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Rspo3 Binds Syndecan 4 and Induces Wnt/PCP Signaling via Clathrin-Mediated Endocytosis to Promote Morphogenesis

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SUMMARY

The *R-Spondin* (*Rspo*) family of secreted Wnt modulators is involved in development and disease and holds therapeutic promise as stem cell growth factors. Despite growing biological importance, their mechanism of action is poorly understood. Here, we show that Rspo3 binds syndecan 4 (Sdc4) and that together they activate Wnt/PCP signaling. In *Xenopus* embryos, Sdc4 and Rspo3 are essential for two Wnt/PCP-driven processes—gastrulation movements and head cartilage morphogenesis. Rspo3/PCP signaling during gastrulation requires Wnt5a and is transduced via Fz7, Dvl, and JNK. Rspo3 functions by inducing Sdc4-dependent, clathrin-mediated endocytosis. We show that this internalization is essential for PCP signal transduction, suggesting that endocytosis of Wnt-receptor complexes is a key mechanism by which R-spondins promote Wnt signaling.

INTRODUCTION

Wnt growth factors play a pivotal role in development and disease, and understanding their complex signaling mechanisms and biological roles is of wide interest (Grigoryan et al., 2008; Nusse, 2005). Besides Wnts, *R-spondins* (*Rspo1-4;* roof plate–specific spondin) encode a family of secreted proteins in vertebrates, which can potently activate β-catenin signaling (Kazanskaya et al., 2004; Kazanskaya et al., 2008; Kim et al., 2005). *R-spondins* show specific embryonic expression patterns and are often coexpressed with and induced by Wnts (Kamata et al., 2004; Kazanskaya et al., 2004; Nam et al., 2007), suggesting that they serve as positive feedback modulators of local Wnt signals. They are involved in embryonic patterning and differentiation in frogs and mice (Aoki et al., 2006; Blaydon et al., 2006; Kazanskaya et al., 2004; Kim et al., 2005; Kishigami 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 (Blaydon et al., 2006; Kim et al., 2005; Parma et al., 2006; Zhao et al., 2009). Of importance for this study is Rspo3, which is involved in vasculogenesis and angiogenesis in *Xenopus* and mouse development (Aoki et al., 2006; Kazanskaya et al., 2008).

R-spondins synergize with Wnts and Fz and indeed require the presence of Wnts to activate β-catenin signaling (Kazanskaya et al., 2008; Kim et al., 2008b; Nam et al., 2006). Their involvement in other Wnt pathways, notably the Wnt/PCP pathway, has not been reported. R-spondin family members encode ~30 kDa proteins, which show high structural similarity and about 60% overall sequence homology. They all contain a C-terminal thrombospondin I domain and two N-terminal Furin-like cystein rich domains, which are present in certain proteases and growth factors, such as IGF.

The mechanism of R-spondin signaling is poorly understood; notably, the identity of their receptor is controversial. Initially, it was suggested that R-spondins interact with Fz8 (Nam et al., 2006), but we and others were unable to detect significant Fz interaction (Wei et al., 2007) (see also Figure 1A). More recently, the Dickkopf1 (Dkk1) receptor Kremen was reported to bind Rspo1 (Binnerts et al., 2007), but this may not be physiologically relevant, because first, unlike most R-spondin single mutant mice, even Kremen1,2 double homozygous mutants are viable and second, Kremen1,2 double homozygous mutant fibroblasts respond normally to R-spondin (Ellwanger et al., 2008). Third, the Wnt coreceptor LRP6 was proposed as R-spondin receptor (Wei et al., 2007), but the reported binding was not confirmed (Binnerts et al., 2007). Notably, none of these studies addressed whether the presumed receptor was required for R-spondin signaling.

Another group of Wnt coreceptors are the syndecans, a family of four transmembrane proteoglycans, which control cell proliferation, differentiation, adhesion, and migration (Bass et al., 2009; Bellin et al., 2002). Syndecans not only act as coreceptors for various growth factors, but they can also transduce signals via their intracellular domain, by interacting with numerous effectors. Syndecans cluster in response to ligand binding, become endocytosed and ubiquitinated, and may control vesicular trafficking (Carvallo et al., 2010; Oh et al., 1997; Tkachenko and Simons, 2002). Unlike in mouse, in *Xenopus* syndecans are essential for embryonic development (Kramer and Yost, 2002; Kuriyama and Mayor, 2009; Matthews et al., 2008; Munoz et al., 2006; Olivares et al., 2009). Of importance for this study is syndecan-4 (Sdc4), which modulates signaling by FGF and chemokines (Charnaux et al., 2005; Iwabuchi and Goetinck, 2006; Tkachenko and Simons, 2002). Sdc4 promotes cell migration

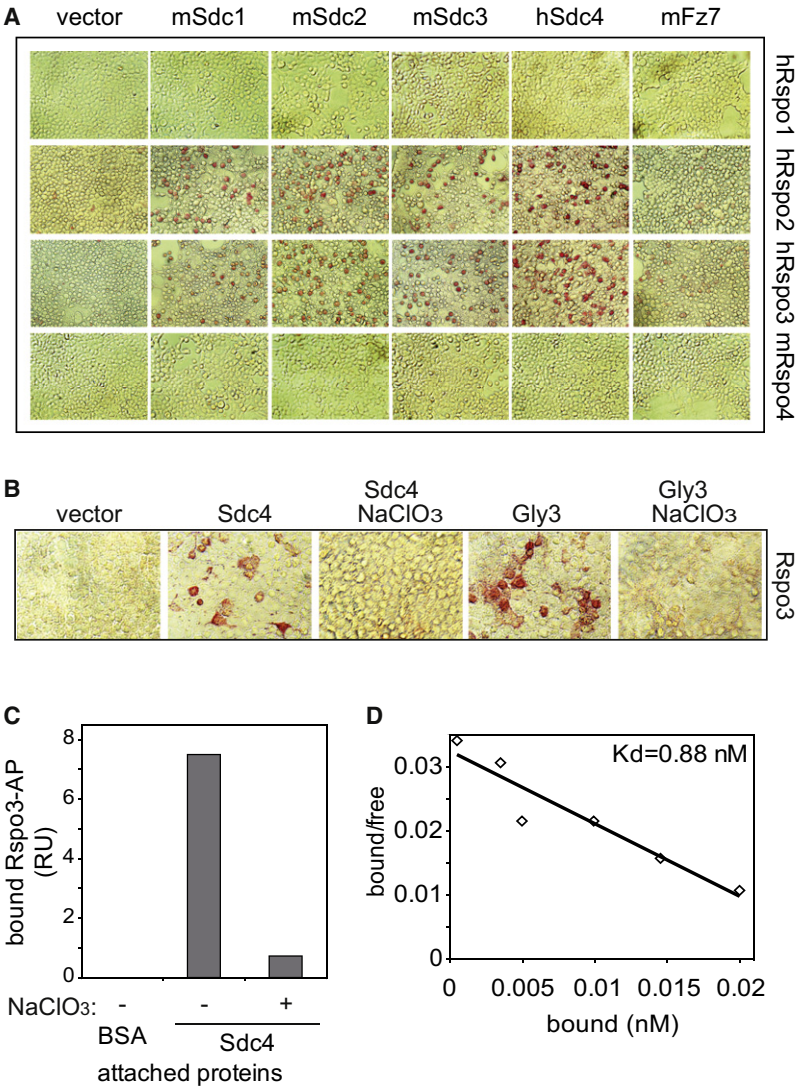


Figure 1. R-Spondin 3 Binds Syndecan 4

(A) Cell-surface binding assay. Cells were transfected with indicated plasmids, and conditioned media containing alkaline phosphatase (AP)–fusion proteins of R-spondins were applied for cell-surface binding and AP staining, as indicated.

