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Mouse Cristin/R-spondin Family Proteins Are Novel Ligands for the Frizzled 8 and LRP6 Receptors and Activate β-Catenin-dependent Gene Expression*

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Wnt signaling plays critical biological roles during normal embryonic development and homeostasis in adults. In the canonical pathway, binding of Wnt ligands to the Frizzled (Fzd) receptor and the low density lipoprotein-related receptor (LRP) 5 or LRP6 coreceptor initiates downstream signaling events leading to gene activation by β-catenin and the T-cell factor (TCF)-lymphoid enhancer factor (LEF) family transcription factor complex. In this study, we provide several lines of evidence that the mouse Cristin/R-spondin family proteins function as Fzd8 and LRP6 receptor ligands and induce the canonical Wnt/β-catenin signaling pathway, leading to TCF-dependent gene activation. First, conditioned medium containing Cristin/R-spondin proteins effectively induced reporter activity in a TCF-binding site-dependent manner. Second, stimulation of cells with Cristin/R-spondin was accompanied by stabilization of endogenous β-catenin proteins and induction of canonical Wnt target genes. Third, Cristin/R-spondin proteins physically interacted with the extracellular domains of the LRP6 and Fzd8 receptors *in vivo* and *in vitro*. Interestingly, unlike canonical Wnt ligands, Cristin/R-spondin failed to form a ternary complex with both LRP6 and Fzd8 receptors, suggesting that R-spondin may activate the canonical Wnt signaling pathway by different mechanisms. Furthermore, Cristin/R-spondin proteins possess an intriguing positive modulatory activity on Wnt ligands, possibly through a direct interaction. Our findings expand the repertoire of ligands that induce β-catenin/TCF-dependent gene activation and implicate the presence of active β-catenin-dependent gene activation in a Wnt-free biological context.

Wnt signaling is one of the key signaling pathways controlling cell proliferation, differentiation, and morphogenesis during embryogenesis and in adults (1, 2). Wnt ligands are secreted glycoproteins and induce multiple intracellular signaling pathways (3–7). In the canonical pathway, Wnt ligands bind to the Frizzled (Fzd)[2] family receptors and their low density lipoprotein receptor-related protein (LRP) 5 or LRP6 coreceptor, leading to recruitment of Dishevelled (Dvl) and Axin proteins from the cytoplasm to the receptors on the plasma membrane (6). Subsequently, the activities of glycogen synthase kinase-3β and casein kinase I are inhibited. As a result, β-catenin proteins, which are normally phosphorylated by these kinases and degraded in the absence of Wnt stimulation, become underphosphorylated and escape from the degradation pathway. Accumulated β-catenin proteins in the cytoplasm are eventually translocated into the nucleus, where they are engaged in gene activation as a complex with T-cell factor (TCF) family transcription factors. Additionally, Wnt ligands also induce β-catenin-independent intracellular signaling pathways, including the Wnt/Ca^{2+}, Wnt/cGMP, and planar cell polarity pathways, in which only the Fzd receptors, not the LRP5 or LRP6 coreceptor, are required (4, 7).

Recent discoveries changed the conventional view of Wnt ligands and Fzd/LRP receptors (8–11). Norrin, a secreted protein unrelated to the Wnt proteins, is encoded by the Norrie disease gene, which is responsible for human hereditary Norrie disease, which is associated with blindness, deafness, and mental retardation (12, 13). Recently, Norrin was discovered as a high affinity ligand specific for the Fzd4·LRP6 receptor complex, which induces β-catenin-dependent signaling (8). The RYK, Derailed, and LIN-18 proteins, receptor tyrosine kinases with unknown function, were determined to be novel Wnt receptors in mouse, *Drosophila*, and *Caenorhabditis elegans*, respectively (9–11). These discoveries expanded the repertoire of ligands and receptors involved in Wnt/Fzd/LRP signaling and suggest the possibility of unknown ligands unrelated to the Wnt proteins and receptors unrelated to Fzd and LRP5 or LRP6.

We provide evidence strongly suggesting that a multigene family of novel secreted proteins, mouse Cristin/R-spondin proteins, may work at the receptor level to activate downstream β-catenin-dependent gene activation and may function as a class of ligands unrelated to Wnt proteins. Moreover, Cristin/R-spondin proteins exhibited intriguing synergistic activity on Wnt ligands, possibly through direct physical interaction. *Xenopus* R-spondin2 and human R-spondin1 protein activity on canonical Wnt signaling was demonstrated recently (14, 15), consistent with our findings. These results suggest that the function of Cristin/R-spondin family proteins is conserved in vertebrates.

MATERIALS AND METHODS

Molecular Biology—Mouse expressed sequence tag cDNA clones encoding full-length Cristin/R-spondin proteins were obtained from the I.M.A.G.E. Consortium. The coding regions of all *Cristin/R-spondin* genes and the deletion mutants of Cristin1/R-spondin3 were PCR-amplified and cloned into the pcDNA3 plasmid carrying a C-terminal hemagglutinin (HA) tag (pcDNA3-HA) or Myc/His epitope tags (pcDNA3.1-Myc/His). The coding sequence of mouse Wnt1 was PCR-amplified and cloned into pcDNA3-HA and the CS2-MT vectors to create HA- and Myc-tagged constructs, respectively. DNA sequence

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY864332.

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[2] The abbreviations used are: Fzd, Frizzled; LRP, low density lipoprotein receptor-related protein; Dvl, Dishevelled; TCF, T-cell factor; HA, hemagglutinin; CRD, cysteine-rich domain; CM, conditioned medium/media; RT, reverse transcription; TSR, thrombospondin type I repeat; HSPGs, heparan sulfate proteoglycans.

encoding a cysteine-rich domain (CRD) of mouse Fzd8 was excised from the Fzd8CRD-IgG plasmid (16) and cloned into pcDNA3-HA to create a C-terminally HA-tagged Fzd8CRD construct (Fzd8CRD-HA).

Cell Culture, DNA Transfection, and Luciferase Assay—Human embryonic kidney 293T cells and mouse L-cells were routinely maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in 5% CO_2 at 37 °C. P19 cells were maintained in α-minimal essential medium supplemented with 7.5% calf serum and 2.5% fetal bovine serum. 293T cells were transfected using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer's protocol. For luciferase assay, 3×10^4 cells were seeded in each well of 24-well plates. The TOPflash or FOPflash reporter (20 ng) and a *Renilla* luciferase-thymidine kinase construct (10 ng) were used along with various amounts of expression plasmids as indicated in the figure legends. Both luciferase activities were measured using a Dual-Luciferase assay kit (Promega) according to the manufacturer's protocol.

Preparation of Conditioned Medium (CM) and Isolation of Cristin/R-spondin Proteins—Wnt3a CM was prepared from the mouse Wnt3a L-cell line (obtained from American Type Culture Collection) as described previously (17). CM containing Cristin/R-spondin proteins was obtained from 293T cells transiently transfected with Cristin/R-spondin expression plasmids. For biochemical assay, serum-free Dulbecco's modified Eagle's medium/nutrient mixture F-12 (1:1) was used to obtain the CM. Soluble heparin (50 μg/ml) was added to the culture medium. Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was used to prepare the CM for the luciferase and β-catenin stabilization assays. For purification of histidine-tagged Cristin/R-spondin proteins, total lysates of 293T cells transiently transfected with histidine-tagged Cristin/R-spondin expression constructs were prepared with lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Nonidet P-40) containing protease inhibitor mixture V (Calbiochem). The cell lysates were incubated with nickel-nitrilotriacetic acid-agarose beads (Qiagen Inc.) for 2 h at 4 °C and washed with 10 mM imidazole buffer three times. The Cristin/R-spondin proteins were eluted with buffer containing 250 mM imidazole. The HA-tagged mouse Fzd8 CRD and human IgG fusions of the mouse Fzd8 CRD and the human LRP6 extracellular domain (LRP6N) were prepared as serum-free CM formats from transiently transfected 293T cells as described previously (18).

Heparin Binding and Cristin/R-spondin Binding Assay—Total cell lysates of 293T cells transfected with Cristin-HA constructs were prepared using lysis buffer containing protease inhibitors and incubated overnight at 4 °C with heparin-Sepharose beads (Sigma). The beads were washed with lysis buffer three times at room temperature, and the Cristin/R-spondin proteins were eluted with buffers with different NaCl concentrations. The presence of Cristin/R-spondin proteins was examined by Western blot analysis.

CM containing IgG, Fzd8CRD-IgG, and LRP6N-IgG were first incubated with protein A-Sepharose beads to conjugate IgG fusion proteins to the beads. Histidine-tagged Cristin/R-spondin proteins were incubated overnight at 4 °C with the beads. After three washings with 0.3 M NaCl/phosphate buffer, the associated Cristin/R-spondin proteins were analyzed by Western blot analysis using anti-His, anti-HA, or anti-Myc primary antibody. The filters were reprobed with anti-human IgG antibody.

