R-Spondin1 regulates Wnt signaling by inhibiting internalization of LRP6

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The R-Spondin (RSpo) family of secreted proteins act as potent activators of the Wnt/β-catenin signaling pathway. We have previously shown that RSpo proteins can induce proliferative effects on the gastrointestinal epithelium in mice. Here we provide a mechanism whereby RSpo1 regulates cellular responsiveness to Wnt ligands by modulating the cell-surface levels of the coreceptor LRP6. We show that RSpo1 activity critically depends on the presence of canonical Wnt ligands and LRP6. Although RSpo1 does not directly activate LRP6, it interferes with DKK1/Kremen-mediated internalization of LRP6 through an interaction with Kremen, resulting in increased LRP6 levels on the cell surface. Our results support a model in which RSpo1 relieves the inhibition DKK1 imposes on the Wnt pathway.

beta-catenin | DKK1 | Kremen

The Wnt family of secreted proteins plays a critical role in diverse biological processes, including development, differentiation, and proliferation (1, 2). Canonical Wnt signaling induces downstream cellular responses by regulating cytosolic levels of β-catenin. In the absence of Wnt, cytosolic β-catenin is phosphorylated by an Axin/GSK3/APC complex and targeted for rapid degradation by the proteasome. Binding of a Wnt ligand to a serpentine Frizzled receptor and LRP6/5 coreceptor leads to inactivation of this complex, resulting in stabilization of cytosolic β-catenin and subsequent nuclear β-catenin/T cell factor (TCF)-mediated target gene transcriptional activation (3, 4). The low-density lipoprotein receptor-related proteins LRP5 and LRP6 are single-pass transmembrane proteins that are required for canonical Wnt signaling (5). Wnt-mediated phosphorylation of the cytoplasmic tail of LRP5/6 has been shown to be important for initiation of the Wnt signaling cascade (6–8). Wnt induces sequential phosphorylation of PPPSP motifs in the LRP6 cytosplasmic tail by GSK3 and casein kinase I and subsequent recruitment of the scaffold protein Axin (6, 7), implicating LRP6-mediated translocation of the Axin/GSK3/APC complex to the plasma membrane in the stabilization of cytosolic β-catenin (5–9).

Cell-surface levels of LRP5/6 are regulated by the secreted Dickkopf (DKK) proteins (10–14). DKK1 and -2 inhibit Wnt signaling by triggering LRP5/6 internalization through formation of a ternary complex with Kremen receptors (10, 12), indicating that the cell-surface levels of LRP5/6 limit cellular responsiveness to canonical Wnt ligands.

We and others recently described the R-Spondin (RSpo) family of secreted ligands that, similar to canonical Wnt family members, activate β-catenin signaling (15–18). Expression of RSpo proteins overlaps with expression of Wnt ligands during development (15, 19), indicating that signaling of RSpo and Wnt proteins may be closely linked. This notion is supported by the recent finding that phenotypes observed in patients or mice with loss-of-function mutations in RSpo1 (20), -3 (21), or -4 (22), are similar to defects seen in mice lacking expression of Wnt ligands and/or receptors (20, 23–25).

RSpo proteins provide a link to the Wnt signaling pathway; however, the precise mechanism by which they induce β-catenin signaling remains unknown. Here we show that Rspo1 regulates Wnt signaling by modulating levels of LRP6 on the cell surface, through inhibition of DKK1-dependent internalization of LRP6.

Results and Discussion

To further investigate the role of RSpo1 in the activation of the Wnt pathway, we generated a HEK-293 stable cell line expressing a TCF luciferase reporter plasmid. In agreement with reported results (16), both RSpo1 and Wnt3A proteins induced a comparable TCF-mediated reporter activity [supporting information (SI) Fig. 7]. However, when Wnt3A was incubated with a suboptimal dose of RSpo1 protein (Fig. 1*A*), a synergistic activity was detected, suggesting that RSpo1 may render cells more sensitive to Wnt signaling (15, 17).

To further explore the link between Wnt and RSpo signaling, we investigated whether known modulators of the Wnt pathway can also regulate RSpo1 activity. Wnt Inhibitory Factor (WIF) and Soluble Frizzled Related Protein 1 (sFRP1) are secreted molecules that bind selectively to Wnt ligands and sequester Wnt activity through a direct interaction with the WIF domain and Frizzled-like cysteine-rich domain, respectively (26–28). As shown in Fig. 1*B*, RSpo1-mediated TCF luciferase activity was blocked by treatment with WIF or sFRP1 recombinant proteins, suggesting a requirement of Wnt ligands for RSpo1 function. Because WIF and sFRP1 directly bind to Wnt proteins (27, 29–31), it is most likely that WIF and sFRP1 inhibit RSpo1-mediated activity by sequestering endogenous Wnt proteins secreted from HEK-293 cells. Consistent with this notion, HEK-293 cells expressed mRNA for the canonical Wnt ligands Wnt3 and Wnt3A (32) and Wnt regulators DKK1 and DKK2, whereas expression was not detected in Rspo1 nonresponsive mouse L- cells (Fig. 1*C* and SI Fig. 8) (16).