(B) Cells were transfected with indicated plasmids and incubated with or without $NaClO_3$. After 24 hr, Rspo3-AP was applied for binding and cells proceeded for staining.

(C) In vitro binding assay with N-streptag Sdc4ΔTMC purified from conditioned media of control or $NaClO_3$ treated cells with purified Rspo3-AP (RU, relative units).

(D) Scatchard plot analysis for in vitro binding assays with purified N-streptag Sdc4ΔTMC and Rspo3-AP.

in a variety of cells, and in *Xenopus* it is essential for gastrulation movements, neural crest migration, and neural induction by promoting Wnt/PCP and FGF signaling (Kuriyama and Mayor, 2009; Matthews et al., 2008; Munoz et al., 2006).

Here we show that R-spondin3 (Rspo3) binds with high affinity to Sdc4 to promote Wnt/PCP signaling. Gain-and-loss-of-function experiments indicate that Sdc4 and Rspo3 functionally interact during *Xenopus* gastrulation and head cartilage morphogenesis. Rspo3-Sdc4 signaling requires Wnt5a, Fz7, and Dvl and it activates JNK. Rspo3 functions by inducing clathrin-mediated endocytosis and we show that this internalization is a prerequisite for Rspo3/PCP signaling. Our study reveals a mechanism of R-spondin signaling, which is mediated by syndecan and required for Wnt/PCP activation.

RESULTS

R-Spondin 3 Binds to Syndecan 4

In our search for an R-spondin receptor, we noted that thrombospondin (TSP1) domains, as they are present in R-spondins, have been reported to bind to HSPGs (Chen et al., 1996). Indeed, in cell-surface binding assays (see Figure S1A available online) alkaline-phosphatase (AP) fusion proteins of full-length Rspo3 or its TSP domain bound specifically to cells transfected with *glypican3* or *Sdc4*. In contrast, no Rspo3-AP binding was detected with *LRP6*, *Kremen1*, or *Frizzled5* (*Fz5*) transfected cells, which however bound recombinant Dkk1 and Wnt5a, respectively. These results prompted us to test binding of AP-fusions of all R-spondins to all syndecans. We found that Rspo2 and Rspo3 bind to all syndecans, whereas Rspo1 and Rspo4 showed no detectable binding under these conditions (Figure 1A). None of the R-spondins bound to *Fz7* transfected cells. All four AP-fusion proteins were secreted (Figure S1B) and active in Wnt reporter assays (data not shown), indicating that they are biologically active. The absence of Rspo1 and Rspo4 binding to syndecans correlates with their significantly lower Wnt signaling activity in reporter assays compared to Rspo2 and Rspo3 (Kim et al., 2008b).

The Rspo3-Sdc4 interaction requires GAGs, because Sdc4 produced from chlorate-treated cells (Keller et al., 1989), which inhibits sulfation (Figure S1C), abolished Rspo3 binding (Figures 1B and 1C). Chlorate was used to impair sulfation, because a quadruple Ser point mutant with mutated putative GAG attachment sites was still sulfated (Figure S1D). Using purified extracellular domain of Sdc4 and purified hRspo3-AP, we determined the apparent Kd as 0.88 nM (Figure 1D). We conclude that R-spondins bind to syndecans but not to Fz5 or Fz7, or LRP6, suggesting that these HSPGs may function as high-affinity receptors or coreceptors for R-spondins.

Rspo3 Functions in Wnt/PCP Signaling During Gastrulation

In *Xenopus*, Sdc4 is prominently involved in Wnt/PCP-mediated morphogenesis (Matthews et al., 2008; Munoz et al., 2006) and notably promotes gastrulation by regulating mediolateral cell intercalation and convergent extension. This raised the question of whether Rspo3 signaling may not exclusively activate Wnt/β-catenin, as is generally believed, but also Wnt/PCP signaling.

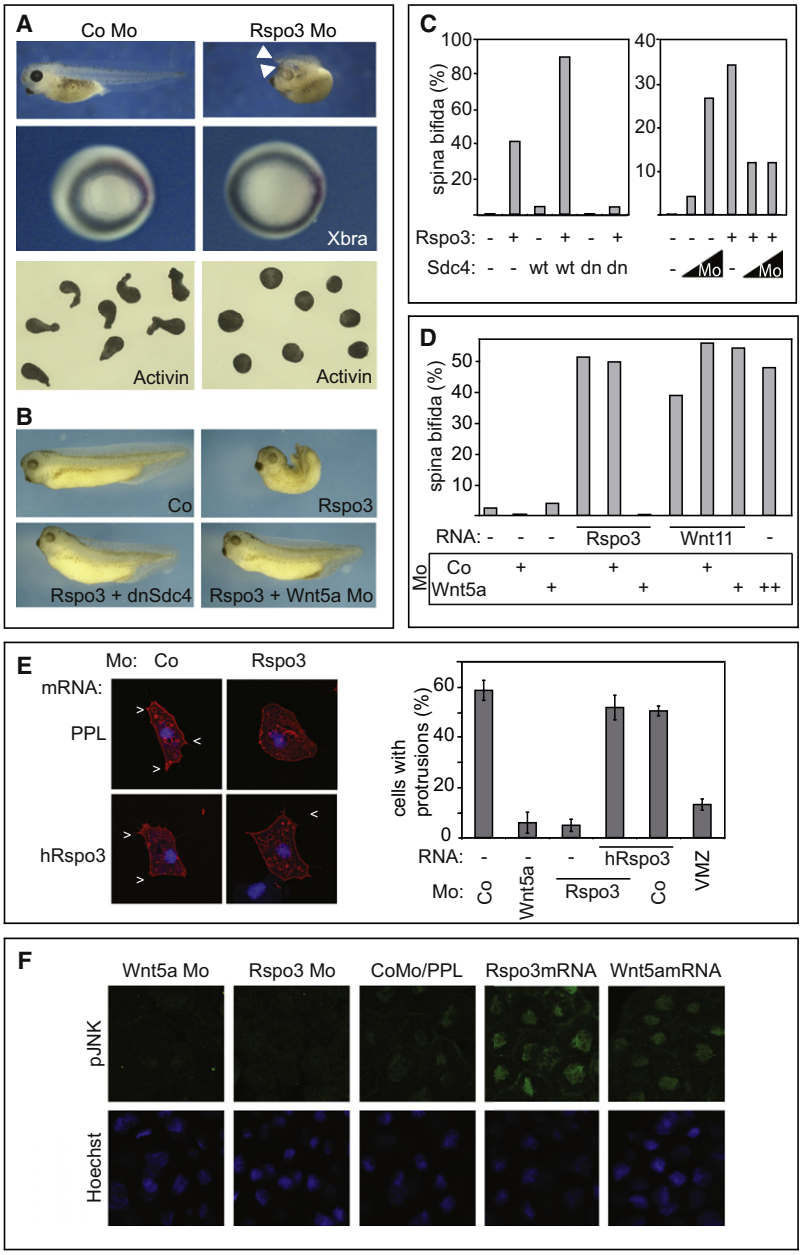


Figure 2. R-Spondin 3 Is Required for *Xenopus* Gastrulation and Noncanonical Wnt Signaling

(A) Loss of *Rspo3* function causes gastrulation defects in *Xenopus* embryos. Top, 4-cell stage embryos were microinjected equatorially into two dorsal blastomeres with 40 ng per embryo of Rspo3 Mo. Note the spina bifida phenotype with two tail ends (arrowheads) in embryos injected with Rspo3 Mo (61%, n = 66) but not control (0%, n = 45) embryos. Middle, in situ hybridization for *Xbra* at gastrula stage (stage 11). Control and Rspo3 Mo embryos showed 100% (n = 20) and 95% (n = 20) normal *Xbra* staining, respectively. However, note the enlarged blastopore in the Rspo3 Morphant. Bottom, Rspo3 Mo inhibits elongation of Activin injected animal caps.

