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IDEAS & SPECULATIONS

Insights & Perspectives

An "R-spondin code" for multimodal signaling ON-OFF states

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Abstract

R-spondins (RSPOs) are a family of secreted proteins and stem cell growth factors that are potent co-activators of Wnt signaling. Recently, RSPO2 and RSPO3 were shown to be multifunctional, not only amplifying Wnt- but also binding BMP- and FGF receptors to downregulate signaling. The common mechanism underlying these diverse functions is that RSPO2 and RSPO3 act as "endocytosers" that link transmembrane proteins to ZNRF3/RNF43 E3 ligases and trigger target internalization. Thus, RSPOs are natural protein targeting chimeras for cell surface proteins. Conducting data mining and cell surface binding assays we report additional candidate RSPO targets, including SMO, PTC1,2, LGI1, ROBO4, and PTPR(F/S). We propose that there is an "R-spondin code" that imparts combinatorial signaling ON-OFF states of multiple growth factors. This code involves the modular RSPO domains, notably distinct motifs in the divergent RSPO-TSP1 domains to mediate target interaction and internalization. The RSPO code offers a novel framework for the understanding how diverse signaling pathways may be coordinately regulated in development and disease.

KEYWORDS
axon guidance, BMP, FGF, Hedgehog, phosphatases, receptor internalization, R-spondin, Wnt

1 | INTRODUCTION

R-spondins (RSPO1-4) are a small family of secreted proteins that were discovered as potent activators of Wnt signaling.[1] RSPOs are best known for their crucial role in enhancing Wnt signaling, and to regulate cellular processes such as cell differentiation, proliferation, tissue homeostasis, and regeneration.[2] Notably, RSPOs facilitate long-term Wnt-driven adult stem cell development, which is crucial for maintaining tissue function and integrity.[3] RSPO2 and RSPO3 have also been implicated as oncogenes where mutation and/or overexpression is associated with various cancers.[4,5]

RSPO proteins have a modular structure containing two Furin-like repeats (FU1, FU2), a thrombospondin type 1 (TSP1, aka TSR) domain and a basic C-terminal region (BR) (Figure 1A). Domain conservation varies between 45% and 65% sequence identity for the FU1, FU2 domains and is lowest for the TSP1 domains (32%–49% sequence identity, Figure 1B).

Evolutionarily, the RSPO family is well-conserved in diverse organisms, from invertebrates to vertebrates, indicating their ancient origin and conserved function throughout evolution, but they are notably absent in ecdyzozoa (e.g., *Drosophila*, *C. elegans*).[6] Furthermore, the modular structure of RSPO proteins, including the presence of furin-like repeats and a TSP1 domain, is also conserved across species, highlighting the significance of these structural elements in their biological activity.

1.1 | RSPO1-4 IN WNT SIGNALING

RSPO proteins interact with cell surface receptors, modulating a variety of signaling pathways, notably the Wnt signaling pathway. RSPO enhances Wnt ligand responses by binding to two types of receptors at the cell surface: one of three leucine-rich repeat-containing G-protein coupled receptors (LGRs), LGRs 4, 5, or 6, and

(B)

FU1 identity (%)

	RSPO1	RSPO2	RSPO3	RSPO4
RSPO1	100	50	54	50
RSPO2	50	100	56	65
RSPO3	54	56	100	48
RSPO4	50	65	48	100

FU2 identity (%)

	RSPO1	RSPO2	RSPO3	RSPO4
RSPO1	100	58	55	45
RSPO2	58	100	56	49
RSPO3	55	56	100	52
RSPO4	45	49	52	100

TSP1 identity (%)

	RSPO1	RSPO2	RSPO3	RSPO4
RSPO1	100	43	49	32
RSPO2	43	100	41	40
RSPO3	49	41	100	43
RSPO4	32	40	43	100

FIGURE 1 **Domain structures and conservation of RSPOs. (A)** Domain structure of human RSPO1-4. The number of amino acids (aa) for each RSPO is indicated (UniProt accession number Q2MKA7 (RSPO1), Q6UXX9 (RSPO2), Q9BXY4 (RSPO3), Q2I0M5 (RSPO4)). Conservation of the FU1, FU2, and the TSP1 domains in RSPO1, -3, and -4 compared to RSPO2 is indicated by the percentage of amino acid identity. SP, signal peptide; FU, Furin-like repeats; TSP, thrombospondin; BR, basic C-terminal region. **(B)** Matrix for amino acid identity of RSPO domains.