To further examine the requirement of endogenously expressed Wnt ligands for RSpo1 activity in HEK-293 cells, we used Wnt3A-specific siRNA. We selected Wnt3A siRNA duplexes that reduced endogenous Wnt3A mRNA levels by 80% (SI Fig. 9*A*), reduced expression of transiently expressed Wnt3A protein (SI Fig. 9*B*), and inhibited reporter activation induced by transfection of Wnt3A plasmid (SI Fig. 9*C*). Interestingly, Wnt3A-specific siRNA duplexes, but not control siRNA duplexes, inhibited RSpo1-mediated TCF reporter activity, even at excess amounts of RSpo1 protein (up to 400 ng/ml) (Fig. 2*A*). In contrast, Wnt3A siRNA had a marginal effect on reporter activity induced by increasing amounts of exog-

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Abbreviation: TCF, T cell factor.

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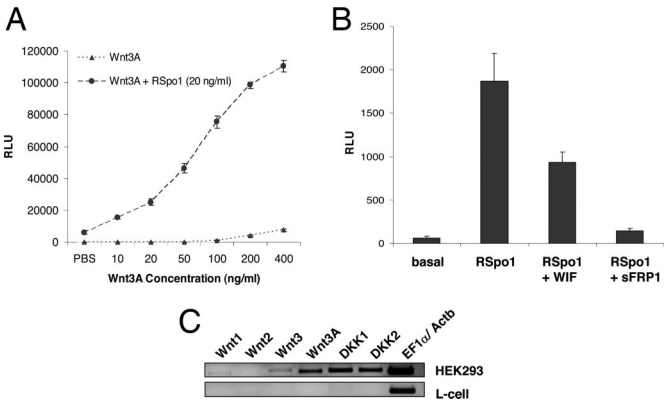

Fig. 1. RSpo1 activity is linked to canonical Wnt signaling. (*A*) HEK-293 cells stably expressing a 16TCF luciferase reporter (HEK-293 A6) were treated with recombinant Wnt3A protein in the absence or presence of a low dose of recombinant RSpo1 protein (20 ng/ml). (*B*) HEK-293 A6 cells were incubated with RSpo1 (200 ng/ml) in the presence or absence of recombinant Wnt pathway inhibitors WIF (1 μg/ml) and sFRP1 (1 μg/ml). (*C*) RSpo1-responsive HEK-293 cells and nonresponsive mouse L-cells were analyzed for mRNA expression of canonical Wnt ligands and Wnt inhibitors by semiquantitative RT-PCR.

enous Wnt3A protein (Fig. 2*B*). Similarly, Wnt3A siRNA duplexes blocked the accumulation of cytosolic β-catenin induced by RSpo1, but not Wnt3A proteins (Fig. 2 *A* and *B*), confirming the requirement of Wnt3A for RSpo1 function in HEK-293 cells.

We next attempted to inhibit Wnt activity in HEK-293 cells by siRNA duplexes specific for Wntless (WLS), a membrane protein required for the secretion of Wnt proteins (33). Wntless siRNA duplexes reduced WLS mRNA levels by 90% (SI Fig. 9*D*) and blocked TCF reporter activity induced by transfected Wnt3A plasmid (SI Fig. 9*E*). Although WLS siRNA inhibited exogenous Wnt3A activity by ≈50% (Fig. 2*D*), it completely blocked RSpo1-mediated TCF reporter activity (up to 400 ng/ml Rspo1; Fig. 2*C*). In addition, WLS siRNA completely blocked RSpo1 but not Wnt3A-mediated stabilization of cytosolic β-catenin (Fig. 2 *C* and *D*). Together, these findings demonstrate that RSpo1 activity in HEK-293 cells depends on an endogenous pool of canonical Wnt proteins and suggest that RSpo1 may render cells more sensitive to low levels of Wnt ligand.

To investigate whether RSpo1 directly activates Wnt receptors, we reconstituted the Wnt signaling pathway in *Drosophila* S2 cells by using human LRP6 and FZD constructs (34, 35) (Fig. 3*A*). Although S2 cells do not respond to Wnt3A or RSpo1 protein treatment, coexpression of FZD1, -2, -4, -5, -7, -8, -9, or -10 with LRP6 was sufficient to reconstitute Wnt3A-dependent TCF reporter activation. In contrast, S2 cells expressing LRP6 and FZD receptors were not responsive to RSpo1. Moreover, RSpo1 did not enhance Wnt3A signaling in LRP6/FZD-expressing S2 cells, confirming that RSpo1 does not directly activate FZD or LRP6 receptors. Although the experiments in S2 cells indicated that LRP6 is not sufficient for RSpo1 function, we wanted to investigate whether it is required for RSpo1 signaling. We therefore analyzed RSpo1 activity in HEK-293 cells overexpressing a dominant-negative LRP6 construct. In agreement with published data (17), overexpression of WT LRP6 in HEK-293 cells enhanced TCF reporter activation induced by both RSpo1 and Wnt3A, whereas expression of a dominant negative LRP6 construct, lacking the C terminus (LRP6ΔC) (36), blocked TCF reporter activity induced by both RSpo1 and Wnt3A proteins (Fig. 3*B*). These data suggest that LRP6 is required for RSpo1 function, and that the RSpo1 and Wnt signaling pathways converge at the level of the LRP6 receptor.