(B–D) Rspo3 signaling requires Sdc4 and Wnt5a to induce gastrulation defects. Embryos were injected into two dorsal blastomeres at 4-cell stage with Morpholinos and/or mRNA as indicated (40 ng Rspo3 Mo; 100 pg wild-type or dominant negative hSdc4 mRNA; 10 or 20 ng of Sdc4 Mo; 2.5 or 10 ng of Wnt5a Mo ["+," and "++"]); 250 pg xRspo3 mRNA per embryo were used).

(E) Confocal microscopy cell protrusion assay. Dorsal marginal zones at stage 10.5 from embryos injected with membrane-RFP mRNA and 40 ng Rspo3 Mo or 10 ng Wnt5a Mo and/or 50 pg hRspo3 mRNA per embryo were dissociated. White arrowheads indicate protrusions. Right, Quantification of protrusions from three independent experiments (between 300 and 600 cells counted for each bar). Standard deviation of the mean is indicated. VMZ, ventral marginal zone for control.

(F) Top, nuclear phospho-JNK immunostaining in stage 10.5 dorsal mesoderm from embryos dorsally injected with indicated Morpholinos or mRNAs. Bottom, nuclear Hoechst stain.

Indeed, injection of an *Rspo3* antisense Morpholino, which was previously characterized (Kazanskaya et al., 2008), induced gastrulation defects (spina bifida) when targeting dorsal mesoderm and at 10-fold higher dose than used previously (Figure 2A). This Morpholino effect was specific and was almost fully rescued by human *Rspo3* mRNA injection (61% spina bifida Rspo3 Mo, n = 66; 4% spina bifida Rspo3 Mo plus hRspo3 mRNA, n = 45).

Gastrulation is driven primarily by the dorsal mesoderm, and *Rspo3* shows prominent expression in this region (Figure S2A). Expression of the mesodermal marker *Xbra* in *Rspo3* Morphants (Figure 2A) and other mesodermal markers, including *Xbra*, *chordin*, *dkk1*, *myoD*, and *ventx2* (*Xvent2*), in activin-injected animal cap tissues were normal (Figure S2G), ruling out that the gastrulation defects were due to reduced mesoderm induction rather than by affecting morphogenesis proper. *Rspo3* Mo also blocked Activin-induced animal cap elongation, consistent with *Rspo3* being required for convergent extension movements (Figure 2A).

As is characteristic for genes regulating gastrulation movements, mRNA overexpression of *Rspo3* also induced gastrulation defects and synergized with *Sdc4* (Figures 2B and 2C). Limiting doses of dominant negative *Sdc4* or *Sdc4* Morpholinos (Munoz et al., 2006) completely abolished this *Rspo3* overexpression effect (Figures 2B and 2C). Similarly, Morpholinos targeting Wnt5a, which promotes embryonic Wnt/PCP signaling and gastrulation in *Xenopus* (Schambony and Wedlich, 2007), also abolished the *Rspo3* overexpression effect (Figures 2B and 2D). In contrast, *Wnt11* mRNA-induced gastrulation defects were not rescued by *Wnt5a* Mo (Figure 2D). This excludes the possibility that Rspo3 signaling simply induces expression of a PCP-Wnt gene such as *Wnt11*.

Convergent extension movements are driven by protrusive activity of mesodermal cells (Wallingford et al., 2000; Winklbauer et al., 1996). *Rspo3* Mo inhibited protrusive activity of dorsal mesodermal cells, and this was rescued by coinjection of low doses of human *Rspo3* mRNA, confirming Morpholino specificity (Figure 2E). Wnt/PCP signaling activates JNK phosphorylation to

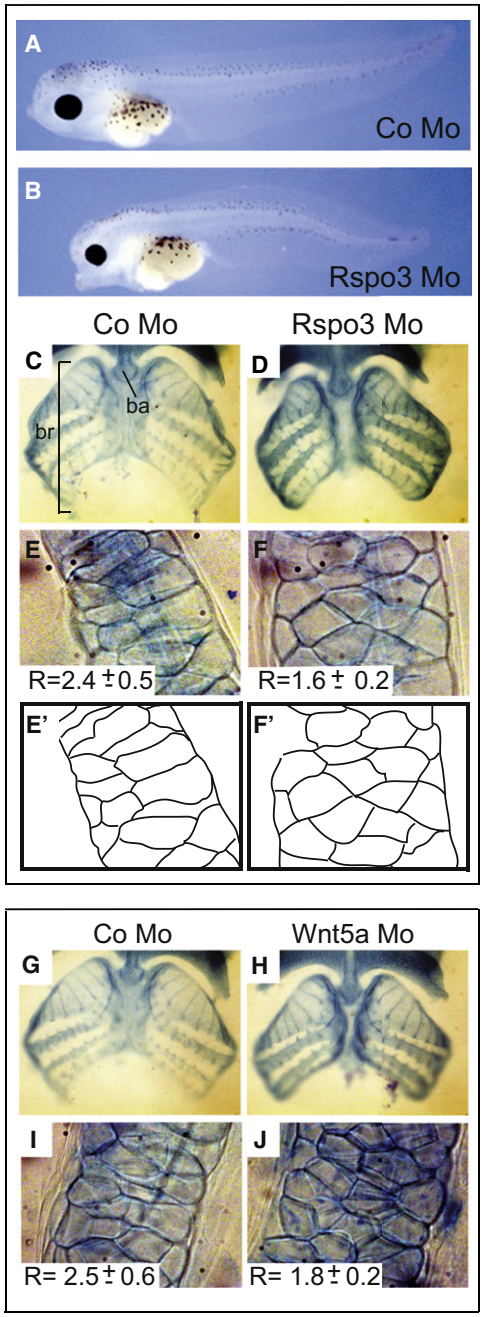


Figure 3. R-Spondin 3 Is Required for Head Cartilage Morphogenesis

(A and B) Tadpole stage *Xenopus* embryos injected at 8-cell stage with 10 ng per embryo of Rspo3 Mo into the animal four blastomeres. Note reduced head in *Rspo3* Morphants (68%, n = 98) compared to Co Mo (0.1%, n = 85). (C, D, G, and H) Ceratobranchial cartilage was stained with Alcian blue and dissected from stage 46 embryos injected at 8-cell stage animally with either 10 ng of Rspo3 Mo or 2.5 ng of Wnt5a Mo per embryo. Note compact cartilage elements. (E, F, I, and J) Ceratobranchial cartilage was dissected from Morpholino injected embryos and flattened. The length-to-width ratios (R) of chondrocytes were determined and are indicated. (E′ and F′) Schematic drawing of cell outlines of (E) and (F). Abbreviations: br, ceratobranchial cartilage; ba, basihyal cartilage.

induce convergent extension movements (Yamanaka et al., 2002). Consistently, *Rspo3* and *Wnt5a* mRNA injection induced JNK phosphorylation, while the respective Morpholinos inhibited JNK phosphorylation in dorsal mesodermal cells (Figure 2F).

Taken together, the results indicate that Rspo3 functions in Wnt/PCP signaling during gastrulation and requires Wnt5a and Sdc4.

Rspo3 Is Required for Head Cartilage Morphogenesis

In the course of our analysis, we discovered a role of Rspo3 in another Wnt/PCP-regulated process, head cartilage morphogenesis. During morphogenesis of the head cartilage, chondrocytes flatten and intercalate to form a column that gives rise to rod- and plate-shaped cartilage elements. This intercalation involves chondrocyte elongation and stacking, very reminiscent of dorsal mesodermal cells during gastrulation (Clement et al., 2008; Piotrowski et al., 1996; Schilling et al., 1996). In zebrafish embryos, it was shown that like gastrulation, head cartilage morphogenesis depends on Wnt/PCP signaling and HSPGs. Mutations in five PCP genes, including *Wnt5a*, *knypek/glypican 4/6*, a transporter of activated sulfate, and two glycosyltransferases required for HSPG synthesis, all interfere with head cartilage morphogenesis, leading to compacted cartilage and stunted head (Clement et al., 2008; Piotrowski et al., 1996; Topczewski et al., 2001).

