β -catenin pathway. Internalization assays with recombinant

Rspo3-horseradish peroxidase (HRP) fusion protein showed that the protein is endocytosed within 1 h of application (Fig 3A,B) and this was competed by unlabelled Rspo3 (Fig 3C). Rspo3 colocalized in vesicular structures with LGR4-green fluorescent protein (GFP, Fig 3D). Internalized Rspo3 colocalized with Clathrin, but not with Caveolin endocytic vesicles (Fig 3E,F). Furthermore, Rspo3 endocytic vesicles were positive for the early

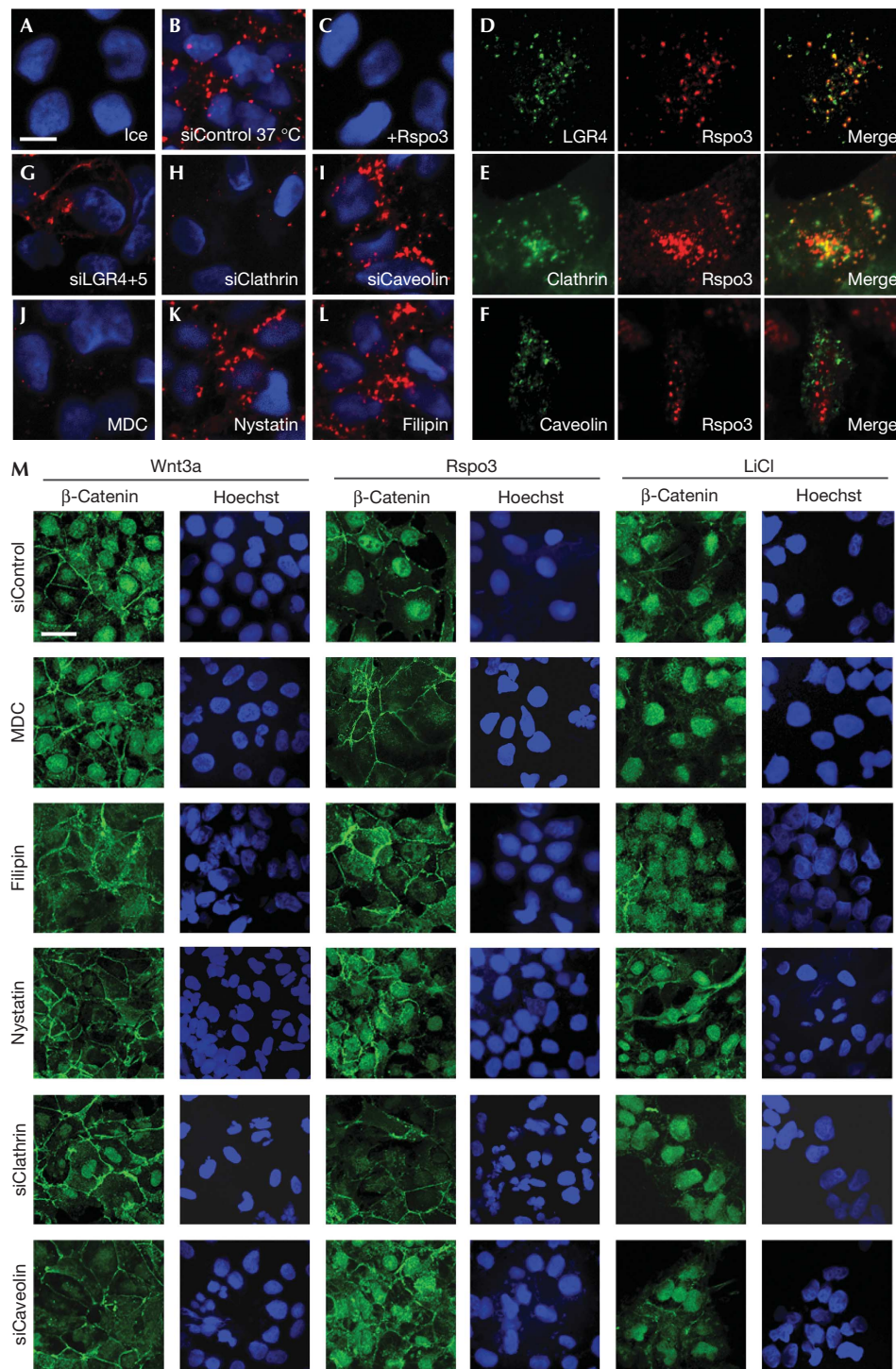


Fig 3 | Clathrin endocytosis is required for Rspo3 signalling. (A–L) Clathrin is colocalized with Rspo3 and is required for Rspo3 internalization. Confocal microscopy of HepG2 cells treated for 1 h with horseradish peroxidase (HRP)-tagged Rspo3-ΔC on ice (A), or at 37 °C (B,D–L), or in the presence of Flag-tagged Rspo3-ΔC (C). HRP-tagged Rspo3-ΔC was visualized by tyramide signal amplification without or with coimmunofluorescence of anti-green fluorescent protein (GFP) antibody against overexpressed *xtLGR4-EYFP* (D), *Clathrin-GFP* (E) and *Caveolin-GFP* (F). Where indicated, cells were pretreated with small interfering RNAs (siRNAs) for 4 days (B,G–I) or with endocytic inhibitors for 1 h (J–L). (M) Clathrin is required for Rspo3-induced nuclear β-catenin accumulation. Confocal microscopy of NTERA2 cells incubated for 1 h with endocytic inhibitors or pretreated with the indicated siRNAs for 3 days. Cells were then treated for 4 h with Wnt3a, or Wnt3a together with Rspo3, or 50 mM LiCl. Scale bars indicate 10 μm. MDC, monodansylcadaverine.