To further understand the functional link between RSpo1 and LRP6, we next tested whether RSpo1 can affect the phosphorylation status of LRP6, because it was recently demonstrated that RSpo1 can induce LRP6 phosphorylation (37). HEK-293 cells were incubated for 4 hours with Wnt3A and RSpo1 proteins and LRP6 phosphorylation analyzed by using a LRP6 phospho-Ser-1490-specific antibody (Fig. 3*C*). Although treatment with either Wnt3A or RSpo1 resulted in an increase in LRP6 phosphorylation, cotreatment of Wnt3A and RSpo1 dramatically increased LRP6 phosphorylation, confirming that RSpo1 regulates Wnt signaling at the level of LRP6. Because Rspo1-mediated β-catenin/TCF signaling depends on endogenous Wnt3A, we next investigated whether RSpo1-mediated phosphorylation of LRP6 also required endogenous Wnt3A ligands. As shown in Fig. 3*C*, induction of LRP6 phosphorylation by RSpo1 was inhibited by Wnt3A siRNA duplexes, whereas LRP6 phosphorylation induced by exogenous Wnt3A was unaffected, indicating that RSpo1 mediated LRP6 phosphorylation also depends on endogenous Wnt3A.

Because LRP6 is tightly regulated by the secreted inhibitor DKK1, we next analyzed the effect of DKK1 on RSpo1 function. DKK1 inhibits LRP6 by coupling LRP6 with Kremen1 and subsequently triggering LRP6 internalization (10, 12–14). As shown in Fig. 4 *A* and *B*, DKK1 inhibited reporter activity induced by either Wnt3A or RSpo1. However, whereas Wnt3A-mediated TCF reporter activation was completely blocked by cotreatment with recombinant DKK1 protein (Fig. 4*A*), DKK1-dependent inhibition of RSpo1 activity was prevented at higher concentrations of RSpo1 protein (200–800 ng/ml; Fig. 4*B*), suggesting that RSpo1 protein can antagonize DKK1 inhibition.

Because RSpo1 interferes with DKK1 function and is sensitive to LRP6 expression levels, we first confirmed that RSpo1 antagonized

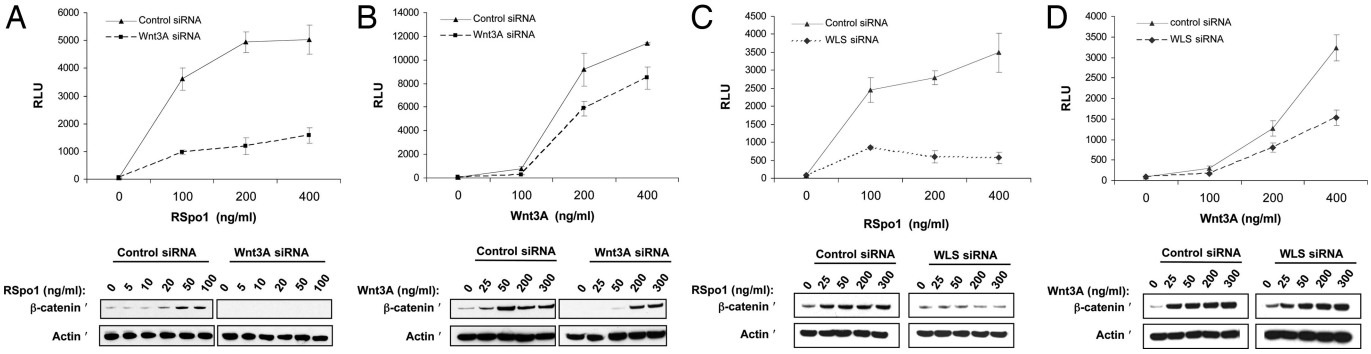

Fig. 2. RSpo1 activity in HEK-293 cells depends critically on an endogenous pool of canonical Wnt ligands. HEK-293 A6 cells were transfected with Wnt3A or control siRNA duplexes (50 nM), treated with increasing amounts of RSpo1 (*A*) or Wnt3A (*B*) proteins, and assayed for luciferase reporter activity or cytosolic β-catenin levels. HEK-293 A6 cells were transfected with WLS or control siRNA duplexes (50 nM), treated with increasing amounts of recombinant RSpo1 (*C*), or Wnt3A (*D*) protein and analyzed as described above.

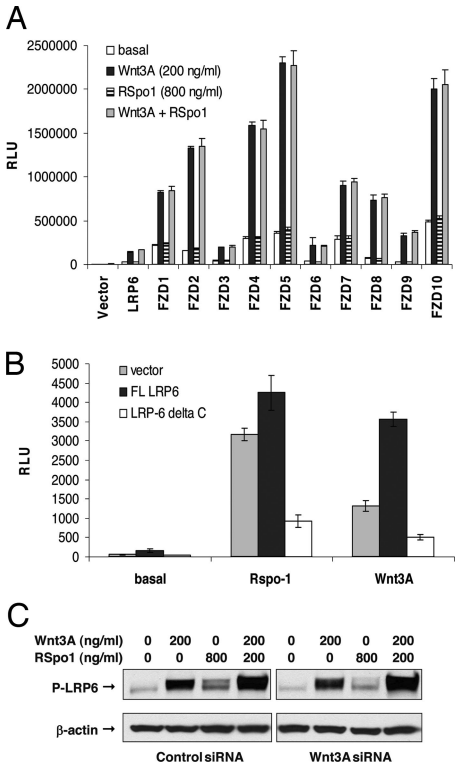

Fig. 3. RSpo1 regulates Wnt signaling at the level of the LRP6 receptor. (*A*) S2 cells were transfected with 16TCF luciferase reporter, TCF4, hLRP6, and hFZD1–10 expression constructs, followed by treatment with Wnt3A (200 ng/ml) or RSpo1 (800 ng/ml) recombinant proteins or a combination of RSpo1 and Wnt3A. (*B*) HEK-293 A6 cells were transfected with WT LRP6 or LRP6ΔC expression constructs and treated with RSpo1 (200 ng/ml) or Wnt3A (200 ng/ml) proteins, and luciferase reporter activity was determined. (*C*) HEK-293 cells transfected with control or Wnt3A siRNA duplexes (50 nM) were treated 48 hours after transfection with RSpo1 (800 ng/ml), Wnt3A (200 ng/ml), or with a combination of RSpo1 and Wnt3A proteins. LRP6 phosphorylation was analyzed 4 hours after treatment by using a phospho-Ser-1490-specific anti-LRP6 antibody.