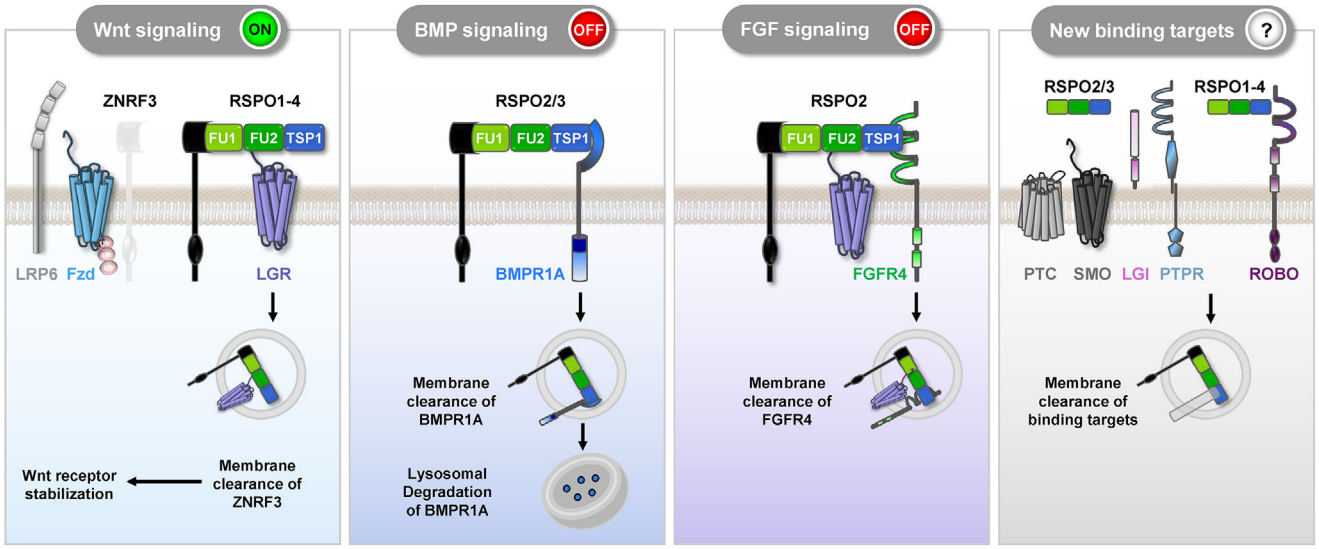

FIGURE 2 **Mechanism of RSPO-mediated regulation of WNT/BMP/FGF signaling receptors and new binding target candidates.** Models showing how RSPOs act by bridging the indicated transmembrane proteins to ZNRF3 (or RNF43, not shown) followed by internalization of the ternary- or quaternary complexes.

one of two transmembrane E3 ubiquitin ligases, ZNRF3 or RNF43.[7–13] ZNRF3/RNF43 directly ubiquitinate and internalize Wnt receptors FZD and LRP5/6 proteins on the cell surface, thereby inhibiting Wnt signaling.[14,15] By inducing ZNRF3/RNF43 auto-ubiquitination and removing ZNRF3/RNF43 from the cell surface, RSPOs potentiate Wnt signaling (Figure 2).[16]

In Wnt signaling, RSPOs form a ternary complex with ZNRF3/RNF43 and LGRs. The FU1 domain binds primarily ZNRF3/RNF43 while the FU2 domain interacts with LGR4-6.[12,17,18] The ternary complex formed by RSPO-LGR-ZNRF3/RNF43 sequesters and internalizes ZNRF3/RNF43. A fragment of RSPOs containing only the FU1 and FU2 domains can promote WNT/β-catenin signaling in cells and support the growth of small intestinal organoids.[1,12,19,20] While this FU1-FU2 construct has full signaling efficacy at sufficiently high concentrations, it is less potent than the full-length protein containing the TSP/BR domains in cells and small intestinal organoids,[20–22]

demonstrating that the TSP/BR domains contribute to signaling even when LGRs are present. Moreover, RSPO2 and RSPO3 may enhance Wnt signaling without LGRs 4, 5, and 6.[21,23,24] This LGR-independent mode of Wnt signaling may involve heparan sulfate proteoglycans (HSPGs) act as alternative co-receptors, which interact with the TSP1/BR domain.[21,22,25–27]

Leveraging the mechanism whereby RSPOs engage ZNRF3/RNF43 E3 ligases, antibody- or nanobody-based chimeras for targeted proteolysis and tissue-specific RSPO surrogates have been developed,[28–32] while our laboratory developed bispecific signaling-disabled RSPO chimera (ROTAC) platform for targeted receptor degradation.[33]