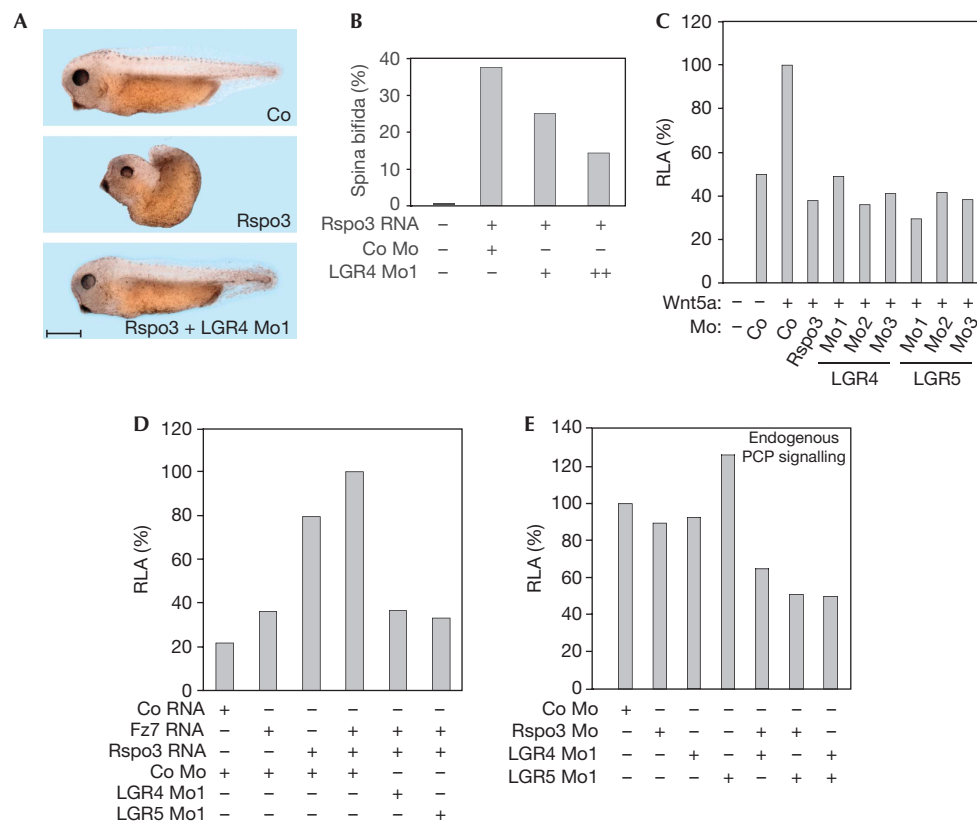


Fig 4 | LGR4 and LGR5 are required for Wnt/PCP signalling in *Xenopus*. (A,B) Rspo3 signalling requires LGR4 to induce gastrulation defects. Embryos were injected equatorially at 4-cell stage with morpholinos and/or messenger RNA (mRNA) as indicated (250 pg *xRspo3* mRNA, 10 ng (+) and 20 ng (++) of *LGR4* Mo1). Scale bar indicates 500 μ m. (C-E) ATF2-luciferase reporter activity is reduced by *LGR4/5* Mos in *Xenopus* embryos. Embryos were injected equatorially with ATF2-luc reporter (100 pg) and *Renilla* reporter plasmids (25 pg) and the indicated morpholinos (20 ng *LGR4* Mo1, 5 ng *LGR5* Mo1 in E) and mRNAs. Luciferase reporter assays were performed from whole embryos collected at gastrula stage. Luciferase activity in embryos injected with either Co Mo or Co Mo plus mRNA of the indicated activators within each condition were set to 100%. RLA, relative luciferase activity. All experiments were performed at least twice with three replicates each. Co, control medium; Mo, morpholino oligonucleotide.

endosomal markers EEA1, but not the late endosomal marker LAMP (supplementary Fig S3A,B online). Rspo3 endocytosis was partially blocked by *siLGR4+5* (Fig 3G). Consistent with colocalization, *siClathrin* and the Clathrin inhibitor monodansylcadaverine (MDC) blocked Rspo3 internalization, unlike inhibitors of Caveolin-mediated endocytosis including filipin, nystatin and *siCaveolin* (Fig 3H-L).

We tested whether Rspo3 internalization was required for Wnt signalling. As a short-term signalling read-out we monitored β -catenin nuclear accumulation. Cells were stimulated either with Wnt3a or Rspo3 in the presence of a tonic Wnt3a dose, which by itself did not induce nuclear β -catenin (not shown). Nuclear β -catenin accumulation following Wnt3a stimulation was inhibited by the Caveolin endocytic inhibitors filipin and