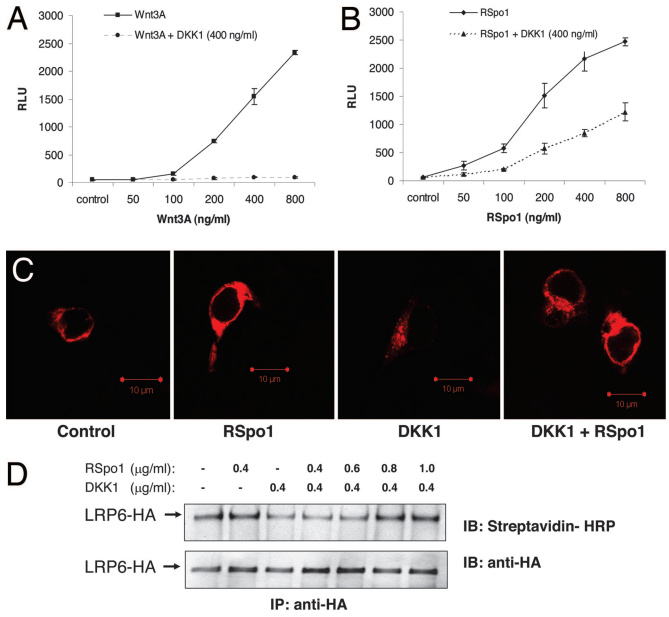

Fig. 4. RSpo1 inhibits DKK1/Kremen1-mediated internalization of LRP6. HEK-293 A6 cells were treated with recombinant DKK1 protein (400 ng/ml) in the presence of increasing amounts of recombinant Wnt3A (*A*) or RSpo1 (*B*) proteins, and TCF reporter activity was determined. (*C*) HEK-293 cells were cotransfected with LRP6-HA and Kremen1 constructs and treated for 10 minutes with recombinant DKK1 (200 ng/ml) in the presence or absence of RSpo1 protein (800 ng/ml). Distribution of immunostained, HA-tagged LRP6 was evaluated by confocal microscopy. (*D*) HEK-293 cells transfected with LRP6-HA and Kremen1 constructs were treated with recombinant DKK1 in the presence or absence of recombinant RSpo1 for 1 hour. Cell-surface proteins were biotinylated and HA-LRP6 was immunoprecipitated with anti-HA mAb and analyzed with streptavidin-HRP or anti-HA mAb.

DKK1 activity in HEK-293 cells overexpressing LRP6 and Kremen1 (SI Fig. 10). We next examined whether RSpo1 regulates DKK1-dependent LRP6 receptor internalization in HEK-293 cells overexpressing LRP6-HA and Kremen1 proteins.

As reported, treatment with DKK1 protein triggered a rapid internalization of LRP6 into endocytic vesicles, resulting in the depletion of LRP6 from the cell surface (10), as visualized by confocal imaging (Fig. 4*C*). Interestingly, DKK1-dependent internalization of LRP6 was prevented by cotreatment with RSpo1 protein (Fig. 4*C*). Quantitation of images indicated that, whereas DKK1 treatment caused a dramatic shift in LRP6 distribution from the cell surface to internal vesicles in 90% of analyzed cells (SI Fig. 11), cotreatment with RSpo1 restored cell surface expression in 50% of cells analyzed. To extend these results, we performed cell-surface biotinylation of cells overexpressing LRP6-HA and Kremen1. In agreement with the confocal imaging analysis, DKK1 treatment reduced LRP6 levels on the cell surface as visualized by immunoprecipitation of biotinylated HA-tagged LRP6, whereas cell-surface levels were increased in cells cotreated with RSpo1 (Fig. 4*D*).

These results indicate that RSpo1 can enhance Wnt responsiveness by regulating DKK1-mediated LRP6 turnover. To understand how RSpo1 regulates LRP6 internalization, we examined the interaction of RSpo1 with Kremen1 or LRP6 receptors. LRP6 and Kremen1 receptors were overexpressed in HEK-293T cells followed by incubation with alkaline phosphatase (AP)-tagged RSpo1 or DKK1 proteins. As shown in Fig. 5*A*, RSpo1-AP protein bound to Kremen1 but not LRP6-expressing cells. In contrast, DKK1-AP bound to cells overexpressing either LRP6 or Kremen1, as demonstrated (10, 36). All binding assays with WT RSpo1-AP were carried out in the presence of heparin to reduce background binding. However, a specific binding to Kremen1-transfected cells was detected in the absence of heparin, with a C- terminally truncated RSpo1 mutant (RSpo1ΔC-AP) lacking the heparin-binding domain (17), whereas no binding to LRP6 was observed under these conditions (Fig. 5*A*). This result indicates that the Furin domain required for RSpo1-dependent activity (17) is also sufficient for binding to Kremen1.