RNA Isolation and Reverse Transcription (RT)-PCR Analysis—Total RNA was isolated from cultured cells and *Xenopus* animal cap explants using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and digested with RNase-free DNase I to remove genomic DNA contamination. The first-strand cDNA was synthesized with SuperScript II (Invitrogen), and one-tenth of the cDNA was used for each PCR. The sequences of the PCR primers were as follows: mouse glyceraldehyde-3-phosphate dehydrogenase (GADPH), 5′-GTGGCAAAGTGGAGATTGTTGCC-3′ (sense) and 5′-GATGATGACCCGTTTGGCTCC-3′ (antisense); mouse *Cristin1*/*R-spondin3*, 5′-GTACACTGTGAGGCCAGTGAA-3′ (sense) and 5′-ATGGCTAGAACACCTGTCCTG-3′ (antisense); mouse *BrachyuryT*, 5′-TGCTGCCTGTGAGTCATAC-3′ (sense) and 5′-ACAAGAGGCTGTAGAACATG-3′ (antisense); mouse *Cdx1*, 5′-GAACCAAGGACAAGTACCGTG-3′ (sense) and 5′-GGTAGAAACTCCTCCTTGACG-3 (antisense); *Xenopus Siamois*, 5′-AAGGAACCCCACCAGGATAA-3′ (sense) and 5′-TACTGGTGGCTGGAGAAATA-3′ (antisense); *Xenopus Xnr3*, 5′-TCCACTTGTGCAGTTCCACAG-3′ (sense) and 5′-ATCTCTTCATGGTGCCTCAGG-3′ (antisense); and *Xenopus Xmax2*, 5′-GTGGAAAGCGACGAAGACTC-3′ (sense) and 5′-CCGAGCTCGAGTAGTTGGAC-3′ (antisense).

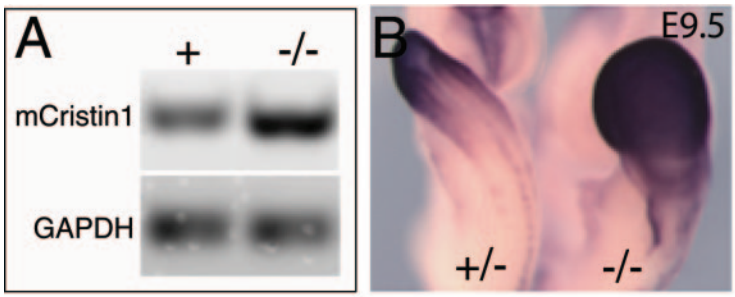
Western Blot Analysis, Immunoprecipitation, and Immunofluorescence Staining—For Western blotting, anti-HA (clone 12CA5), anti-Myc (clone 9E10), anti-His (Rockland Immunochemicals, Inc.), and anti-human IgG (Jackson ImmunoResearch Laboratories, Inc.) antibodies were used at 1:2000, 1:5000, 1:3000, and 1:4000 dilutions, respectively. Anti-β-catenin (Pharmingen) and anti-β-actin (Sigma) antibodies were used at 1:500 and 1:5000 dilutions, respectively. The presence of target proteins was detected by the chemiluminescence method (Amersham Biosciences). For immunoprecipitation, protein A-Sepharose and anti-HA or anti-Myc antibody conjugated to agarose (Sigma) or Sepharose (Santa Cruz Biotechnology, Inc.) beads were used to purify the protein complex. Subcellular localization of Cristin/R-spondin proteins in 293T cells was determined by immunofluorescence staining using mouse anti-HA primary antibody (1:1000 dilution) and Alexa 488-conjugated goat anti-mouse IgG secondary antibody (1:500 dilution; Molecular Probe). Images were acquired by confocal microscopy.

Mouse Embryo Collection and Whole-mount in Situ Hybridization—Wild-type embryos at different stages were collected from the timed matings of ICR mice. The *pMesogenin1* gene mutant embryos were collected from the mating between the heterozygous animals, and the genotypes of collected embryos were determined by genomic DNA PCR analysis with yolk sac DNA as described (19). The collected embryos were immediately fixed in freshly prepared 4% paraformaldehyde/phosphate-buffered saline solution overnight at 4 °C and kept in 100% methanol at −20 °C until used. Digoxigenin-labeled antisense *Cristin1*/*R-spondin3* RNA probes were synthesized *in vitro* from a linearized DNA template utilizing appropriate RNA polymerases in the presence of digoxigenin-labeled CTP. Whole-mount *in situ* hybridization was performed as described previously (19). Stained embryos were photographed with a Zeiss AxioCam digital camera.

Xenopus Embryos and Animal Cap Explants—*Xenopus* embryos were prepared by *in vitro* fertilization of oocytes collected from hormonally induced females by a standard protocol. Capped RNA was synthesized using an Ambion Message Machine kit according to the recommended protocol. Various amounts of RNA were injected into the animal pole of two-cell stage embryos. Animal caps were explanted at the blastula stage and cultured in 1× modified Bart's saline until the companion embryos reached the gastrulation stage.

RESULTS

Multigene Family of Novel Secreted Mouse Cristin/R-spondin Proteins—We previously demonstrated that mouse mutants lacking the *pMesogenin1* gene, which encodes a presomitic mesoderm-specific basic helix-loop-helix transcription factor (20), display severe defects in



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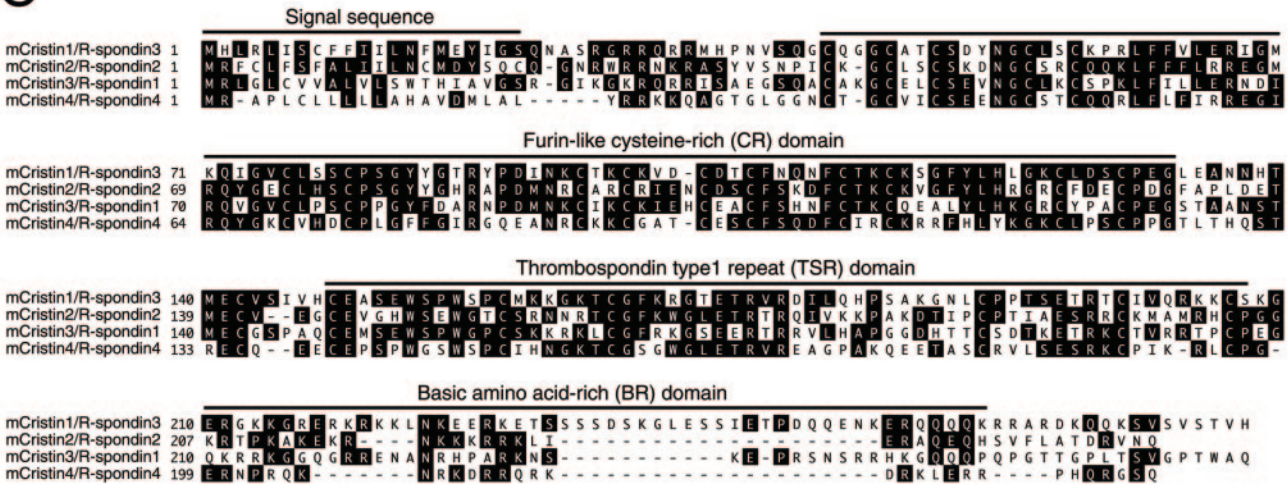

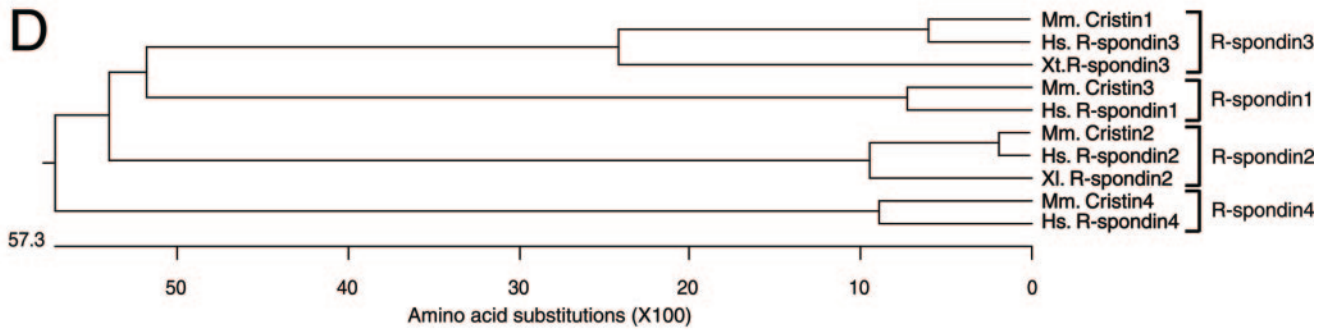

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Gene	Human	Mouse	# of Exons (H/M)
Cristin1/R-spondin3	Chr. 6q22	Chr. 10B5	5/5
Cristin2/R-spondin2	Chr. 8q23	Chr. 15B3	6/6
Cristin3/R-spondin1	Chr. 1p34	Chr. 4D2	8*/6
Cristin4/R-spondin4	Chr. 20p13	Chr. 2G3	5/5