nystatin, as well as by *siCaveolin* treatment (Fig 3M; supplementary Fig S4A,B online). Conversely, Rspo3 signalling was inhibited by MDC and *siClathrin*. Nuclear β -catenin accumulation following LiCl stimulation, which blocks GSK3 intracellularly, was unaffected by any treatment. We confirmed these findings by monitoring soluble β -catenin levels (supplementary Fig S4C online).

Taken together, these results indicate (i) that Rspo3 and LGR4 are co-internalized, (ii) that internalization occurs by

Clathrin-mediated endocytosis, which (iii) is an obligatory step in Rspo3 β -catenin signalling. In contrast, Wnt3 signalling requires Caveolin-mediated internalization.

LGR4 and LGR5 mediate PCP signalling in *Xenopus*

We recently showed that Rspo3 amplifies not only β -catenin, but also Wnt/PCP signalling, namely in *Xenopus* embryos (Ohkawara et al, 2011). To test whether LGR4 and LGR5 are required for R-spondin PCP signalling we analysed *Xenopus tropicalis* embryos. *LGR4* and *-5* are maternally expressed and their zygotic transcripts increased after gastrula stage (supplementary Fig S5A online). *LGR4* was expressed in all three germ layers, while *LGR5* was expressed predominantly in endoderm (supplementary Fig S5B online).

A hallmark of Rspo3 signalling in early *Xenopus* embryos is that its messenger RNA (mRNA) injection induces misgastrulation and spina bifida, due to exaggerated PCP signalling (Ohkawara et al, 2011; Fig 4A). Importantly, the spina bifida phenotype was rescued by coinjection with an antisense morpholino oligonucleotide (Mo1) targeting the 5' untranslated region of *LGR4* (Fig 4A,B). This indicates that LGR4 is required for Rspo3 signalling *in vivo*. *LGR5* Mo1 failed to rescue the *Rspo3*

overexpression phenotype (not shown), probably because the gene is expressed predominantly in endoderm, while gastrulating cells are mesodermal.