1.2 | RSPO2 AND RSPO3 IN BMP SIGNALING

We previously introduced a novel role and mechanism of RSPO signaling, independent of LGRs but mediated by ZNRF3/RNF43 in BMP signaling. RSPO2 and RSPO3, but not RSPO1 or RSPO4, antagonize BMP signaling by directly interacting with ZNRF3 and BMPR1A, triggering internalization and lysosomal degradation of BMPR1A (Figure 2). This RSPO-BMP regulation is important in development and cancer. In early *Xenopus* embryos, Rspo2 is a negative feedback inhibitor in the BMP4 synexpression group and regulates dorsoventral axis formation.[34] In acute myeloid leukemia, RSPO2 acts as an autocrine BMP antagonist to promote cancer cell renewal.[35]

RSPO2 binds via its TSP1 domain to the BMPR1A ectodomain with high affinity (K_d ~5 nM).[34] Accordingly, a RSPO1 chimera harboring the TSP1 domain of RSPO2 binds BMPR1A and inhibits BMP signaling[34] Analogous to the situation in Wnt signaling, RSPO2 bridges BMPR1A and ZNRF3 to form a ternary complex, causing BMP receptor removal from the cell surface.[34] In RSPO2-expressing cells, BMPR1A is depleted from the cell surface and colocalizes in endosomal and lysosomal vesicles with ZNRF3. Knocking down RSPO2 disrupts endosomal and lysosomal localization, causing BMPR1A to accumulate at the cell surface. For their BMP antagonist function, the FU2 domain and LGR proteins are dispensable.[34]

To further uncouple RSPO2's BMP antagonist- from Wnt agonist function, we performed peptide scanning of the TSP1 domain using overlapping synthetic TSP1-domain peptides and identified a 10-mer peptide (RNNRTCGFKW, "RW") (Figure 3A). When oligomerized as a dendrimer, RW blocks BMPR1A–RSPO2 binding and derepresses BMP signaling without altering Wnt signaling, both in vitro and in *Xenopus* embryos.[36]

1.3 | RSPO2 IN FGF SIGNALING

RSPO2 was recently shown to also act as antagonist of FGF signaling,[37] notably via receptor 4 (FGFR4).[38] Here, RSPO2 forms a quaternary complex with ZNRF3, LGRs, and FGFR4 via its FU1, FU2 and TSP1 domains, respectively. Thereby, RSPO2 promotes membrane clearance of FGFR4 via clathrin-mediated endocytosis and desensitizing cells to FGF signaling. One would assume that in analogy to BMPR1A inhibition, ternary complex formation between RSPO2, ZNRF3 and FGFR4 should suffice for internalization. Hence, it is surprising and remains unclear why LGR engagement (mediated via the RSPO2-FU2 domain) is required for internalization of FGFR4 but not of BMPR1A. Possibly, LGRs enhance FGFR4 endocytosis. Notably, the effect of RSPO-mediated ZNRF3/RNF43 auto-ubiquitination on BMPR1A and FGFR4 internalization has not been tested. Physiologically, FGFR4 inhibition by RSPO2 is important during establishment of the left-right (LR) body axis in *Xenopus* embryos, where Rspo2 produces an FGF signaling gradient that governs LR-symmetry breakage.[38]

Among RSPOs, FGFR4 inhibition is specific to RSPO2, which binds FGFR4 with high affinity (K_d ~2 nM).[38] TSP1 domain swap between RSPO2 and RSPO1 confers FGFR4 binding also to RSPO1, corroborating the importance of the TSP1 domain. To identify potential binding motifs in the TSP1 domain, we conducted synthetic peptide scanning, which revealed a peptide (TRQIVKKPVK, "TK") harboring a 5-amino acid motif (KKPVK) that competes RSPO2-FGFR4 binding.[38] This motif is non-conserved in other RSPOs and does not overlap with the RW motif required for BMPR1A binding (Figure 3A). In the TSP1 domain, the TK peptide motif lies on opposite end of the BMPR1A binding peptide motif (RW; Figure 3B). TK peptide derepresses FGF signaling both in vitro and in vivo, without affecting Wnt or BMP signaling. Thus, the three signaling modes of RSPO2 – Wnt activation, BMP and FGF inhibition – can be uncoupled using the inhibitory RW and TK peptides.