FIGURE 1. **Cristin/R-spondin multigene family proteins are novel secreted proteins.** *A* and *B*, mouse (*m*) *Cristin1/R-spondin3* RNA expression in the tail buds of *pMesogenin1*-null mutant embryos was analyzed by both RT-PCR and whole-mount *in situ* hybridization, respectively. For total RNA isolation, the trunk regions below the hind limb levels of normal (+, wild-type and heterozygous embryos combined) and homozygous null mutant (−/−) embryos at embryonic day 9.5 (*E9.5*) were dissected in ice-cold phosphate-buffered saline. More than 40 individuals were combined to isolate RNA. Both heterozygous (+/−) and homozygous (−/−) *pMesogenin1* mutant embryonic day 9.5 embryos were collected and used for whole-mount *in situ* hybridization after genotyping of yolk sac genomic DNA. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *C*, shown is a comparison of mouse Cristin/R-spondin family protein sequences. The predicted protein sequences were aligned using the ClustalW program of the Lasergene package (DNAStar, Inc., Madison, WI). Identical amino acids are shown in *black boxes*. *Hyphens* indicate a sequence gap. The GenBank™ accession numbers for mouse R-spondin proteins are as follows: mouse Cristin3/R-spondin1, NP_619624; mouse Cristin2/R-spondin2, NP_766403; mouse Cristin1/R-spondin3, AY864332; and mouse Cristin4/R-spondin4, XP_130619. *D*, shown is a phylogenetic analysis of vertebrate R-spondin family proteins. The discovery of human and *Xenopus* R-spondins was reported while this manuscript was in preparation. The GenBank™ accession numbers as follows: human R-spondin1, NP_775911; human R-spondin2, NP_116173; human R-spondin3, AAQ88533; human R-spondin4, XP_297816; *Xenopus* R-spondin2, AY753198; and *Xenopus* R-spondin3, AY753199. *Mm*, *Mus muscularis*; *Hs*, *Homo sapiens*; *Xl*, *Xenopus laevis*. *Xt*, *Xenopus tropicalis*. *E*, the syntenic relationship between human (*H*) and mouse (*M*) *R-spondin* gene loci is shown. The predicted protein sequences of human R-spondin1 do not contain any notable signal sequences. It is unclear whether this particular DNA sequence represents an alternatively spliced form or an artifact of cDNA cloning. Therefore, the number of exons of the human *R-spondin1* gene is provisional. *Chr.*, chromosome.

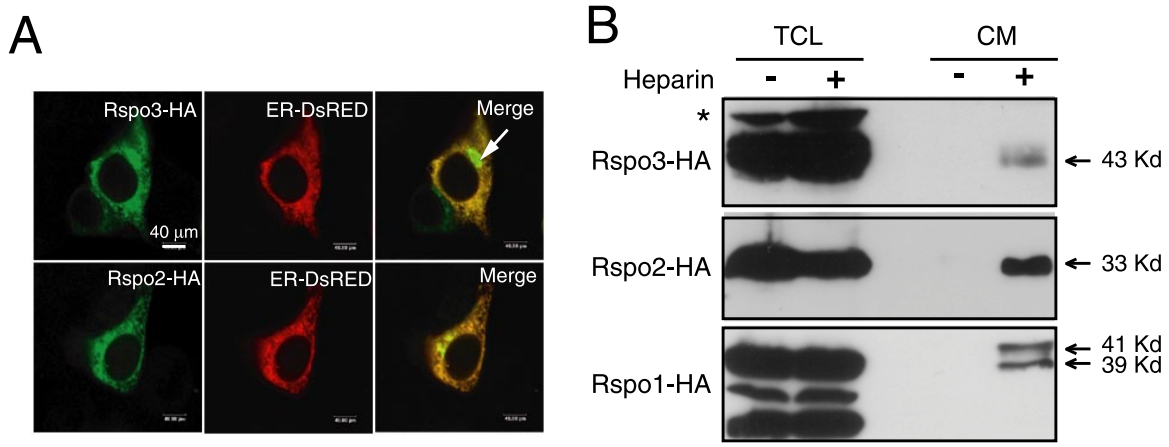

FIGURE 2. **Cristin/R-spondins are secreted proteins.** *A*, mouse Cristin/R-spondins (*Rspo*) are localized in the endoplasmic reticulum and Golgi apparatus in 293T cells. 293T cells were cotransfected with Cristin/R-spondin-HA expression plasmids and pDsRed2-ER (Clontech). The majority of Cristin/R-spondin proteins co-localized with the DsRed proteins residing in the endoplasmic reticulum. The *white arrow* indicates Cristin/R-spondin expression in the Golgi apparatus. *B*, mouse Cristin/R-spondins are secreted proteins. 293T cells were transfected with expression plasmids encoding HA-tagged Cristin/R-spondin proteins. Total cell lysates (*TCL*) and serum-free CM were analyzed by Western blotting using anti-HA antibody.

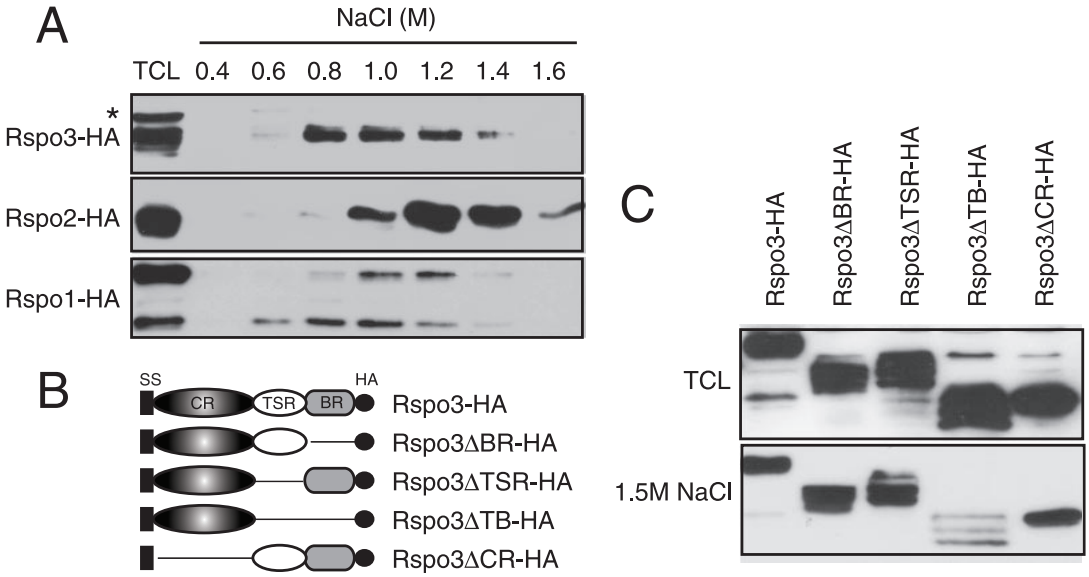

FIGURE 3. **Cristin/R-spondins are heparin-binding proteins.** *A*, total cell lysates (*TCL*) containing Cristin/R-spondin (*Rspo*) proteins were incubated with heparin-agarose beads, and the bound proteins were eluted with buffers containing different concentrations of NaCl. The presence of Cristin/R-spondin proteins was analyzed using 0.1 volume of each eluted fraction by Western blot analysis using anti-HA antibody. *B*, shown is a schematic diagram of Cristin1/R-spondin3-HA deletion constructs. *BR*, basic amino acid-rich domain; *CR*, CRD; *SS*, single sequence; *TB*, TSR plus BR. *C*, total cell lysates containing various Cristin1/R-spondin3 deletion constructs were incubated with heparin-agarose beads, and the bound proteins were eluted with 1.5 M NaCl buffer. 0.1 volume of eluted protein was analyzed by Western blotting as described for *A*. To monitor protein expression levels, the Cristin1/R-spondin3 protein expression level was measured in total cell lysates.

posterior paraxial mesoderm development (19). In an attempt to understand the molecular mechanisms by which pMesogenin1 regulates paraxial mesoderm development, potential target genes for pMesogenin1 were screened by analyzing gene expression profiles in the presomitic mesoderm of *pMesogenin1*-null and wild-type embryos. One gene whose RNA expression was increased by ~4-fold in the *pMesogenin1*-null mutant samples encoded a novel secreted protein (Fig. 1*A*). Based on the protein structure derived from the predicted peptide sequences, we named this gene *Cristin1* (for cysteine-rich and single thrombospondin domain-containing protein-1) (Fig. 1*C*). Whole-mount *in situ* hybridization analysis showed prominent RNA expression of *Cristin1* in the tail buds of wild-type mouse embryos (Fig. 1*B*). *Cristin1* expression within the tail buds of homozygous *pMesogenin1*-null mutants was significantly expanded, increased, and tightly associated with the tail bud defects, consistent with RT-PCR data. Thus, aberrant *Cristin1* expression may be related to the *pMesogenin1*-null phenotype. *Cristin1* expression was also detected in the primitive streak, dorsal neural tube, forebrain, and migrating neural crests of mouse embryos (Fig. 1*B*). In searching sequence data bases, we identified three genes homologous to *Cristin1* in the mouse genome (designated as *Cristin2*, *Cristin3*, and *Cristin4*) (Fig. 1*C*). We also identified four homologous genes in the human genome as well as expressed sequence tag clones with high sequence homology among other vertebrates, including chicken, *Xenopus*, and zebrafish. Phylogenetic analysis of predicted protein sequences, syntenic relationships of gene loci in human and mouse chromosomes, and comparisons of genomic structures determined the orthologous relationships among the human and mouse genes (Fig. 1, *D* and *E*). Interestingly, searches of the *Drosophila* and *C. elegans* genome data bases failed to identify *Cristin* homologs. Thus, *Cristin* genes may be unique to vertebrates, although functional homologs may exist in invertebrate species. Recently, the isolation of mouse *R-spondin*, which is identical to mouse *Cristin3*, and two *Xeno-*