Similarly, we found that in *Xenopus* embryos, injection of *Rspo3* Mo into animal blastomeres targeting the ectoderm (and thus future neural crest) instead of primary mesoderm, induces stunted heads and compacted cartilage (Figures 3A and 3B). All cartilage elements were present in the Morphants, but they were shorter and thicker (Figures 3C and 3D), indicating that the phenotype was not due to differentiation or neural crest migration defects. Consistent with this finding, there was no effect of *Rspo3* Mo on neural crest marker expression (Figure S3). Instead, stacking and elongation of chondrocytes was impaired. The average length-to-width ratio of individual chondrocytes was reduced from 2.4 in wild-type to 1.6 in Morphant tadpoles (Figures 3E and 3F). Compacted head cartilage with impaired stacking and elongation of chondrocytes was also obtained following injection of a previously characterized *Wnt5a* Mo (Figures 3G–3J) (Schambony and Wedlich, 2007).

Moreover, low *Rspo3* Mo doses synergized in inducing this phenotype either when coinjected with low doses of dominant-negative *Sdc4* mRNA, or *Sdc4* Mo, which by themselves elicited no phenotype (Figures S4A–S4C and S4E), or when combined with mild chlorate treatment, which inhibits HSPG sulfation (Figure S4D). *Rspo3* and *Sdc4* are coexpressed in head cartilage during head mesoderm development, consistent with a direct role in this tissue (Figures S2B–S2F). These results indicate that Rspo3 is required for head cartilage morphogenesis, where it also functionally interacts with Sdc4.

Cooperation of Rspo3 and Wnt5a Is Required for Sdc4-Mediated PCP Signaling

During Wnt/PCP signaling, certain Wnt/Fz combinations activate Rho, Rac, and JNK through Dvl (Angers and Moon, 2009; Tada and Kai, 2009), leading to activation of the transcription factor ATF2 (Schambony and Wedlich, 2007; Zhou et al., 2007). To molecularly corroborate Wnt/PCP activation, we therefore

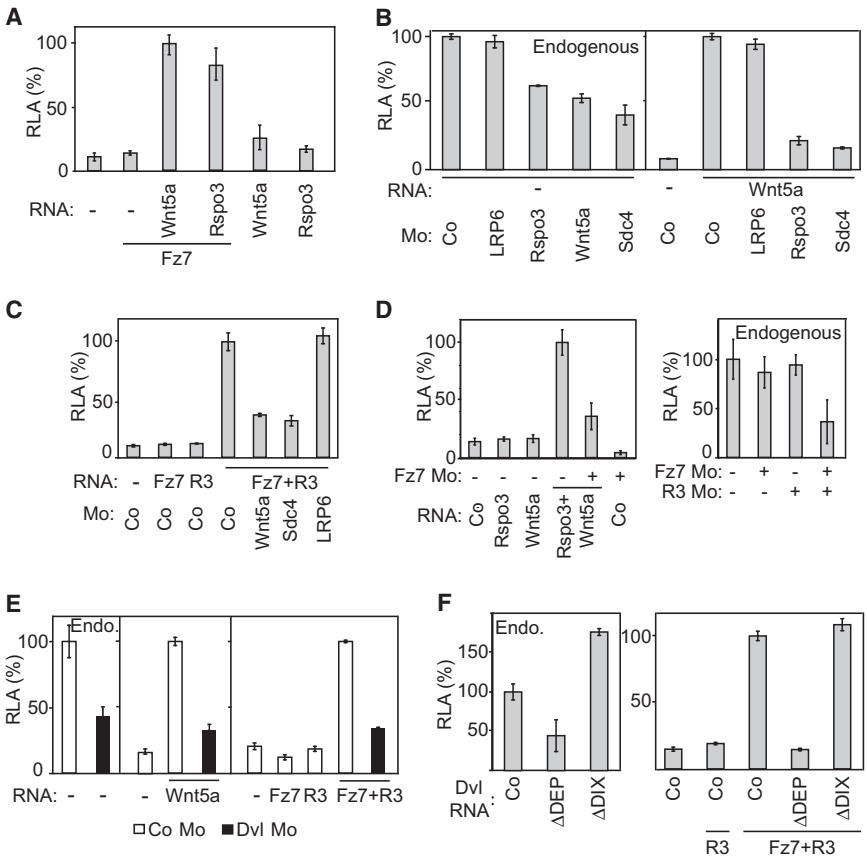


Figure 4. Rspo3/PCP Signaling Requires Wnt5a, Fz7, Sdc4, and Dvl

(A–F) ATF2-Luciferase reporter assay in *Xenopus* embryos. 4-cell stage embryos were injected equatorially with ATF2-Luc reporter and Renilla reporter plasmids and the indicated antisense Morpholinos and mRNAs. Luciferase reporter assays were performed from whole embryos harvested at gastrula stage (stage 12). Luciferase activity in embryos injected with either Co Mo only or Co Mo plus mRNA of the indicated activators within each conditions were set to 100%. RLA, relative luciferase activity. The mRNA and Mo doses for injections were (per embryo) as follows: Wnt5a mRNA (100 pg in A and D; 500 pg in B and E); xRspo3 (250 pg) and Rspo3 Mo (20 ng in B, 5 ng in D); and Wnt5a Mo (5 ng), Sdc4 Mo (20 ng), and Fz7 Mo (20 ng D left, 5 ng D right). Part of the data in (E) and (F) are from Ohkawara and Niehrs (2011). Data with standard error of the mean are shown.

used a JNK responsive ATF2-luciferase reporter (van Dam et al., 1995), which specifically monitors Wnt/PCP stimulation in *Xenopus* embryos (Ohkawara and Niehrs, 2011). This ATF2 reporter was activated by microinjected *Wnt5a* mRNA in combination with *Fz7*, a receptor-ligand combination that mediates Wnt/PCP signaling in *Xenopus* (Kim et al., 2008a). It was also activated by *Rspo3* mRNA in combination with *Fz7* (Figure 4A), confirming that Rspo3 is able to stimulate Wnt/PCP signaling in vivo.