We designed two more antisense Mos each against *LGR4* and *LGR5* to validate Mo specificities, as the mRNA rescue of the *LGR4/5* Mos proved technically difficult (not shown). Mos2 targeted different 5' regions for translation inhibition and Mos3 each targeted a splice site of the *LGR4* and *LGR5* pre-mRNAs. We individually injected the six *LGR4/5* Mos into *Xenopus* embryos and monitored Wnt/PCP signalling by analysing expression of ATF2-luciferase, which in early *Xenopus* embryos is a specific reporter for Wnt/PCP signalling (Ohkawara & Niehrs, 2011). When PCP signalling was stimulated by coinjection with *Wnt5a* mRNA, reporter activity was inhibited by *Rspo3* Mo (Fig 4C), as recently described (Ohkawara *et al*, 2011). Likewise, all six *LGR4* and *LGR5* Mos inhibited ATF2-luciferase activity (Fig 4C). Similarly, PCP signalling stimulated by coinjection of *Rspo3* and *Fz7* mRNAs was inhibited by *LGR4/5* Mos (Fig 4D). In contrast, neither *LGR4* nor *LGR5* Mo inhibited signalling by Nodal or BMP4 (supplementary Fig S5C,D online), further attesting specificity. Finally, low Mo doses were injected and endogenous PCP signalling from unstimulated embryos was monitored: reporter activity was inhibited when *Rspo3* Mo was combined with either *LGR4* or *LGR5* Mo, indicating functional interaction, or when *LGR4* and *LGR5* Mo were coinjected, indicating functional redundancy (Fig 4E). This Mo synergy corroborates once again their specificity. We conclude that *LGR4* and *LGR5* are required for R-spondin signalling by the Wnt/PCP pathway in *Xenopus* embryos.

We provide independent lines of evidence that *LGR4* and *LGR5* function as *Rspo3* receptors, including reporter assay epistasis experiments, cell surface binding, recombinant protein binding, coendocytosis and functional interaction in *Xenopus*. While this study was underway two reports came to the same conclusion (Carmon *et al*, 2011; de Lau *et al*, 2011). As a minor difference, inhibition of Wnt3a signalling by si*LGR4* was not observed by de Lau *et al* (2011), but we used an siRNA pool, which might be more effective.

We provide further evidence that *LGR4/5* not only mediate R-spondin signalling by the Wnt/ β -catenin but also the Wnt/PCP pathway. SDC4 mediates PCP signalling and binds to the R-spondin TSP1 domain, while *LGR4/5* bind to the Furin domains. This suggests that R-spondins bridge between syndecans and *LGR4/5* in the case of PCP signalling. As *LGR4/5* associate with Fz and LRP6 in β -catenin signalling (Carmon *et al*, 2011; de Lau *et al*, 2011), the specificity of R-spondin to signal either by β -catenin or the PCP pathway might be determined by the presence of available Wnt/Fz combinations as well as of syndecans.

LGR4 is cointernalized with *Rspo3* by Clathrin-mediated endocytosis. Our finding that Clathrin is essential for *Rspo3*-triggered β -catenin signalling is consistent with the model that GSK3 bound to LRP6 signalosomes needs to be sequestered in multivesicular bodies to allow accumulation of β -catenin (Taelman *et al*, 2010). If internalization is a rate-limiting step in Wnt signalling, it is likely that the used endocytic route has an important role in determining the rate and/or effectiveness of GSK3 sequestration and hence downstream signalling. As we show, *Rspo3* and Wnt3a have a differential requirement for Clathrin- and Caveolin-mediated endocytosis, respectively. This raises the possibility that R-spondin/*LGR4/5* amplify low-level

Wnt signalling because GSK3 is more effectively sequestered if LRP6 signalosomes internalize by Clathrin than by Caveolin. The fact that LRP6 phosphorylation at the GSK3 site is enhanced by R-spondin (Carmon *et al*, 2011) is not inconsistent with this possibility.

METHODS

Constructs. Human *LGR5* (BC096324) and *Xenopus tropicalis LGR4* (BC158183) complementary DNAs were obtained from Source BioScience. Tagged *LGR4/5* constructs were generated by inserting *LGR4/5* into a pCS-based vector containing a V5 or Flag epitope after the signal peptide of mouse *krm2*.