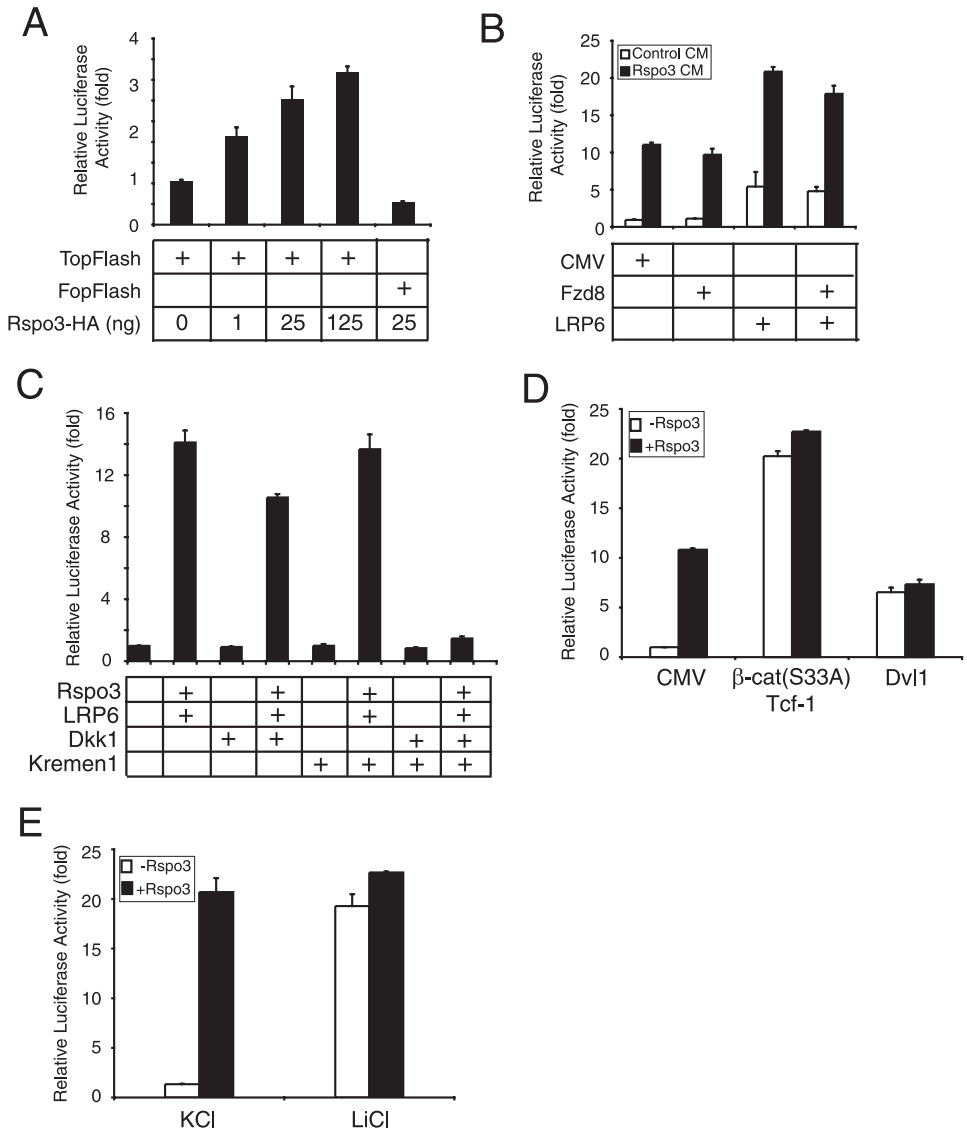

FIGURE 4. **Cristin/R-spondin proteins activate canonical Wnt/β-catenin signaling.** *A*, mouse Cristin1/R-spondin3 (*Rspo3*) induced β-catenin-dependent gene activation in a dose-dependent manner. 20 ng of TOPflash or FOPflash reporter plasmid was cotransfected with the indicated amounts of Cristin1/R-spondin3 expression plasmid into 293T cells grown in 24-well plates. 10 ng of *Renilla* luciferase driven by the thymidine kinase gene promoter was included as a transfection control. *B*, Cristin/R-spondin functioned at the ligand/receptor level. 293T cells were transfected with mouse Fzd8 (25 ng) and human LRP6 (10 ng) expression plasmids and stimulated with CM containing Cristin1/R-spondin3 for 24 h. The TOPflash reporter activities were measured. *CMV*, cytomegalovirus. *C*, Dkk1 and Kremen1 reversed the activation of the TOPflash reporter that was induced by both Cristin1/R-spondin3 and LRP6. 293T cells in 24-well plates were transfected with various combinations of Cristin1/R-spondin3 (25 ng), LRP6 (10 ng), Dkk1 (10 ng), and Kremen1 (10 ng) expression plasmids with the TOPflash reporter (20 ng). *D*, no synergy between Cristin1/R-spondin3 and intracellular components of the Wnt signaling pathway in the activation of the TOPflash reporter was observed. Expression plasmids encoding human β-catenin(S33A) (*β-cat(S33A)*; 80 ng), human TCF1 (10 ng), and human Dvl1 (50 ng) were cotransfected into 293T cells for the TOPflash reporter assay as indicated. *E*, Cristin1/R-spondin3 failed to potentiate the TOPflash reporter activation induced in 293T cells by treatment with LiCl, an inhibitor of glycogen synthase kinase-3β. 293T cells were transfected with the R-spondin1 expression plasmid and the TOPflash reporter. At 24 h after transfection, the cells were treated with either LiCl (40 mM) or NaCl (40 mM) for 8 h.

pus homologs of the mouse *R-spondin* genes was reported (14, 21). Thus, for consistency in nomenclature, we renamed the *Cristin* genes to *R-spondin* as indicated in Fig. 1*D*.

Comparisons of the predicted mouse R-spondin amino acid sequences revealed significant homologies (Fig. 1*C*). First, all R-spondin proteins contain an N-terminal 20–25-amino acid hydrophobic region, which likely serves as a signal sequence for secretion. Three additional conserved protein domains are evident: (i) a CRD with homology to the CRDs of furin and the insulin-like growth factor receptor, (ii) a thrombospondin type I repeat (TSR) (22), and (iii) a C-terminal basic amino acid-rich domain (Fig. 1*C*).

The presence of a putative signal sequence and the lack of a notable transmembrane domain suggest that R-spondins may be secreted proteins. To test this possibility, we transfected expression plasmids encoding HA-tagged R-spondins into human embryonic kidney 293T cells and examined the subcellular localization of R-spondins by immunofluorescence staining. R-spondin proteins were detected mainly in the endoplasmic reticulum and the Golgi apparatus (Fig. 2*A*), indicating that R-spondin proteins are in the secretory pathway. In similarly transfected cells, the expression of R-spondin proteins and their secretion into the CM were monitored by Western blot analysis. The majority of the R-spondin proteins were associated with total cell lysates and were barely detected, if at all, in the CM (Fig. 2*B*). Interestingly, the addition of soluble heparin to the medium in culture significantly enhanced the presence of R-spondins in the CM and confirmed their nature as secreted proteins (Fig. 2*B*). The addition of sodium chlorate, an inhibitor of sulfation, increased the level of R-spondin proteins in the CM.[3] These results suggest that secreted R-spondins may be associated, in part, with the heparan sulfate proteoglycans (HSPGs) of the plasma membrane and extracellular matrix.

R-spondin Binds to Heparin with High Affinity—We next examined whether R-spondin can bind to heparin. We prepared lysates from 293T cells transfected with R-spondin-HA expression plasmids. The cell lysates were incubated with heparin-Sepharose beads, and the bound proteins were eluted with a series of buffers with different salt concentrations. All R-spondins tested efficiently bound to the heparin-Sepharose beads and were eluted from the heparin beads between 0.8 and 1.2 M NaCl (Fig. 3*A*). Thus, all R-spondins bind to heparin with high affinity comparable with fibroblast growth factors (23).

To determine which domains within R-spondin3 are required for heparin binding, we generated a set of R-spondin3 constructs contain-

[3] J.-S. Nam, T. J. Turcotte, and J. K. Yoon, unpublished data.

ing various deletions of the identified domains (Fig. 3*B*). Total lysates of 293T cells transfected with these constructs were prepared, and the heparin binding of each protein construct was determined. It appears that both the basic amino acid-rich and TSR domains contain the majority of heparin binding capability, as deletion of both domains significantly decreased R-spondin3 binding to heparin (Fig. 3*C*). In contrast, R-spondin3 with a CRD deletion showed efficient binding to heparin comparable with wild-type R-spondin3, indicating that the CRD may not be necessary for heparin binding. We concluded that R-spondin3 is a heparin-binding protein and that both the TSR and basic amino acid-rich domains are necessary for binding to heparin.