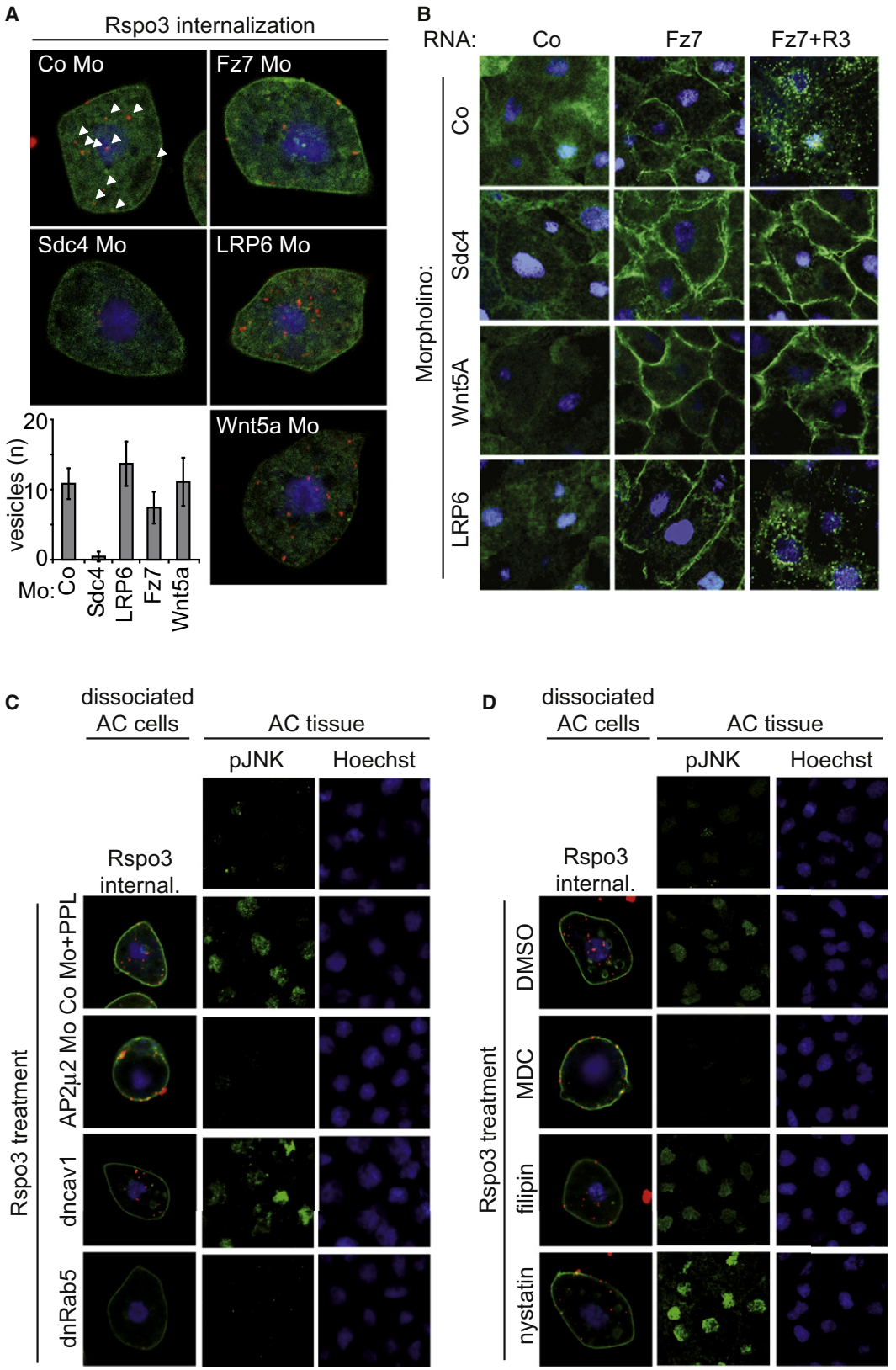
In unstimulated embryos, ATF2 reporter activity resulting from endogenous signaling was reduced by Morpholinos targeting *Wnt5a* or *Sdc4*, but not *LRP6* (Figure 4B, left). Importantly, endogenous reporter activity was reduced by *Rspo3* Mo to a similar extent as by *Wnt5a* and *Sdc4* Mo, consistent with the effect of *Rspo3* Mo on gastrulation, mesodermal cell protrusive activity, and JNK phosphorylation (Figure 4B, left). This effect of Rspo3 Mo on endogenous ATF2 reporter activity was rescued by human Rspo3 mRNA injection, again confirming Mo specificity (Figure S5).

Reporter activity in exogenously stimulated *Wnt5a* mRNA–injected embryos was blocked by Morpholinos targeting *Rspo3* and *Sdc4*, showing that this ligand-receptor pair is essential for Wnt5a signaling (Figure 4B, right). An *LRP6* Mo, which potently inhibits canonical Wnt signaling (Hassler et al., 2007), had no effect on *Wnt5a* mRNA signaling, confirming reporter specificity. The phenotypic data (Figure 2D) suggested that also the reverse is true—namely, that Rspo3 signaling relies upon endogenous Wnt5a. Indeed, *Wnt5a* Mo inhibited PCP signaling resulting from *Rspo3*/*Fz7* mRNA costimulation (Figure 4C). Morpholinos targeting *Sdc4* but not *LRP6* also blocked this signal (Figure 4C). Thus, Rspo3 and Wnt5a are codependent during Wnt/PCP signaling.

Rspo3 mRNA also strongly cooperated with *Wnt5a* mRNA to induce reporter activity, and this was expectedly inhibited by *Fz7* Mo (Figure 4D, left). Moreover, *Rspo3* Mo and *Fz7* Mo synergistically inhibited endogenous PCP signaling (Figure 4D, right), showing the physiological relevance of their functional interaction. Thus, Rspo3 signaling requires this Wnt receptor.

Dvl plays a critical role in Wnt/PCP signaling. Notably, Dvl binding to Sdc4 is required for Fz7-PCP signaling in *Xenopus* (Munoz et al., 2006). Hence we tested whether Rspo3/ATF2 signaling was affected by Dvl knockdown. Injection of a *Dvl2* Mo (Sheldahl et al., 2003) reduced endogenous *Wnt5a*-stimulated as well as *Fz7*/*Rspo3*-stimulated ATF2 reporter activity (Figure 4E). Dvl contains three conserved domains: the N-terminal DIX domain is essential for β-catenin signaling but not for PCP activation, and the central PDZ and C-terminal DEP domains are both required for the PCP function. Dvl deletions lacking the DEP and DIX domain act as dominant negatives for PCP and β-catenin signaling, respectively, and can be used to experimentally distinguish between both pathways (Boutros et al., 1998; Habas et al., 2001; Heisenberg et al., 2000; Moriguchi et al., 1999; Rothbächer et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000; Yamamoto et al., 1999). Consistent with Wnt/PCP activation, we found that Rspo3/ATF2 signaling is inhibited *ΔDEP* but unaffected by *ΔDIX* mRNAs (Figure 4F).

From these gain-and-loss-of-function data, we conclude that in early *Xenopus* embryos (1) Rspo3 functionally interacts with Wnt5a and Fz7 during Wnt/PCP signaling; (2) Rspo3 signals through Fz7 and its downstream effector Dvl, (3) that its signaling


A
Rspo3 internalization
Co Mo
Fz7 Mo
Sdc4 Mo
LRP6 Mo
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vesicles (n)
20
10
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Mo: Co Sdc4 LRP6 Fz7 Wnt5a
B
RNA: Co Fz7 Fz7+R3
Morpholino: Co Sdc4 Wnt5A LRP6
C
dissociated AC cells
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pJNK
Hoechst
Rspo3 internal.
Rspo3 treatment
Co Mo+PPL
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Hoechst
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Rspo3 treatment
DMSO
MDC
filipin
nystatin

requires Sdc4, consistent with a Rspo3 receptor function, and (4) that Wnt5a and Rspo3 mutually require each other during Wnt/PCP signaling.

Rspo3 Induces Clathrin-Mediated Endocytosis, Which Is Required for Wnt/PCP Signaling

A hallmark of syndecans is their ability to induce endocytosis following ligand binding, which is essential, for example, for FGF signal transduction (Fuki et al., 2000; Li et al., 2006; Tkachenko et al., 2004; Wittrup et al., 2009). Moreover, there is a body of evidence that Wnt signaling proceeds via an endocytic compartment and that Wnt-receptor complex internalization is an essential step both in canonical and noncanonical Wnt signaling (reviewed in Kikuchi and Yamamoto, 2007). This raised the possibility that the molecular mechanism by which Rspo3 promotes Wnt/PCP signaling is by binding Sdc4 and inducing internalization of the Wnt-receptor complex. Internalization assays with recombinant SNAP549-labeled Rspo3 showed that the protein is indeed internalized within 1 hr of application in *Xenopus* animal cap cells, where it was found in intracellular vesicles (Figure 5A). Endocytosis of Rspo3 was inhibited by Morpholinos against *Sdc4* and *Fz7* but not *Wnt5a* or *Lrp6*, consistent with its internalization proceeding via an Sdc4/Fz7 complex. To rule out unspecific effects of the Morpholinos on endocytosis, we monitored fluorescent-Dextran uptake, which remained completely unaffected (Figures S6A and S6B).