1.4 | NEW R-SPONDIN TARGET CANDIDATES: HEDGEHOG SIGNALING, AXON GUIDANCE, AND PHOSPHATASES

RSPO2 alone binds four different transmembrane proteins (ZNRF3/RNF43, LGRs, BMPR1A, FGFR4) and one of its target binding domains, TSP1, is multivalent and sequence divergent in other RSPOs (Figure 1A). This suggests that there may be more transmembrane targets for RSPO family members. To identify novel target candidates by data mining, we exploited the guilt-of-association paradigm, whereby co-expressed genes tend to interact in common molecular pathways (synexpression groups, co-expressed gene clusters).[39,40] We screened co-expression data bases[41–43] for genes whose expression pattern is highly correlated with individual *RSPO* family members and that encode transmembrane proteins. We identified several new candidates that we validated in cell-surface binding assays for direct interaction with recombinant RSPO proteins. Validated hits include Hedgehog (Hh)—and axon guidance receptors, and receptor tyrosine phosphatases (PTPRs) (Figure 3C). Among RSPOs, RSPO2 was the most promiscuous and in cell surface binding assays bound to all of the tested hits. Conversely, among candidate ligands, the axon guidance receptor ROBO4 bound to all four RSPOs. The two hedgehog receptors, PTC1 and -2 are actually negative receptors that keep the Hh pathway silenced in the absence of a ligand, while SMO promotes Hh signaling, suggesting potentially a dual role of RSPOs in Hh signaling. These results raise the possibility that RSPOs have a surprising degree

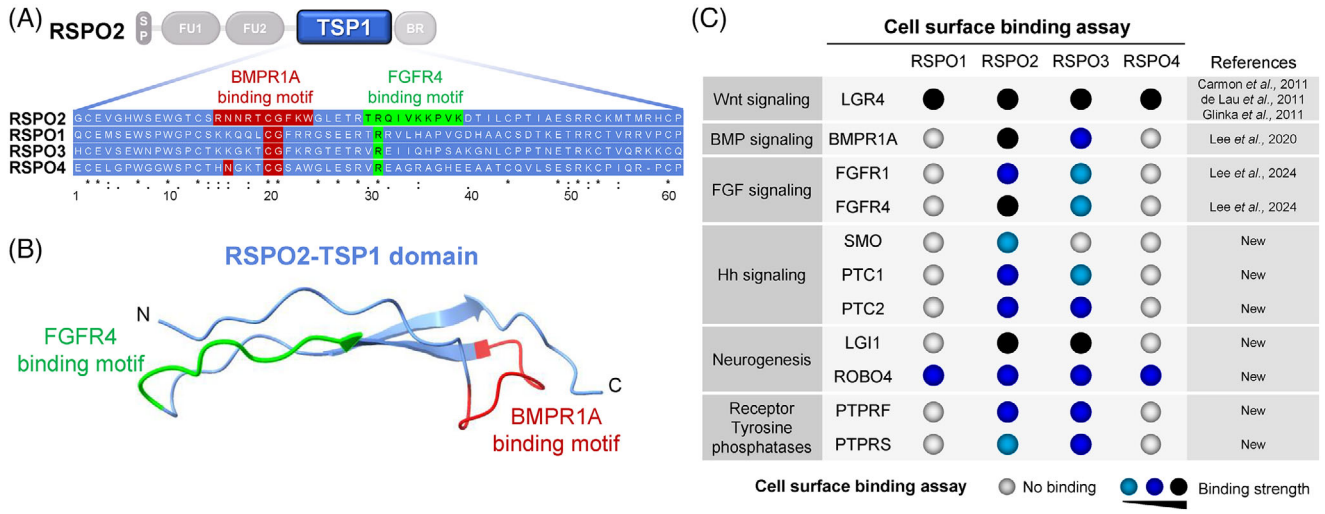

FIGURE 3 **Identification of new RSPOs target candidates. (A)** Sequence alignment of human RSPO1-4 TSP1 domains. Note that RSPO2 motifs for BMPR1A (red boxes) and FGFR4 (green boxes) binding reside in variable regions that in other RSPOs might interact also with unknown targets. **(B)** AlphaFold 3 predicted structure of the TSP1 domain (blue) from human RSPO2. Motifs for FGFR4 (green) and BMPR1A (red) binding are indicated.[52] **(C)** List of R-spondin target candidates identified by datamining and cell-surface binding assays.[34] In brief, candidate transmembrane protein genes co-expressed with RSPOs (*Source*: GeneFriends,[42] Coexpedia,[43] COXPRESdb[41]) were transfected into HEK293T cells and tested for cell surface binding assays with recombinant human RSPO1-4 proteins as described.[34] Binding strength was estimated by the percentage of binding-positive cells.

of promiscuity. We note though that these analyses provide only a preliminary candidate list. Indirect effects in the cell surface binding assays cannot be excluded, there may be no direct RSPO interaction with the noted candidates at all. For example, the overexpression of the "positive" candidates may actually increase levels of HSPGs on the cell surface, leading to overall stronger RSPO cell surface binding. Yet, since RSPO2 alone is an established direct binder of LGR4,5,6, BMPR1a, ZNRF3, RNF43, Syndecans, and FGFR4, the existence of additional direct interactors seems likely.