R-spondin Activates Canonical Wnt/β-Catenin Signaling—Two observations led us to evaluate the possible involvement of R-spondin in Wnt signaling. First, embryonic expression of *R-spondin* genes highly overlaps with known *wnt* gene expression domains (24). Second, CCN (CYR61/connective tissue growth factor/NOV) family proteins, which contain domains structurally similar to those of R-spondins such as the cysteine-rich and TSR domains (25), were recently demonstrated to either antagonize or agonize Wnt signaling in a context-dependent manner (26, 27). The steady-state level of β-catenin protein and the activity of reporter constructs such as the TOPflash reporter carrying TCF1-binding sites in their upstream regulatory regions were extensively used to analyze canonical Wnt signaling. In 293T cells, overexpression of R-spondin3 strongly induced TOPflash reporter activity in a dose-dependent manner (Fig. 4*A*). Activation was dependent on the TCF1-binding site because a reporter carrying nonfunctional TCF1-binding sites (FOPflash) was not activated by R-spondin3. In addition, other mouse R-spondin family members and *Xenopus* R-spondin2 showed similar activities (14).[3] Interestingly, CM collected from the culture of 293T cells transfected with R-spondins effectively induced TOPflash reporter activity, suggesting that R-spondin may act in the extracellular environment (Fig. 4*B*).

To determine the target position for R-spondin activity in the Wnt signaling axis, we examined reporter activity under conditions of R-spondin3 coexpression with various Wnt signaling components. R-spondin3 activity was synergistically potentiated by the LRP6 receptor (Fig. 4*B*). However, unlike Wnt ligands, R-spondin3 failed to show any significant synergy with Fzd8 (Fig. 4*B*). Fzd8 failed to further enhance the reporter activity co-induced by R-spondin3 and LRP6 (Fig. 4*B*). Therefore, Fzd8 does not appear to actively contribute to the intracellular transmission of R-spondin signals. Furthermore, the reporter activity induced by both LRP6 and R-spondin3 was sensitive to the presence of both Dickkopf1 (Dkk1) (28) and Kremen1 (29), a condition that presumably enhanced LRP6 receptor endocytosis and inhibited intracellular signaling through the LRP6 receptor (Fig. 4*C*) (29). However, Dkk1 or Kremen1 alone marginally affected or had no effect on reporter activity induced by R-spondin3 and LRP6.

In contrast, R-spondin3 failed to show any synergistic activation of the reporter when coexpressed with other intracellular components of Wnt signaling, including Dvl1 or β-catenin/TCF1 (Fig. 4*D*). In addition, when cells were treated with LiCl, which mimics Wnt activation by inhibiting glycogen synthase kinase-3β, no synergistic activation with R-spondin3 of reporter activity was observed (Fig. 4*E*).

R-spondin Stabilizes Endogenous β-Catenin and Induces the Known Canonical Wnt/β-Catenin Target Genes—We examined R-spondin activity on stabilization of β-catenin, a landmark intracellular event upon activation of canonical Wnt signaling. R-spondin1 and R-spondin3 CM effectively increased steady-state β-catenin levels in 293T cells compared with Wnt3a CM (Fig. 5*A*), consistent with the reporter assay results. This result clearly shows that degradation of cytoplasmic β-catenin was prevented by R-spondins and that accumulated β-catenin may lead to the activation of reporter activity as shown in Fig. 4*A*. Both *Xenopus* R-spondin2 and human R-spondin1 also induce the stabilization of β-catenin (14, 15), consistent with our results.

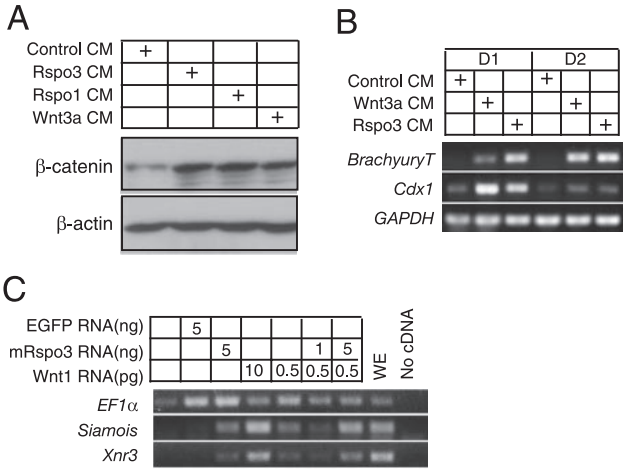

FIGURE 5. *A*, stabilization of β-catenin proteins by R-spondins. 293T cells were treated with CM containing R-spondin1 (*Rspo1*), R-spondin3 (*Rspo3*), or Wnt3a for 12 h. The steady-state level of endogenous β-catenin protein was analyzed by Western blotting. β-Actin expression was measured by Western blotting to provide a control for total sample quantity. *B*, induction of Wnt3a target genes by Cristin1/R-spondin3 in embryonic carcinoma P19 cells. Monolayer cultured P19 cells were stimulated with Cristin1/R-spondin3 CM (1:2) or Wnt3a CM (1:10) for 1 (*D1*) and 2 (*D2*) days, respectively. RNA expression of the *BrachyuryT* and *Cdx1* genes was analyzed by semiquantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was also measured to ensure the quality and quantity of cDNA samples. *C*, induction of Wnt target genes in *Xenopus* animal cap explants injected with mouse *R-spondin1* RNA. Injection of mouse (*m*) *Cristin1*/*R-spondin3* RNA (5 ng) or mouse *wnt1* RNA (10 pg) induced both *Siamois* and *Xnr3* RNA expression effectively. Co-injection of *Cristin1*/*R-spondin3* (1 ng) and *wnt1* (0.5 pg) RNAs at suboptimal concentrations synergistically activated target gene expression. To ensure the quantity and quality of cDNA samples, elongation factor-1α (*EF1α*) gene expression was measured. *EGFP*, enhanced green fluorescent protein.

We further investigated whether R-spondin can induce the expression of genes known as canonical Wnt signaling targets. Mouse embryonic carcinoma P19 cells were stimulated with Wnt3a or R-spondin3 CM for 2 and 3 days, respectively, and expression of *BrachyuryT* and *Cdx1*, two known Wnt3a target genes, was examined by semiquantitative RT-PCR analysis. Robust induction of both genes was observed in cells incubated with R-spondin3 and Wnt3a CM, respectively (Fig. 5*B*). Because both genes are expressed in the tail bud regions of mouse embryos (30, 31), where *R-spondin3* is also expressed, it is likely that both *BrachyuryT* and *Cdx1* are direct downstream targets of R-spondin3 signaling.

Axis duplication assay in *Xenopus* embryos is a signature assay to test Wnt/β-catenin activation *in vivo*. We next examined whether ectopic *R-spondin* expression in *Xenopus* embryos induces axis duplication. Injection of up to 5 ng of *R-spondin1* and *R-spondin3* RNAs into one of the ventral blastomeres in four-cell stage embryos did not induce any significant axis duplication phenotype, whereas similarly injected *wnt1* RNA in a picogram quantity produced a clear duplication of axis (data not shown). Our results are consistent with those recently reported for the *Xenopus R-spondin2* gene (14). The majority of embryos injected with a high concentration of *R-spondin3* RNA displayed severe gastrulation failure. Additionally, we occasionally observed tail duplication in a small number of the *R-spondin3* RNA-injected embryos. Interestingly, unlike whole embryo injection, transcription of two known Wnt target genes, *Siamois* and *Xnr3*, was significantly enhanced in *Xenopus* animal cap explants injected with mouse *R-spondin3* RNA compared with uninjected and control enhanced green fluorescent protein RNA-injected cap explants (Fig. 5*C*). We concluded that the R-spondin family of