Dvl plays a key role in endocytosis during Wnt/PCP signaling and upon Wnt signaling is recruited to endocytic vesicles (Chen et al., 2003; Kim et al., 2008a; Kishida et al., 2007; Yu et al., 2007). We therefore monitored the accumulation of Dvl-GFP in vesicles upon stimulation. In animal cap cells, Dvl-GFP shows a diffuse staining but upon coinjection of *Fz7* and *Rspo3* mRNA it accumulated in punctate structures. This accumulation was blocked by Morpholinos against *Sdc4* and *Wnt5a* but not *Lrp6* (Figure 5B). These results support that Rspo3 induces endocytosis of the Wnt receptor complex and Dvl.

The findings raised the question whether Rspo3-mediated endocytosis is merely an epiphenomenon (e.g., of receptor clearance) or whether the internalization is required for Wnt/PCP signaling to proceed. To answer this question we treated animal cap cells with Rspo3 protein and after 1 hr we monitored either Rspo3 internalization (in dissociated cells) or stained for phospho-JNK (in intact explants) as a read-out for Wnt/PCP activation. Following Rspo3 treatment, the labeled protein was internalized, and this was accompanied by nuclear phospho-JNK induction, consistent with Wnt/PCP activation (Figures 5C and 5D, top). Importantly, blocking clathrin-mediated endocytosis using a Morpholino targeting the clathrin adaptor *AP2μ2* (Borner et al., 2007; Motley et al., 2003; Yu et al., 2007) led to Rspo3 clustering at the cell surface and impaired internalization and JNK phosphorylation (Figure 5C). This uncoupling of Rspo3 binding from endocytosis provides compelling evidence for the importance of the internalization for signaling.

Dominant negative *Rab5* mRNA, which inhibits both clathrin- and caveolin-mediated endocytosis (Shin et al., 2005), also blocked internalization as well as nuclear phospho-JNK induction. Likewise, the clathrin endocytosis inhibitor monodansylcadaverine (MDC) (Schlegel et al., 1982) blocked Rspo3 internalization and phospho-JNK induction (Figure 5D). In contrast, inhibitors of caveolin-mediated endocytosis, including dominant negative *caveolin* mRNA (Sanguinetti and Mastick, 2003), filipin, and nystatin treatment (Rothberg et al., 1992), had no effect on Rspo3 internalization and phospho-JNK accumulation, whereas they significantly reduced Fluorescein-Dextran uptake (Figures S6C and S6D).

We conclude that Rspo3 induces clathrin-mediated endocytosis and that this internalization is essential for Wnt/PCP signaling.

DISCUSSION

Rspo3 Binds Syndecan 4

We provide independent lines of evidence that Sdc4 functions as Rspo3 receptor or coreceptor, including cell-surface binding, recombinant protein binding, functional cooperation, and requirement of Sdc4 for Rspo3/PCP signaling in vivo. The affinity of Rspo3 to Sdc4 is similar to Sdc4 binding to other ligands, e.g. bFGF to Sdc3 (Kd = 0.5 nM), and this binding requires heparin chains, (Chernousov and Carey, 1993). Another example is pleiotrophin, which binds Sdc3 with 0.6 nM Kd (Raulo et al., 1994). On the other hand, a number of ligands bind syndecans with 10–100-fold lower affinity, such as cathepsin G (56 nM), elastase (35 nM) (Kainulainen et al., 1998), or interleukin 8 (23 nM) (Halden et al., 2004). Thus, Rspo3 can be added to the list of high-affinity ligands of syndecans. Our results do not prove that syndecans are universally required for Rspo3 signaling, and there may be additional R-spondin receptors or coreceptors, including glypicans. However, the results highlight that such candidates will need to be tested in loss-of-function experiments. Likewise, although we failed to detect cell-surface binding between Rspo1 and Rspo4 with syndecans, we cannot rule out that there

Figure 5. Rspo3 Requires Clathrin-Mediated Endocytosis to Induce Phospho-JNK

(A) Confocal microscopy of dissociated animal cap cells treated with hRspo3ΔC-SNAP549 protein for 1 hr (orange, arrowheads in CoMo). 4-cell stage embryos were injected animally with membrane-bound Venus mRNA (green) and indicated Morpholinos (Mo) (20 ng of Sdc4; 20 ng of Fz7; 5 ng of Wnt5a). The average number of vesicles per cell is indicated in the graph. Data with standard error of the mean are shown.

(B) Confocal microscopy of Dvl-GFP (green) in stage 8 *Xenopus* animal caps. 4-cell stage embryos were injected animally with indicated Mo and/or mRNAs together with Dvl-GFP mRNA.

(C and D) Confocal microscopy of animal cap (AC) cells from embryos injected at 4-cell stage with indicated Mo or mRNA (C) or treated with endocytosis inhibitors (D) as described in Experimental Procedures. "Dissociated AC cells," embryos were injected animally with membrane-bound Venus mRNA (green); ACs were explanted and dissociated at stage 8, incubated for 1 hr with hRspo3ΔC-SNAP549 (red), fixed, and analyzed. "AC tissue," whole stage 8 ACs were treated either with Protein A (top two panels) or recombinant hRspo3ΔC-streptag-PrA_2 ("Rspo3 treatment") and immunostained with anti-pJNK antibody, as described in Experimental Procedures. Cells and tissues were counter-stained with Hoechst (blue). Note that clathrin inhibitor–treated cells (AP2μ2 Mo, MDC) bind and cluster Rspo3 at the surface but fail to internalize the protein and to induce phospho-JNK.

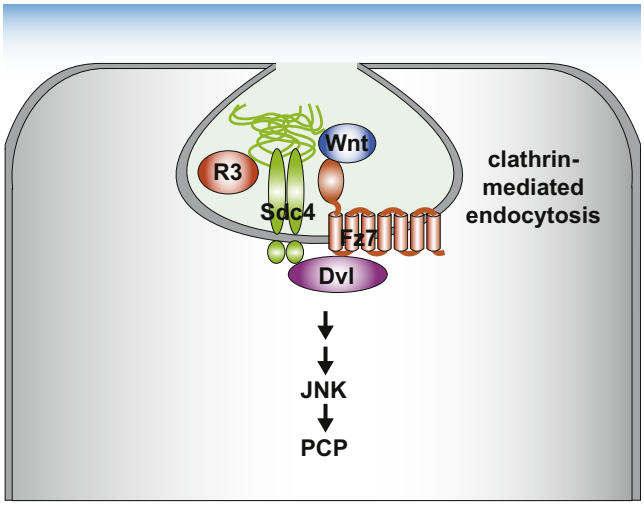

Figure 6. Model for Rspo3 Association with a Wnt Receptor Complex Mediating PCP Signaling

Endocytosis of Wnt receptor complexes is essential for Wnt/PCP signal transduction. Rspo3 binding to Sdc4 promotes clathrin-mediated endocytosis of the Wnt receptor complex and thereby signaling via the PCP pathway.

is physiologically relevant binding between them, but of lower affinity than for Rspo3 and Sdc4, which may be below our detection limit.

Considering the numerous studies implicating syndecans in growth factor signaling and extracellular matrix interaction, it is surprising that mice mutants lacking *Sdc1*, *3*, or *4* develop without gross developmental abnormalities (Alexander et al., 2000; Ishiguro et al., 2000; Kaksonen et al., 2002). In contrast, R-spondin mutant mice show quite obvious abnormalities, including sex reversal (*Rspo1*), limb and craniofacial defects (*Rspo2*), embryonic lethality (*Rspo3*), and anonychia (*Rspo4*). One likely reason for the rather benign mutant phenotypes in syndecan mutants may be their ubiquitous and high expression, which may promote functional compensation. Yet, closer examination reveals a number of overlapping biological roles between R-spondins and syndecans in vertebrates beyond those described in our study. *Rspo3* and *Sdc2* are essential for angiogenesis (Chen et al., 2004; Kazanskaya et al., 2008), and *Rspo2*, *Sdc3*, and *Sdc4* regulate myogenic differentiation (Cornelison et al., 2004; Kazanskaya et al., 2004). *Sdc1* is required for keratinocyte activation during wound healing and *Rspo1* deficient patients show skin defects (Parma et al., 2006; Stepp et al., 2002). Finally, *Rspo2*, *Rspo3*, and *Sdc1* all promote mouse mammary tumorigenesis (Alexander et al., 2000; Lowther et al., 2005; Theodorou et al., 2007). Thus, it will be interesting to analyze R-spondin/syndecan double mutants for functional interaction in mammals.