Because it was recently reported that RSpo1 directly binds to LRP6 ECD (37), we further examined the interaction of RSpo1 with LRP6. Recombinant monomeric mouse LRP6 ECD was mixed with recombinant monomeric RSpo1 or DKK1 proteins, and the mobility of the proteins was analyzed on a size-exclusion chromatography column. As shown in Fig. 5*B*, whereas DKK1 migrated as a monomer of ≈44 kDa when injected alone, it coeluted with LRP6 ECD (≈670 kDa) when injected together with LRP6. In contrast, elution of RSpo1 mixed with LRP6 extracellular domain (ECD) was identical to elution of RSpo1 injected alone, confirming and extending our findings that RSpo1 does not directly interact with LRP6.

Together, our results indicate that RSpo1 antagonizes DKK1 through an interaction with Kremen1. To further understand how the interaction of RSpo1 with Kremen1 regulates DKK1 activity, we examined whether RSpo1 can interfere with DKK1-mediated association between LRP6 and Kremen. As shown in Fig. 5*C*, LRP6 coimmunoprecipitated with Kremen1 only in the presence of DKK1, indicating that association between Kremen1 and LRP6 is

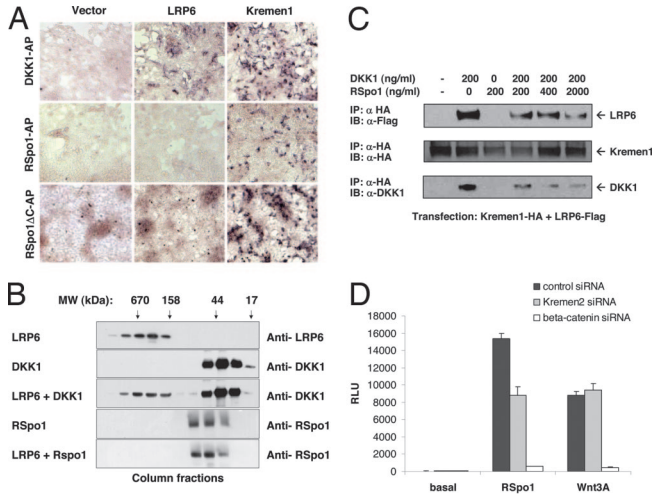

Fig. 5. RSpo1 interacts with Kremen. (*A*) HEK-293T cells transiently transfected with LRP6-HA, Kremen1, or control plasmids were incubated with DKK1-AP (15 nM) conditioned medium or with RSpo1-AP (5 nM) conditioned medium in the presence of heparin (2 μg/ml) and were stained for bound AP activity. HEK-293T cells transfected as described above were incubated with RSpoΔC-AP (180 nM) in the absence of heparin and stained for bound AP activity. (*B*) Recombinant DKK1 or RSpo1 proteins were mixed with monomeric recombinant LRP6 ECD, and complex formation was analyzed by monitoring coelution of injected proteins on a analytical size-exclusion chromatography column. (*C*) HEK-293 cells stably expressing LRP6-Flag were transfected with Kremen1-HA and subjected to coimmunoprecipitation with anti-HA antibodies. Immunoprecipitates were analyzed by Western blotting by using the indicated antibodies. (*D*) HEK-293 A6 reporter cells were transfected with Kremen2 siRNA complexes (2 nM), treated with recombinant RSpo1 (200 ng/ml) or Wnt3A (200 ng/ml) 48 hours after transfection, and analyzed for reporter activity 18 hours after treatment.

DKK1-dependent. Interestingly, the DKK1-dependent association between LRP6 and Kremen1 was reduced in the presence of RSpo1, which correlated with a reduction of DKK1 in the complex. These results suggest that RSpo1 can regulate LRP6 cell surface levels by uncoupling LRP6 from the DKK1/Kremen internalization machinery. To address whether this activity is sufficient for enhancement of Wnt signaling observed with RSpo1, we analyzed Wnt3A and RSpo1 activities in cells transfected with Kremen2-specific siRNA. HEK-293 cells transfected with Kremen2-specific siRNA showed a 40–50% reduction in transiently expressed Kremen2 protein levels (SI Fig. 12), which correlated with a 50% reduction in RSpo1 but not Wnt3A-dependent reporter activity (Fig. 5*D*), indicating that RSpo1 activity in HEK-293 cells requires the presence of Kremen2.

To extend these findings, we next analyzed RSpo1 function in mouse L-cells, because these cells were shown previously to be nonresponsive to RSpo1, but sensitive to Wnt3A-dependent activation of the β-catenin pathway (16). As shown in Fig. 1*C*, mRNA expression of several Wnt ligands, DKK1 and -2 were detected in HEK-293 but not in L-cells, whereas Kremen1 and -2 were expressed in both cell lines (Fig. 1*C* and SI Fig. 8). These data are consistent with the notion that RSpo1 activity is restricted to cells expressing endogenous Wnt ligands and DKK proteins. Interestingly, LRP6 phosphorylation was detected in L-cells treated with Wnt3A but not with RSpo1 (Fig. 6*A*), confirming our Wnt3A siRNA results in HEK-293 cells that RSpo1-mediated LRP6 phosphorylation depends on Wnt ligands. We next tested whether RSpo1 activity in L-cells could be reconstituted with exogenous Wnt3A and DKK1 proteins. As shown in Fig. 6 *B* and *C*, Wnt3A-induced β-catenin accumulation and TCF reporter activity were only marginally enhanced by RSpo1, confirming that RSpo1 has a limited effect on Wnt activity in the absence of DKK. We then cotreated cells with recombinant DKK1 and Wnt3A proteins and tested whether RSpo1 protein could restore Wnt3A activity. Interestingly, addition of RSpo1 protein inhibited DKK1 function and partially restored β-catenin stabilization and TCF reporter activity induced by Wnt3A, supporting the notion that RSpo1 responsiveness may require expression of both Wnt ligands and DKK inhibitors.