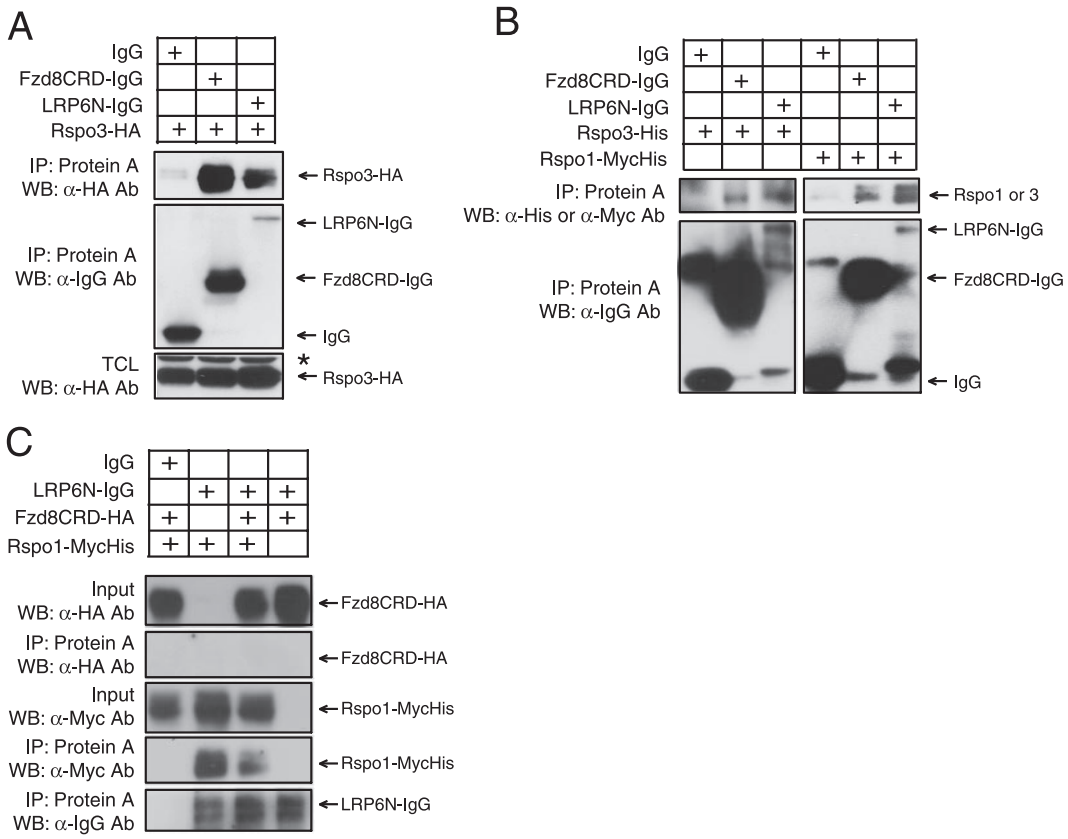

FIGURE 6. *A*, *in vivo* interaction of Cristin1/R-spondin3 (*Rspo3*) with mouse Fzd8 and human LRP6. Expression plasmids encoding the mouse Fzd8 CRD (Fzd8CRD-IgG) and the human LRP6 ectodomain (LRP6N-IgG) as human IgG Fc fusion proteins and control IgG were cotransfected with the Cristin1/R-spondin3-HA plasmid into 293T cells. Total cell lysates (*TCL*) were prepared and subjected to immunoprecipitation (*IP*) using protein A-Sepharose. Immunoprecipitated proteins were further analyzed by Western blot (*WB*) analysis using anti-HA or anti-human IgG antibody (*Ab*). To confirm the presence of Cristin/R-spondin protein, total cell lysates were also analyzed by anti-HA Western blotting. The *asterisk* indicates the nonspecific protein band detected by anti-HA antibody. *B*, *in vitro* interaction of Cristin/R-spondin with the Fzd8 or LRP6 receptor. R-spondin1-Myc/His and R-spondin3-His proteins were added after IgG, Fzd8CRD-IgG, or LRP6N-IgG in CM was prebound to protein A-Sepharose. *C*, No Ternary complex formation was detected in R-spondin1-Myc/His, Fzd8 CRD-HA, and LRP6N-IgG. *Input* represents one-tenth of the proteins used in the binding reactions.

proteins possesses signaling activities that induce β-catenin-dependent gene activation.

Binding of R-spondin to the Extracellular Domains of Fzd8 and LRP6 Receptors—To biochemically characterize R-spondin activity in the Fzd•LRP receptor complexes, we examined whether R-spondin3 binds to the extracellular domain of Fzd8, LRP6, or both. First, we utilized a cotransfection/co-immunoprecipitation format. Expression plasmids encoding mouse *R-spondin3* tagged with the HA epitope and human IgG fusions of the CRD of mouse Fzd8 (Fzd8CRD-IgG) (16) and the extracellular domain of human LRP6 (LRP6N-IgG) (32) were cotransfected into 293T cells. Cell lysates were subjected to immunoprecipitation followed by Western blot analysis. The R-spondin3 protein was effectively co-immunoprecipitated with Fzd8CRD-IgG and LRP6N-IgG, but not with IgG (Fig. 6*A*). Although much less concentrated than Fzd8CRD-IgG, the LRP6N-IgG protein co-immunoprecipitated a comparable amount of R-spondin3. Thus, R-spondin3 may bind to the LRP6 ectodomain at a higher affinity than to the CRD of Fzd8 *in vivo*. In contrast, more R-spondin3 molecules may bind to the LRP6 ectodomain than to the CRD of Fzd8.

We next examined these interactions under cell-free liquid-phase conditions. We isolated histidine-tagged R-spondin1 and R-spondin3 proteins from total lysates of 293T cells transiently transfected with R-spondin expression plasmids. Fzd8CRD-IgG and LRP6N-IgG fusion proteins were prepared in a CM format as described (32). We found that both R-spondin1 and R-spondin3 were specifically coprecipitated with Fzd8CRD-IgG and LRP6N-IgG, but not with IgG (Fig. 6*B*). Similar to the cotransfection/co-immunoprecipitation results, R-spondin proteins appeared to bind to LRP6N-IgG more efficiently than to Fzd8CRD-IgG, as similar amounts of R-spondins were co-immunoprecipitated with a reduced amount of LRP6N-IgG.

Because the Wnt1 ligand has been shown to form a ternary complex with Fzd8 and LRP6 under *in vitro* binding conditions (32), we next examined whether R-spondin can form a similar ternary complex with both receptors. LRP6N-IgG and Fzd8 CRD-HA failed to form a complex (Fig. 6*C*), consistent with the previously reported results (32). When the R-spondin1-Myc/His protein was incubated with both LRP6N-IgG and Fzd8 CRD-HA, we could not detect any Fzd8 CRD-HA protein in the immunoprecipitated LRP6N-IgG construct, whereas R-spondin1-Myc/His was present in the construct (Fig. 6*C*). This result suggests that, unlike Wnt1 ligands, the R-spondin, LRP6, and Fzd8 proteins do not form a ternary complex. Interestingly, the presence of Fzd8 CRD-HA in the binding reaction significantly reduced the binding of R-spondin1 to LRP6N-IgG (Fig. 6*C*), indicating that Fzd8 CRD-HA may compete with LPR6-IgG for binding to R-spondin1. The same or overlapping domain of the R-spondin1 protein may be engaged in binding to both LRP6 and Fzd8 receptors.

To further investigate which domain of R-spondin is involved in these interactions, we cotransfected R-spondin3 deletion mutants with Fzd8CRD-IgG or LRP6N-IgG and analyzed their interactions. We determined that the CRD of R-spondin3 was essential for the interaction with both Fzd8 and LRP6 receptors (Fig. 7, *A* and *B*). The R-spondin3 construct lacking the CRD failed to signal through the LRP6

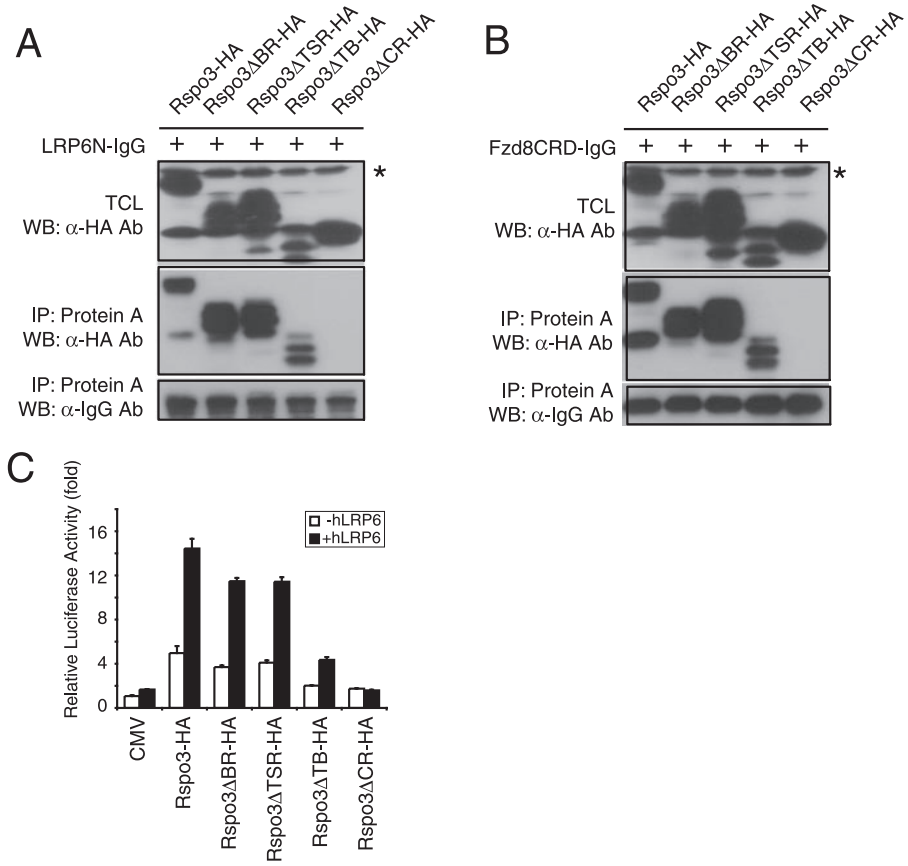

FIGURE 7. **CRD of Cristin1/R-spondin3 is a primary region for interacting with the Fzd8·LRP6 receptor complex.** *A*, 293T cells were cotransfected with the expression plasmid encoding LRP6N-IgG and R-spondin3 (*Rspo3*) derivatives carrying various deletions. Total cell lysates (*TCL*) were prepared and subjected to immunoprecipitation (*IP*) with protein A-Sepharose, followed by Western blot (*WB*) analysis using anti-HA and anti-human IgG antibodies (*Ab*). Total cell lysates were also analyzed by anti-HA Western blotting to confirm the presence of proteins in the cell lysates. The *asterisk* indicates the nonspecific protein band detected by anti-HA antibody. *BR*, basic amino acid-rich region; *CR*, CRD; *TB*, TSR plus BR. *B*, 293T cells were cotransfected with the expression plasmid encoding Fzd8CRD-IgG and Cristin1/R-spondin3 derivatives carrying various deletions. Immunoprecipitation and Western blot analyses were performed as describe for *A*. *C*, the CRD of Cristin1/R-spondin3 mediated a synergistic activation of β-catenin-dependent gene expression by LRP6 and Cristin1/R-spondin3. 293T cells were cotransfected with the TOPflash reporter, the human (*h*) LRP6 expression plasmid, and various Cristin1/R-spondin3 deletion constructs and assayed for reporter luciferase activity. The expression level of the Rspo3ΔTB-HA construct was lower than that of other deletion constructs in most of the tested cases. *CMV*, cytomegalovirus.

receptor in the reporter assay (Fig. 7*C*), consistent with the co-immunoprecipitation results. Interestingly, R-spondin3 lacking both TSR and basic amino acid-rich domains also showed reduced activation. However, this result appears to reflect the lower level of expression, not the activity, of this construct.