Rspo3 Functions in Wnt/PCP Signaling in *Xenopus* Embryogenesis

In lower vertebrates, HSPGs play a prominent role in Wnt/PCP signaling and morphogenesis. Zebrafish, *glypican4/6* (*Knypek*), and *Xenopus Sdc4* regulate gastrulation movements, neural tube closure, and neural crest migration (Munoz et al., 2006; Topczewski et al., 2001). In *Xenopus* gastrulation, Sdc4 functions together with Fz7 to mediate convergent extension and PCP signaling (Munoz et al., 2006). We show that Sdc4/PCP signaling in gastrulae requires Rspo3, revealing that R-spondin function is not limited to Wnt/β-catenin signaling, and consistent with the proposed Rspo3 receptor/coreceptor function of Sdc4. In line with the notion that R-spondins require Wnts to signal, we find that Rspo3 relies upon Wnt5a, which is known to control cell shape and movement during zebrafish and *Xenopus* gastrulation by engaging the PCP pathway to activate JNK and ATF2 (Cha et al., 2008; Kilian et al., 2003; Ma and Wang, 2007; Schambony and Wedlich, 2007).

Besides gastrulation, we show that Rspo3 and Sdc4 function together in another process known to be regulated by Wnt/PCP signaling, namely head cartilage morphogenesis. In zebrafish mutant for various Wnt/PCP components, morphogenesis of chondrocytes is impaired, which fail to polarize and intercalate, and hence show reduced elongation of larval cartilage elements (Clement et al., 2008; LeClair et al., 2009; Topczewski et al., 2001). We show that the same phenotype is exhibited by *Xenopus* embryos with reduced Rspo3, Sdc4, or Wnt5a function, raising the possibility that these genes may be involved more generally in PCP signaling, possibly including adult skeleton formation.

Rspo3 Induces Syndecan 4 Endocytosis to Activate Wnt/PCP Signaling

There is now accumulating evidence that Wnt-induced endocytosis is not only a mechanism for clearing receptor-ligand complexes or transporting Wnt proteins by transcytosis, but that internalization is an obligatory step in activating β-catenin signal transduction (Blitzer and Nusse, 2006; Seto and Bellen, 2006) (Yamamoto et al., 2006). Internalization is also required for PCP signaling (Yu et al., 2007; Chen et al., 2003; Kim and Han, 2007). Similarly, a hallmark of syndecans is that their binding to ligands induces endocytosis (Fuki et al., 1997; Fuki et al., 2000; Tkachenko et al., 2004) and this is intimately linked to signaling initiation (Li et al., 2006). We provide evidence that Rspo3 promotes Wnt signaling via clathrin-mediated endocytosis, an internalization route that has previously been implicated in Wnt signaling (Blitzer and Nusse, 2006; O'Connell et al., 2010, ; Yu et al., 2007).

Our data corroborate the importance of endocytosis in Wnt signal transduction and they suggest a model (Figure 6) whereby syndecan acts as a Wnt-Frizzled coreceptor whose internalization is regulated by R-spondin. Together with the previous evidence for the importance of endocytosis, this begs the question of what it is about internalization that activates Wnt signal transduction. Recent evidence suggests that endocytosis is required for the Wnt-receptor complex to reach an acidified compartment, both in Wnt/β-catenin and Wnt/PCP signaling (Buechling et al., 2010; Cruciat et al., 2010; Hermle et al., 2010; Niehrs and Boutros, 2010; Taelman et al., 2010).

Although the present study discloses its role in Wnt/PCP signaling, Rspo3 is also a potent activator of Wnt/β-catenin signaling. This raises the question of what is the common functional basis for Rspo3 promoting both pathways. Our data clearly indicate that Rspo3 signals independently of Lrp6 in Wnt/PCP signaling. This is consistent with the notion that Lrp6 directs Wnt signaling toward the β-catenin pathway. Conversely, neither

syndecans nor glypicans have been directly implicated in β-catenin signaling but rather in noncanonical Wnt pathways. Thus, neither Lrp6 nor syndecan binding appears as likely universal feature of R-spondin's mechanism of action. On the other hand, we and others have previously shown that R-spondins act synergistically with Wnts to stimulate Wnt/β-catenin signaling. Moreover, knockdown of Wntless impairs R-spondin signaling (Kim et al., 2008b). The Wnt ligand dependence also holds for Rspo3/PCP signaling, because we found endogenous Wnt5a to be essential. Taken together, these results indicate that R-spondins serve to amplify Wnt ligand signaling in β-catenin as well as PCP signaling. We speculate that the common mechanism of R-spondins to achieve this rests in their ability to induce endocytosis of Wnt-receptor complexes. Which R-spondin receptor substitutes for Sdc4 in β-catenin signaling remains to be resolved.

EXPERIMENTAL PROCEDURES

Cell Culture, Conditioned Media, and Transfections

HEK293T cells were maintained in DMEM (Lonza), 10% FCS, and 10% CO_2. Wherever recombinant proteins are mentioned, conditioned media were used. For recombinant protein production, to avoid any contamination of preparations with polycations, 293T cells were transiently electroporated with corresponding DNA using Neon™ Transfection System (Invitrogen). If indicated, 24 hr after transfection or electroporation, the cells' culture medium was supplemented with 25 mM $NaClO_3$ and was incubated another 24 hr.

Luciferase Reporter Assays, and siRNA Transfections

For luciferase reporter assays in *Xenopus* embryos, embryos were injected with 200 pg of ATF2-luciferase (van Dam et al., 1995) and 50 pg per embryo of pRenilla-TK plasmid DNA plus Morpholinos and/or synthetic mRNA. Three pools of seven embryos each were lysed with passive lysis buffer (Promega) and assayed for luciferase activity using the Dual luciferase system (Promega).

Cell-Surface Binding

Recombinant protein conditioned media were produced by transient electroporation of HEK293T cells with hRspo1-AP, hRspo2ΔC-AP, hRspo3ΔC-AP, mRspo4ΔC-AP hRspo3TSP-AP, and dkk1-AP in complete DMEM. XWnt8-hFz5 fusion was produced in serum-free medium (OPTIMEM I, GIBCO), and conditioned medium was concentrated about 100-fold using Centricon Plus-20 filters (Millipore). Wnt5aV5 protein conditioned media was produced using L cells stable transfected with pWnt5aV5. For cell-surface binding experiments, 293T cells were transfected using FuGENE 6 (Roche) for total 48 hr, incubated with conditioned media for 2 hr on ice, washed with Hank's buffer, fixed for 30 min with 0.5 mM DSP (Pierce) in Hank's-100 mM HEPES (pH 7.2), washed with 0.1 M Tris (pH 8.0), and stained with Fast Red (Roche).

In Vitro Binding Assay

For in vitro binding experiments, hRspo3ΔC-AP was partially purified from conditioned medium in two steps. hRspo3ΔC-AP from conditioned medium was absorbed to Heparin agarose beads (Sigma, Type I), washed with TBS, and eluted with 0.5 M NaCl and 20 mM Tris (pH 7.5). Eluted material was loaded on Concanavalin A agarose (Sigma), washed with 0.5 M NaCl and 20 mM Tris (pH 7.5), and eluted with 0.5 M NaCl, 1 mM $MgCl_2$, 100 mM methyl-α-D-manno-pyranoside, and 20 mM Tris (pH 7.5). Concentration of eluted fusion protein was determined from Coomassie stained SDS PAGE gels using BSA as standard.