Together, our results suggest a model in which RSpo1 regulates Wnt signaling by antagonizing Kremen/DKK-dependent LRP6 internalization (Fig. 6*D*). However, it is not clear from our experiments whether this activity is sufficient for the enhancement of Wnt signaling induced by RSpo1. Thus it remains possible that RSpo1 may regulate Wnt signaling through additional mechanisms and/or unidentified components. Consistent with the notion that RSpo1 antagonizes DKK activity is the fact that neither RSpo family members nor Kremen or DKK orthologues are found in *Drosophila*, indicating these genes may have coevolved as a functional regulatory unit to control Wnt signaling in more complex organisms.

An important implication from our model is that RSpo1 acts as a Wnt enhancer and therefore should have activity only in tissues in which the Wnt pathway is already activated. This prediction is consistent with the expression patterns observed for RSpo family members and Wnt ligands in *Xenopus* and mouse embryos (15, 19–22, 38). Moreover, we have shown that injection of Rspo proteins into adult mice has a strong mitogenic effect on crypt epithelial cells, whereas no obvious proliferative effects are ob-

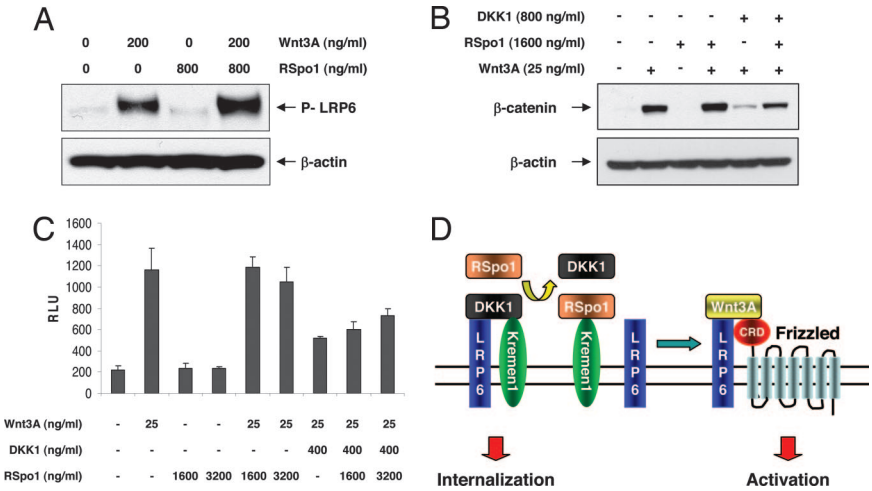

Fig. 6. Analysis of RSpo1 signaling in RSpo1 nonresponsive L-cells. (*A*) Mouse L-cells were treated with recombinant Wnt3A (200 ng/ml), RSpo1 (800 ng/ml), or a combination of RSpo1 and Wnt3A and analyzed for LRP6 phosphorylation 4 hours after treatment, as described above. Mouse L-cells were treated with Wnt3A, DKK1, or RSpo1 proteins as indicated and analyzed for cytosolic β-catenin levels (*B*) or 16TCF reporter activation (*C*). (*D*) Model of RSpo1 function. Wnt/β-catenin signaling is initiated upon binding of a canonical Wnt ligand to Frizzled and association with LRP5/6 receptors. In the absence of RSpo1, Wnt signaling is limited by the amount of LRP6 on the cell surface, which is kept low by DKK1/Kremen1-mediated internalization of LRP6. RSpo1 enhances Wnt signaling by antagonizing DKK1/Kremen-mediated LRP6 turnover, resulting in increased cell surface levels of LRP6.

served in other tissues (16, 18). Intestinal crypt proliferation depends on Wnt signaling activity (39–43), and thus *in vivo* responses to RSpo proteins appear to be restricted to organs undergoing constitutive Wnt signaling. This notion is supported by the recent finding that loss-of-function mutations in RSpo1 (20), RSpo3 (21), or RSpo4 (22) result in phenotypes similar to defects seen in mice lacking expression of Wnt ligands and/or receptors (20, 23–25).

Although our data show that RSpo1 enhances Wnt responses by antagonizing DKK1 activity, expression of DKK1 was not detected in human and mouse intestine (44, 45). However, DKK1-encoding ESTs have been isolated from human intestinal libraries (Unigene Hs.40499, National Center for Biotechnology Information), and numerous publications reported silenced DKK1 expression in colorectal adenocarcinoma (46, 47), supporting the notion that DKK1 is expressed in intestinal tissues. It is conceivable that DKK1 expression in the intestine is restricted to a subset of cells, such as the stem cell niche, and thus below detection of the methods used. Finally, it is also possible that DKK1 originates from a source distinct from the intestinal tissue itself, because recent reports have shown that intestinal crypt proliferation can be disrupted by adenoviral expression of DKK1 (43).