R-spondin Positively Modulates Wnt Ligand Activity—We unexpectedly observed that cotransfection of canonical Wnt proteins, including Wnt1 and Wnt3a, and R-spondin3 also induced TOPflash reporter activity at a significantly higher level than either Wnt or R-spondin3 individually (Fig. 8*A*). R-spondin1, R-spondin2, and R-spondin4 showed similar synergistic activities with Wnt proteins on the induction of reporter activity.[3] Both the CM stimulation and DNA-mediated transfection formats generated similar synergistic activation by R-spondin3 and Wnt3a (Fig. 8*A*), indicating that synergistic activation of Wnt signaling by R-spondin occurs in the extracellular environment. We examined the steady-state level of β-catenin protein in mouse L-cells after exposure to Wnt3a CM, R-spondin CM, or a mixture of Wnt3a and R-spondin CM. Stimulation of cells with both R-spondin and Wnt3a synergistically enhanced β-catenin stability (Fig. 8*B*), consistent with reporter assay results. Similar synergistic activation of signaling was observed in the activation of *Siamois* and *Xnr3* gene transcription in *Xenopus* animal cap explants (Fig. 5*C*).

To investigate the biochemical nature of this synergistic activity between R-spondin and Wnt proteins, we examined whether Wnt1 and R-spondin proteins physically interact with each other in cultured cells. In the lysates of 293T cells cotransfected with Wnt1-Myc and R-spondin-HA expression plasmids, Wnt proteins were efficiently co-immunoprecipitated with R-spondin1, R-spondin2, and R-spondin3 proteins (Fig. 8*C*). R-spondin2 seemed to be co-immunoprecipitated with Wnt1 less effectively compared with R-spondin1 and R-spondin3. Thus, R-spondins appear to be physically associated with Wnt proteins, possibly through direct interaction.

To further determine which domain of R-spondin3 interacts with the Wnt1 protein, a series of R-spondin3 deletion constructs were tested for their ability to bind to the Wnt1 protein by a cotransfection/co-immunoprecipitation format in 293T cells. The R-spondin3 construct lacking the CRD did not co-immunoprecipitate with the Wnt1-Myc protein (Fig. 8*D*), indicating that the CRD of R-spondin3 is required for binding to Wnt1. Consistent with this result, cotransfection of Wnt1 and R-spondin3 lacking the CRD did not cause the synergistic activation of the TOPflash reporter, whereas other R-spondin3 constructs synergistically activated the reporter in the presence of Wnt1 (Fig. 8*E*). We concluded that the CRD of R-spondin3 is necessary for its positive modulatory activity on the Wnt1 protein.

DISCUSSION

R-spondins as Novel Ligands for Canonical Wnt Signaling—The canonical Wnt signaling pathway consists of Wnt ligands and a receptor complex consisting of Fzd family and LRP5/6 receptors at the plasma membrane (6). Several recent studies have expanded our knowledge of the repertoire of ligands and receptors involved in Wnt/β-catenin signaling. First, Norrin, a non-Wnt related protein, was identified as a specific ligand for the Fzd4·LRP6 receptor complex (8). Second, the RYK receptor and its invertebrate homologs Derailed (*Drosophila*) and LIN-18 (*C. elegans*) were demonstrated to be receptors for Wnt ligands (9–11). In both cases, the ligand binding to the receptors induced effective β-catenin-dependent gene activation in signal recipient cells.

Our data clearly demonstrate that R-spondins are novel ligands for the Fzd8 and LRP6 receptors and induce β-catenin/TCF-dependent

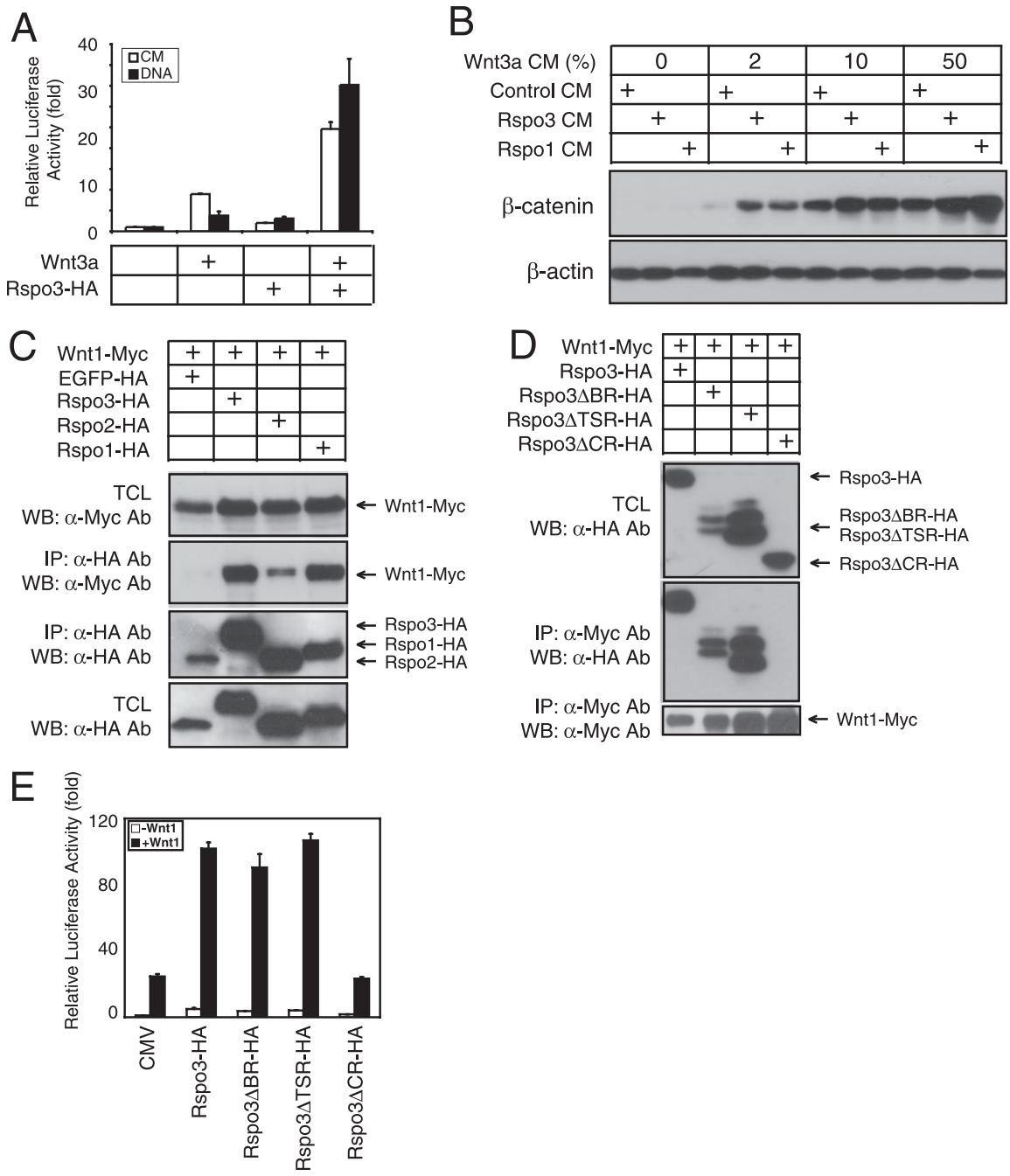

FIGURE 8. **Cristin/R-spondin proteins function as a positive modulator of Wnt ligands.** *A*, Cristin1/R-spondin3 (*Rspo3*) CM and Wnt3a CM synergistically induce TOPflash reporter activity. 293T cells were transfected with the TOPflash reporter and stimulated with Wnt3a CM (1:2), Cristin1/R-spondin3 CM (1:2), or both for 8 h. Control CM was used for diluting the CM or as a background. In a parallel experiment, expression plasmids encoding Wnt3a and Cristin1/R-spondin3 were cotransfected into 293T cells and tested for synergism. *B*, synergistic increase in the steady-state level of β-catenin protein in mouse L-cells stimulated with various concentrations of Wnt3a (1:2–50), Cristin1/R-spondin3 CM (1:2), or both for 12 h. The β-catenin protein level was determined by Western blot analysis. The β-actin level was also measured to confirm an equivalent amount of sample loading. *C*, Wnt1 and Cristin/R-spondin proteins are associated with each other in 293T cells. 293T cells were cotransfected with Wnt1-Myc and different Cristin/R-spondin-HA expression plasmids. As a negative control, the enhanced green fluorescent protein (*EGFP*)-HA expression plasmid was cotransfected with the Wnt1-Myc plasmid. Total cell lysates (*TCL*) were prepared and subjected to immunoprecipitation (*IP*) using anti-HA antibody (*Ab*)-agarose beads. The immune complexes were analyzed by Western blotting (*WB*) using anti-Myc antibodies. To determine protein expression, total cell lysates were also analyzed by Western blotting using anti-Myc and anti-HA antibodies. *D*, the CRD of R-spondin3 is required for Wnt1 binding. R-spondin1 deletion constructs tagged with the HA epitope were cotransfected with the Wnt1-Myc plasmid into 293T cells. Anti-Myc antibody-agarose beads were used to immunoprecipitate the Wnt1-Myc protein from cell lysates. Anti-HA and anti-Myc antibodies were used to detect R-spondin1 derivatives and Wnt1 protein, respectively, within the immune complex by Western blot analysis. Expression of the R-spondin1 derivatives was also determined by Western blot analysis of total cell lysates using anti-HA antibody. *BR*, basic amino acid-rich domain; *CR*, CRD. *E*, the CRD of R-spondin3 is necessary for the synergistic regulation of TOPflash reporter activity induced by Wnt1. Wnt1-Myc (25 ng) and R-spondin1-HA deletion constructs (25 ng) were cotransfected with the TOPflash reporter (20 ng) into 293T cells.