N-streptag Sdc4ΔTMC protein was partially purified from conditioned medium by absorbing on Streptavidin Agarose (Thermo Scientific), washing with 0.5 M NaCl and 20 mM Tris (pH 7.5), and elution with 2 mM biotin in same buffer. Eluted protein was diluted to 150 mM NaCl and bound to DEAE Sephadex A-50. Beads were washed with TBS to remove biotin and eluted with 1 M NaCl and 20 mM Tris (pH 7.5). Eluted protein was dialyzed against TBS and kept at −20°C before use.

For in vitro binding assay, white high-binding ELISA 96-well plates (Greiner) were coated overnight at 4°C with 100 μl of 2 μg/ml Streptavidin in bicarbonate buffer (50 mM $NaHCO_3$ [pH 9.6]). Wells were washed six times with 230 μl of TBST and incubated with 230 μl of blocking buffer (5% BSA and 1 mM $MgCl_2$ on TBST) on a shaker for 1 hr at room temperature. The wells were loaded with 100 μl N-streptag Sdc4ΔTMC protein in blocking buffer and allowed to bind overnight on a shaker at 4°C. Wells were washed six times with 230 μl of TBST and incubated with 230 μl of blocking buffer for 1 hr. For the binding experiments, 100 μl of serially diluted hRspo3ΔC-AP were placed in wells coated with blocking buffer or N-streptag Sdc4ΔTMC. After 2 hr of incubation at room temperature, wells were washed six times with 230 μl of TBST. Bound AP activity was measured using chemiluminescent SEAP Reporter Gene Assay (Roche). For each dilution, background binding value was subtracted from hRspo3ΔC-AP value. Binding data were analyzed by Scatchard plot using Excel.

In Vivo [^{35}S] Sulfate Metabolic Labeling

HEK293T cells transiently transfected with N-streptag Sdc4ΔTMC or GFP control were metabolically labeled with 0.2 mCi/ml [^{35}S] sulfate, according to a method described by Tooze (2001).

After 24 hr of labeling, conditioned medium was harvested, supplemented with 1% NP40 and 10 mM cold Na_2SO_4, and was incubated overnight with streptavidin beads. Beads were washed with 0.5 M NaCl, 1 mM Na_2SO_4, 1% NP40, and 20 mM Tris (pH 7.5). Bound labeled N-streptag Sdc4ΔTMC was eluted with 2 mM biotin in the same buffer and analyzed by western blot and autoradiography.

SNAP Tag Labeling

Conditioned medium containing hRspo3ΔC-SNAP was partially purified at 4°C on Heparin agarose as described for hRspo3ΔC-AP, with the modification that all buffers were supplemented with 1 mM mercaptoethanol. Eluted protein was concentrated on Amicon filter (Millipore) and labeled for 3 hr at room temperature with SNAP-surface 549 substrate, according to the manufacturer (New England Biolabs). Labeled hRspo3ΔC-SNAP549 protein was separated from free substrate on a Sephadex G50 column (final concentration, A_{556} = 0.93) and kept in aliquots at −20°C.

General *Xenopus* Methods

In vitro fertilization, embryo culture, preparation of mRNA, microinjection, and culture of embryo explants were performed as described elsewhere (Gawantka et al., 1995). Whole-mount in situ hybridization was performed according to Bradley et al. (1996). The mRNA doses for injections were as indicated in the legends and otherwise were, per embryo, 250 pg Fz5, 100 pg XWnt11, 250 pg XFz7, 200 pg XDsh-GFP, 250 pg membrane-bound RFP or Venus (gift from N. Kinoshita), 500 pg ΔDIX and ΔDEP of *Xenopus Dishevelled*, and 500 pg dominant negative Rab5 mutant (gift of M Zerial) and dominant negative caveolin-1 (YF mutant, gift of C. Mastik).

The antisense Morpholino oligonucleotides were described as follows and used as indicated in the legends and otherwise as follows (per embryo): Rspo3 (Kazanskaya et al., 2008), Sdc4 (Munoz et al., 2006), Wnt5a (Schambony and Wedlich, 2007), and Fz7 (Winklbauer et al., 2001) as indicated; 2.5 ng LRP6 (Hassler et al., 2007); 25 ng Dsh (Sheldahl et al., 2003); and 20 ng AP2μ2 (Borner et al., 2007). Equal amounts of total Mo were injected by adjustment with the standard control Mo (Gene Tools), where necessary. For protrusion assays, dorsal or ventral mesoderm cells from stage 10.5 embryos injected as described in legend were dissected, dissociated in Ca^{2+}-free MBS, and cultured in 0.5× Barth including 1% BSA (BSA-Barth) for 1 hr on a fibronectin-coated plate (50 μg/ml). Alcian Blue staining of head cartilage was performed as described elsewhere (Berry et al., 1998). To prepare head cartilage, tadpoles were treated with Proteinase K (100 μg/ml in PBS) for 2 hr followed by 0.05% Trypsin (GIBCO) for 3 hr, and head cartilage was manually dissected out under a stereo microscope.

Immunostaining, Internalization Assays, and Rspo3 Treatment of *Xenopus* Embryonic Cells

To monitor JNK phoshorylation, stage 10.5 dorsal marginal zones were dissected and fixed in Dent's fixative overnight at −20°C. After rehydration, explants were blocked in 20% horse serum and 1% blocking reagent (Roche

Molecular Biochemicals) in PBST (0.1% Tween in PBS). Incubation with the primary anti-pJNK antibody (V7931, Promega, 1:1000) overnight at 4°C was followed by anti-rabbit Alexa488 (1:1000) for 4 hr at room temperature and Hoechst staining. To monitor Rspo3 internalization, stage 8 animal caps were dissected from animally injected embryos, dissociated in Ca^{2+}-free MBS and cultured in BSA-Barth together with either hRspo3ΔC-SNAP549 protein (1:40) or Fluorescein-Dextran (1 μg/ml) (Molecular Probes) for 1 hr. Cells were washed twice with BSA-Barth, fixed with MEMFA for 15 min, and Hoechst-stained. For endocytosis inhibitor treatments, dissociated animal cap cells were pretreated with MDC (monodansylcadaverine, 1 mM, Sigma), filipin III (300 ng/ml, Sigma), nystatin (25 μg/ml, Sigma), or 2% DMSO for 45 min and continued for 1 hr in the presence of hRspo3ΔC-SNAP549 (1:40) in BSA-Barth. To induce and detect phospho-JNK in animal caps, microscopy cover-glasses were pretreated with IgG (4.5 μg/ml IgG [Sigma] in 50 mM $NaHCO_3$ [pH 9.6]) overnight at 4°C, washed with TBST, blocked with 5% BSA in TBST for 1 hr, and then loaded with hRspo3ΔC-streptag- PrA_2 conditioned medium or Protein A (1 μg/ml, Amersham) proteins overnight at 4°C. The glass was used after washing with BSA-Barth. Stage 8 animal caps were dissected from injected embryos and pretreated for 45 min open face-up under a BSA-treated cover-glass with endocytosis inhibitors at the mentioned doses. The cover-glass was then replaced by a hRspo3ΔC-strep-tag-PrA_2– or Protein A–loaded cover-glass, and incubation in presence of inhibitors was continued for 1 hr. Animal caps were removed, fixed in Dent's fixative overnight at −20°C, rehydrated, and immunostained with anti-pJNK antibody as described. Confocal laser scanning was done on a Nikon e-C1plus microscope.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and may be found with this article online at doi:10.1016/j.devcel.2011.01.006.

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