In summary, we have provided a mechanism by which RSpo proteins can regulate Wnt signaling by antagonizing DKK1-dependent internalization of LRP6.

Methods

Reagents. ORF nucleotide sequences for hKremen1 [BC063787; American Type Culture Collection (ATCC), Manassas, VA], Kremen2 (BF312414; ATCC), hLRP6 (NM_002336; Open Biosystems, Huntsville, AL), and mWnt3a (X56842; ATCC) were amplified from indicated cDNA clones and inserted into the pcDNA3.1/Intron plasmid (16). mLRP6ΔC was generated by deleting the LRP6 cytoplasmic domain as described (36). LRP6-HA was generated by in-frame fusion of the LRP6 ORF sequence with a HA-epitope tag (YPYDVPDYA) encoding sequence. ORF sequences for hFZD1–7, 9–10 (Origene, Rockville, MD), mTCF4 (BC014293; Invitrogen, Carlsbad, CA), and rFZD8 (BC100088; Open Biosystems) were amplified from the indicated cDNA clones and inserted into the pAc5.1/V5 HIS plasmid (Invitrogen) to generate S2 expression constructs. RSpo1 and DKK1 ORF sequences were cloned into the pAPtag-5 vector (GenHunter, Nashville, TN) to generate AP-tagged fusion protein expression constructs. RSpo1ΔC-AP was generated by cloning nucleotides 1–444 from the RSpo1 ORF into pAPtag-5.

Recombinant hDKK1, mWnt3A, hsFRP1, WIF, mLRP6 ECD, and anti-DKK1 antibodies were purchased from R&D Systems (Minneapolis, MN). Recombinant RSpo1 was purified as described (16) from conditioned medium derived from stable RSpo1 expressing CHOK1 cells.

Anti-DKK1 polyclonal antibodies were purchased from R&D Systems, an LRP6-specific monoclonal antibody was raised against hLRP6 purchased from R&D Systems, and a RSpo1-specific polyclonal antibody was raised against recombinant RSpo1 by Covance (Denver, PA).

Luciferase Reporter Assays, siRNA Experiments, and Cytosolic β-Catenin Assays. A 16TCF luciferase reporter construct was genereated by cloning 16 repeats of a TCF consensus site (AGATCAAAGG) (48) into the pTA-Luc vector (Clontech, Mountain View, CA). A geneticin selectable marker was inserted into the vector and used to select a stable HEK-293 clone (A6) exhibiting minimal basal reporter activation. HEK-293 A6 cells were seeded in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Invitrogen), starved for 8 hours in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% FBS, treated in triplicate for 18 hours with the indicated proteins, and reporter activity was determined by using a Veritas Luminometer (Turner Biosystems, Sunnyvale, CA). Gene-specific and nontargeting control siRNA duplexes were purchased from (Dharmacon, Chicago, IL) [Wnt3A: GGAACUACGUGGAGAUCAU, β-catenin: GCAGUGACACGCUCAUGUG, Kremen2: GUCCAGGCCUGUCCGAAUGUU, WLS: GCGUCAGUCCAAGUGAA (33)]. HEK-293 A6 reporter cells were transfected in triplicate in 96-well plates with the indicated siRNA duplexes by using transfection with Neofect (Ambion, Austin, TX) or Lipofectamine RNAiMAX (Invitrogen). Cells were treated as indicated 48 hours after siRNA transfection and analyzed for reporter activity as described above or for cytosolic β-catenin levels as described (16).

S2 cells seeded in 96-well plates in *Drosophila* Schneider medium (Invitrogen) containing 10% heat-inactivated FBS were transfected with 6 ng of TCF4-pAc5.1 plasmid, 6 ng of 16TCF luciferase reporter plasmid, and 18 ng of test DNA per well by using Cellfectin transfection reagent (Invitrogen). Twenty hours after transfection, cells were cultured for 8 hours in medium containing 1% FBS and then treated in triplicate for 18 hours with the indicated proteins. Luciferase activity was determined 20 hours later, as described above.

L-cells were treated with the indicated proteins and analyzed for cytosolic β-catenin content as described (16). Mouse L-cells seeded in 96-well plates in DMEM containing 10% FBS were transfected with 16TCF reporter and TCF4 plasmids (each at 35 ng/well) by using FuGENE HD (Roche, Indianapolis, IN), starved and treated as described above, and analyzed for reporter activity 48 hours after transfection.

Semiquantitative RT-PCR Analysis. RNA was isolated from HEK-293 or L-cells by using an RNAeasy minikit (Qiagen, Valencia, CA), and cDNA was synthesized by using cloned AMV RT and oligo-DT primers (Invitrogen). PCR was carried out for 40 cycles on an ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA), and PCR products were resolved on 2% agarose gels.

Primers used are: hWnt1 (forward: tcctgctcagaaggttccat; reverse gcctcgttgttgtgaaggtt), hWnt2 (forward: cgtgtgtgcaacctgacttc; reverse: ttgtccagtcagcgttcttg), hWnt3 (forward: tgtgaggtgaagacctgctg; reverse: aaaagttgggggagttctcg), hDKK1 (forward: caccttggatgggtattcca; reverse: tccatgagagccttttctcc), hDKK2 (forward: gagatcgaaaccacggtcat; reverse: tacagacttcccctgatgg), hEF1α (forward: cacacggctcacattgcat; reverse: cacgaacagcaaagcgacc), mWnt1 (forward: atgaaccttcacaacaacgag; reverse: ggttgcctcggttg), mWnt2 (forward: ctggctctggctccctctg; reverse: ggaactggtgttggcactctg), mWnt3 (forward: caagcacaacaatgaagcaggc; reverse: tcgggactcacggtgtttctc); mWnt3A (SuperArray, Frederick, MD); mDKK1 (forward: ctgaagatgaggagtgcggctc; reverse: ggctgtggtcagagggcatg), mDKK2 (forward: gccaaactcaactccatcaagtcc; reverse: tcttactgccgccgaaagcc); and mActb (forward: aggtgacagcattgcttctg; reverse: gctgcctcaacacctcaac).