gene activation. Recently, the discovery of *Xenopus R-spondin2* and *R-spondin3* genes was reported (14). *Xenopus* R-spondin2 and human R-spondin1 exhibit a positive signaling activity on canonical Wnt signaling (14, 15), consistent with our results. However, both studies lacked any evidence showing the interactions between R-spondin and the Fzd and LRP5/6 receptors. In their report on *Xenopus* R-spondin2, Kazanskaya *et al.* (14) suggested that their attempt to determine binding between R-spondin and the Fzd or LRP6 receptor failed. However, we

convincingly demonstrated that R-spondin interacts with the extracellular domains of the Fzd8 and LRP6 receptors both *in vivo* and *in vitro* (Fig. 6). It is difficult to understand the reason for the different results. It is possible that *Xenopus* R-spondin may have a significantly weaker binding activity for the receptors, which made it difficult to detect the interaction in their assay. Nonetheless, our results confirm that mouse R-spondin is indeed a novel ligand for both Fzd and LRP receptors.

Interestingly, the Fzd8 receptor was incompetent to mediate a downstream signaling event leading to β-catenin-dependent gene activation despite the efficient binding to the R-spondin protein (Figs. 4*B*, 6, and 7*B*). Two explanations are possible for these puzzling data. First, an increasing body of evidence indicates that signaling activities through the LRP6 receptor may be dissociated from those through the Fzd receptors (33–36). Thus, preferential R-spondin signaling through the LRP6 receptor may be another example of dissociation of intracellular signaling events at the receptor level. Therefore, R-spondin binding to the LRP6 receptor may be sufficient to induce β-catenin-dependent signaling without any contribution from Fzd (Fig. 9, *Model 3*). Our result from the *in vitro* binding assay supports this possibility. Unlike canonical Wnt ligands that could induce ternary complex formation with the Fzd8 and LRP6 receptors (Fig. 9, *Model 1*), the R-spondin1 protein failed to form a ternary complex (Fig. 6*C*). Because the same CRD of R-spondin was required for binding to the Fzd and LRP6 receptors (Fig. 7), it is likely that R-spondin binding to both receptors may be competitive. Consistent with this notion, Fzd8 CRD-HA significantly inhibited the binding of R-spondin1 to the LRP6N-IgG protein (Fig. 6*C*). Because the association of Fzd and LRP6 mediated by canonical Wnt ligands appears to be critical for activating downstream events (18), this result suggests that R-spondin induces β-catenin-dependent gene activation by mechanisms distinct from Wnt signaling. Furthermore, it is possible that the R-spondin and Fzd interaction may induce only non-canonical signaling independent of β-catenin (Fig. 9, *Model 4*).

Second, R-spondin may preferentially bind to and transmit the signal through other Fzd receptors along with the LRP6 receptor (Fig. 9, *Model 2*). Although it is currently unknown whether other Fzd receptors transduce R-spondin signals, this is a very plausible scenario given that the Norrin ligand transduces the signal exclusively through the Fzd4·LRP6 receptor complex (8).

Positive Modulation of Wnt Ligand Activity by R-spondin—Surprisingly, we discovered that R-spondin plays a positive modulatory role in Wnt ligand activity presumably by directly interacting with Wnt ligands (Fig. 8). It appears that the CRD of R-spondin3 is necessary for this activity (Fig. 8, *D* and *E*). How can R-spondins modulate Wnt activity? First, it is plausible that the Wnt·R-spondin complex is a higher affinity ligand than Wnt or R-spondin individually. Second, R-spondin may stabilize the Wnt ligand/receptor interaction without increasing the binding affinity of the Wnt ligand for the receptor. Third, Wnt may be protected from degradation or turnover when it is associated with R-spondin. The molecular mechanisms underlying this activity will be a focus of future studies.

A large number of Wnt antagonists are known. Some Wnt antagonists, including secreted Frizzled-related proteins (37) and Wnt inhibitory factor (38), likely inhibit Wnt signaling activity by direct interaction with Wnt proteins to prevent their receptor binding. In contrast, Dkk1 may not directly interact with Wnt proteins, but may regulate LRP5/6 receptor endocytosis or availability (29). Because R-spondin seems to directly interact with Wnt proteins, how R-spondin activity is regulated by other Wnt-binding antagonists will be a focus of future investigation. To this end, the CCN family proteins, which primarily act as Wnt antagonists (26, 27), are of particular interest. The presence of similar protein motifs such as TSRs and CRDs in both R-spondins and CCN proteins (25) strongly suggests that they may belong to the ligand superfamily and have opposing activities for modulating Wnt signaling activity.

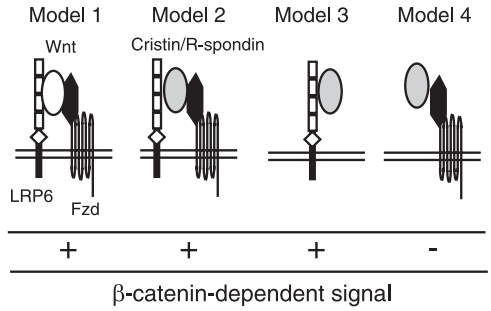

FIGURE 9. **Proposed models for Cristin/R-spondin and Wnt signaling through the Fzd and LRP6 receptors.** *Model 1* is a current view of canonical Wnt signaling. In *Model 2*, Cristin/R-spondin proteins generate β-catenin-dependent signaling through the Fzd/LRP6 receptors, similar to canonical Wnt signaling. In contrast, Cristin/R-spondin may transduce its signal through the LRP6 receptor (*Model 3*). Cristin/R-spondin also induces non-canonical Wnt signaling through the Fzd receptor (*Model 4*). Wnt and Cristin/R-spondin ligands are indicated by *white* and *gray ovals*, respectively.

Possible Regulatory Role of HSPGs in R-spondin and Wnt Signaling—The majority of R-spondin proteins expressed in the cells appear to reside within the endoplasmic reticulum and Golgi apparatus, whereas a relatively small portion is secreted from cells (Fig. 2). The secreted R-spondin seems to be associated with the plasma membrane and extracellular matrix. We found that R-spondins are heparin-binding proteins with high affinity and that both the TSR and basic domains of R-spondin3 are required for this binding (Fig. 3). Therefore, it is highly likely that secreted R-spondin is associated with HSPGs on the plasma membrane and extracellular matrix.

Heparin or HSPGs play important regulatory roles in growth factor/cytokine signaling (39, 40). The role of HSPGs in Wnt signaling was recently demonstrated (41–43). Two models have been proposed for a HSPG regulatory role in Wnt signaling based primarily on studies on the *Drosophila* Wnt homolog Wingless (44). First, HSPGs control either degradation or diffusion of Wingless to regulate its availability. Second, HSPGs serve as low affinity receptors for the Wingless ligand and may facilitate its binding to the Fzd/LRP receptors. It was demonstrated recently that heparin binding to Wnt ligands inhibits Wnt signaling activity in mammalian cells (41). Furthermore, Chinese hamster ovary cell mutants for heparan sulfate biosynthesis are defective in the activation of Fzd receptor signaling (41). It would be intriguing to determine whether HSPGs regulate R-spondin signaling activity and, if so, what the underlying mechanisms are. In our preliminary studies, we found that soluble heparin exhibited an inhibitory activity on R-spondin signaling, similar to Wnt signaling.[3]

Fine Control of Canonical Wnt Signaling Activity—Our study has provided evidence that R-spondin family proteins are novel ligands for the Fzd8 and LRP6 receptors. Furthermore, R-spondin proteins can induce β-catenin-dependent gene activation likely through the LRP6 receptor by mechanisms different from those in canonical Wnt signaling. Interestingly, under R-spondin and Wnt coexistence conditions, the signaling activity of the Wnt·R-spondin ligand complex is significantly higher than that of either Wnt or R-spondin alone. Thus, combinatorial expression of R-spondin and Wnt proteins may produce differential signaling activities that lead to differential activation of a battery of genes, which eventually govern the outcome of cellular processes. Future studies will be focused on determining the molecular mechanisms by which the differential signaling activity is regulated. In addition, the co-presence of different secreted Wnt antagonists (37) will

further define the level of signaling activity, although whether any known Wnt antagonists affect R-spondin activity is unknown.

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