Cell culture, conditioning of media, cell surface binding, Wnt reporter and *Xenopus* assays were carried out as described (Cruciat *et al*, 2010; Ohkawara *et al*, 2011). In Wnt reporter assays performed in HEK293T cells, if not indicated elsewhere, the amounts of transfected plasmids per 96-well were 1 ng *Xenopus tropicalis LGR4*; 5 ng *Flag-LGR5*; 1, 2, 5 ng *Dkk1*; 5, 10, 20 ng *CK1 γ 1(K73R)* and *GSK3 β* and 1, 5 ng mouse *Axin-HA*. For stimulation with recombinant proteins, a limiting amount of Wnt3a, *Rspo3*- Δ C, *Rspo3*- Δ C + Δ TSP and *Rspo3*- Δ C + Δ Furin-conditioned medium was used. siRNAs targeting the open reading frame of *LGR4* and *LGR5* (supplementary Table S2 online) were from Thermo Scientific Dharmacon.

In vitro binding assay. N-V5-tagged *LGR4/5* were transfected in HEK293T cells using Xtreme 9 (Roche). After 2 days, membrane fractions were prepared and extracted with buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2% NP40, proteinase inhibitor mix (Roche)). Binding, purification, blocking and washing steps were done as described (Ohkawara *et al*, 2011). In brief, for binding, 96-well white plates (Greiner) were coated overnight at 4 °C with 100 μ l of 2 μ g/ml anti-V5 antibody (Invitrogen) in bicarbonate buffer (50 mM NaHCO₃, pH 9.6). After blocking and washing steps, N-V5-*LGR4/5*-containing extracts were applied to a plate overnight at 4 °C. Following extensive washing, partially purified h*Rspo3*- Δ C-AP fusion protein of known concentration was applied for 2 h. After washes, AP activity was measured and analysed by Scatchard plot using Excel.

Rspo3 internalization assay, endocytic inhibitors and immunostaining. For immunofluorescence, cells were grown in 24-well plates on collagen-treated glass coverslips. siRNA transfection was carried out with DharmaFECT1 (Dharmacon) and either 500 nM (si*Clathrin* and si*Caveolin*) or 1,000 nM (si*LGR4* + 5) siRNAs were used. DNA transfection was performed by Eugene 6 (Roche) with the following amounts of DNA per well: 10 ng *Caveolin-GFP*, 10 ng *Clathrin-GFP* and 100 ng *Xenopus tropicalis LGR4-EYFP*. For endocytosis inhibition, cells were pretreated with MDC (100 μ M, Sigma), filipin III (3 μ g/ml, Sigma), nystatin (25 μ g/ml, Sigma), or 2% dimethylsulphoxide for 1 h, and this treatment continued for 1 h in the presence of h*Rspo3*- Δ C-HRP. For the *Rspo3* internalization assay, h*Rspo3*- Δ C-HRP (25 U/ μ l) was used and detected by tyramide signal amplification (TSA; Dubois *et al*, 2001; Speel *et al*, 2006). After 1 h incubation with *Rspo3* at 37 °C, cells were washed three times with cold Hank's buffer and fixed with 0.5 mM dithiobis[succinimidyl propionate] (DSP) in Hanks's 10 mM HEPES, pH 7.2, for 30 min and then were permeabilized with 0.1% saponin in TSA buffer (100 mM Tris, pH 8.8, 10 mM imidazole). The TSA reaction was carried out for 30 min in dark with the following development solution:

30 μ M tyramide-Rhodamine and 0.003% H₂O₂ in TSA buffer. Cells were washed with TSA buffer, followed by immunofluorescence staining where indicated, stained with Hoechst and embedded in Mowiol. For immunostaining, anti-GFP (Invitrogen), anti- β -catenin (BD Bioscience) and Alexa-488-conjugated antibodies (Invitrogen) were used.

Xenopus mRNA and Morpholino injections. The mRNA doses for injections were as follows (per embryo): 250 pg *Fz7*, 250 pg *Rspo3*, 500 pg *Wnt5a*, 400 pg *BMP4* and 400 pg *xnr1*. The antisense *Mos* (supplementary Table S2 online) were used as follows, if not described otherwise (per embryo): 40 ng *LGR4* Mo1, 10 ng *LGR4* Mo2/3, 10 ng *LGR5* Mo1, 5 ng *LGR5* Mo2, 2.5 ng *LGR5* Mo3, 10 ng *Rspo3* Mo (Ohkawara *et al*, 2011) and 2.5 ng *LRP6* Mo (Hassler *et al*, 2007). Equal amounts of total Mo/RNA were injected by adjustment with the standard control Mo (Gene Tools) or *PPL* RNA, where necessary.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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