LRP6 Internalization Assays. Confocal microscopy analysis was used to analyze the cellular distribution of LRP6 in HEK-293 cells transfected with LRP6-HA and Kremen1. Cells were seeded onto 12-mm glass coverslips coated with polyD lysine (BD Biosciences, Bedford, MA). The next day, cells were transfected with LRP6-HA and Kremen1 plasmids by using FuGENE 6 reagent (Roche). Cells were serum-starved overnight 24 hours after transfection and treated with recombinant RSpo1 and/or DKK1 proteins at 37°C for 10 min. Cells were washed three times with ice-cold PBS, fixed in 4% paraformaldehyde in PBS for 20 min on ice, and permeabilized in permeabilization buffer (50 mM NaCl/300 mM sucrose/3 mM $MgCl_2$/10 mM Pipes, pH 6.8/0.5% TX-100). After blocking for 30 min in Superblock (Pierce, Rockford, IL), cells were incubated with Alexa-594-conjugated anti-HA-mAb (Invitrogen). Stained specimens were mounted in antifade reagent (Prolong Gold antifade reagent, Invitrogen) and examined by using a Zeiss confocal

laser-scanning microscope LSM510 with ×63 objective (Zeiss, Thornwood, NY).

LRP6 cell-surface distribution in LRP6-HA/Kremen1-transfected HEK-293 cells was also monitored by using biotinylation of cell-surface proteins. HEK-293 cells were seeded in 10-cm dishes and transfected with LRP6-HA and Kremen1 constructs, as described above. Transfected cells were starved overnight and treated with RSpo1 and/or DKK1 proteins for 1 hour at 37°C. Cells were placed on ice and washed three times with ice-cold PBS and subjected to cell-surface biotinylation by using EZ-Link Sulfo-NHS-LC-Biotin (Pierce), according to the manufacturer's instructions. Cells were lysed in lysis buffer (Cell Signaling, Danvers, MA) and HA-tagged LRP6 was immunoprecipitated by using anti-HA sepharose (Covance, Berkeley, CA).

Immunoprecipitates were washed in lysis buffer and analyzed by Western blotting by using goat-anti-HA antibodies (Abcam, Cambridge, MA) or HRP-conjugated streptavidin (Pierce).

Analysis of LRP6 Phosphorylation. HEK-293 cells or mouse L-cells were seeded in six-well dishes and treated with recombinant RSpo1 or Wnt3A for 4 hours. Cells were placed on ice, rinsed with ice-cold PBS, and lysed in cell lysis buffer containing phosphatase inhibitors (Invitrogen). Lysates were resolved on 3–8% SDS/PAGE gels and LRP6 phosphorylation analyzed by using a phospho-Ser-1490 specific anti-LRP6 polyclonal antibody (Cell Signaling).

Binding Assays. AP-tagged fusion protein expression constructs were transfected into HEK-293T cells to generate conditioned media containing AP-fusion proteins. Relative concentrations of AP-tagged fusion proteins were determined by Western blotting by using anti-AP antibodies and purified AP protein standards (GenHunter). HEK-293T cells were transfected with LRP6-HA, Kremen1 or vector control plasmids and incubated with AP-fusion protein containing conditioned media for 30–90 min at room temperature 48 hours after transfection. Binding of RSpo1-AP was carried out in the presence of 2 μg/ml of Heparin Sulfate (Sigma, St. Louis, MO) and binding of RSpo1ΔC-AP at 37°C in the absence of Heparin. Cells were washed five times and surface binding visualized by using the AP substrate BCIP (Genhunter), as described (49).

Analytical size-exclusion chromatography was carried out with a Superdex 200 column (PC2.3/30 SMART column; GE Healthcare, Piscataway, NJ) equilibrated with PBS at 0.05 ml/min at room temperature. The column was calibrated by using the following protein standards: Thyroglobulin, M_r 670,000; γ-globulin, M_r 158,000; ovalbumin, M_r 44,000; Myoglobin, M_r 17,000. For the protein–protein interaction analysis, 20 pmol of mLRP6 ECD was incubated for 20 min at room temperature with 50 pmol either DKK1 or Rspo1 in PBS buffer. The reactions were applied to the column and eluted with PBS. Fractions of 0.1 ml were collected and analyzed by Western blotting by using the indicated antibodies.

Coimmunoprecipitation. HEK-293 cells stably expressing LRP6-Flag were seeded in 10-cm dishes and transfected with 5 μg of Kremen1-HA expression construct by using FuGENE 6 (Roche). Forty-eight hours after transfection, cells were washed with PBS and lysed in lysis buffer (Cell Signaling). Cleared lysates were subjected to immunoprecipitation with anti-HA antibodies (Abcam) and Protein-G magnetic beads (Dynal Invitrogen). Immunoprecipitates were analyzed by Western blotting by using the indicated antibodies.

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