1.5 | AN "R-SPONDIN CODE" CONFERS MULTIMODAL SIGNALING ON-OFF STATES

The specificity of RSPO2 and RSPO3 for FGFR4 and BMPR1A binding resides in the TSP1 domain, a structural motif found in numerous extracellular proteins, including thrombospondin-1, F-spondin, CCN proteins, members of the semaphorin 5 family, UNC-5, and others.[44] Many of these proteins exhibit expression patterns and characteristics that point to functions in cell- and growth cone migration, consistent with the identification of ROBO4 and LGI1 as RSPO target candidates.

The TSP1 domain also binds glycosaminoglycans (GAG) and other matrix molecules.[45,46] RSPOs contain the six cysteines and the **WSXW** and **CSXXCG** motifs characteristic for TSP1 domains. The TSP1 structure has an elongated fold with three antiparallel strands, with the N- and C-termini at opposing ends of the domain.[47] Clusters of basic amino acids are thought to confer GAG binding to the TSP1 domain, properties shared by RSPOs. The BMPR1A and FGFR4 binding sites (Figure 3B) may confer independent binding of both targets. Indeed, in cell surface binding experiments, recombinant FGFR4-ECD effectively replaced RSPO2 binding to FGFR4 but did not affect BMPR1A binding.[38] Conversely, BMPR1A-ECD competed RSPO2 binding to BMPR1A but not FGFR4. This indicates that RSPO2 can indeed bind both receptors simultaneously.

We conclude that RSPOs are multimodal signaling proteins that selectively amplify signals (e.g., Wnt) while inhibiting others (e.g., BMP, FGF). Hence, we suggest that there is an "R-spondin code", the combination of distinct motifs, notably in the divergent RSPO-TSP1 domains, that mediate target interaction and internalization, which imparts combinatorial ON-OFF signaling states of multiple growth factors simultaneously. Depending on the function of target receptors endocytosed by RSPOs, the signaling outcome is either up- or downregulation of the pathway. Thus, the minimal RSPO2 code is Wnt (ON) – BMP (OFF) – FGF (OFF). With the new candidates the RSPO2 code might be expandable to PTC/Hh (ON) – SMO/Hh (OFF) – LGI1/AMPA (OFF) – ROBO4/SLIT (OFF) – PTPRF (OFF).

In electronics, such elements are known as "narrow-band amplifier" combined with "notch filter". These electronic devices are used to enhance system performance and reliability, for example, to eliminate specific hums, buzzes, or unwanted tones from audio signals without affecting the overall sound quality. Correspondingly, the ability of RSPOs to modulate multiple signaling pathways may have important implications for cell differentiation and development. For instance, RSPO2's role in both promoting Wnt signaling and inhibiting BMP and FGF signaling pathways can influence processes like dorsoventral- and LR patterning, and organogenesis,[34,37,48–50] ensuring that cells respond appropriately to developmental cues. By balancing different signaling pathways, the RSPO code may play crucial roles in maintaining tissue homeostasis and promoting regeneration. RSPO's function as

an endocytoser critically relies on *ZNRF3/RNF43* E3 ligases, which are themselves Wnt target genes, as are *RSPO* genes.[1,14,51] Hence, RSPOs may act principally in a Wnt (ON) context.

Dysregulation of RSPOs leads to disease, including cancer, developmental disorders, and tissue degeneration.[4,5] Understanding the RSPO code can provide insights into disease mechanisms and potential therapeutic targets, where modulating RSPO activity could restore normal signaling balance. Given the modularity of RSPO target binding sites, inhibitors may be designed that block target interactions selectively, as exemplified with BMP and FGF-inhibitory RW and TK peptides.

2 | CONCLUSIONS

We propose that the RSPO-ZNRF3/RNF43 system is a multimodal modulator and switch of diverse signaling pathways including Wnt and beyond. Underlying this multifunctionality is an RSPO code, the combination of distinct binding sites, notably in the divergent RSPO-TSP1 domains, which mediate target interaction and internalization. The code imparts combinatorial ON-OFF signaling states, principally in a Wnt-(ON) context, to orchestrate a wide range of cellular behaviors critical for development and adult tissue homeostasis.

AUTHOR CONTRIBUTIONS

Christof Niehrs conceptualized and wrote the manuscript, and acquired funding. Hyeyoon Lee generated figures, analyzed and curated data, and wrote part of the manuscript. Carina Seidl performed datamining and cell surface binding assays.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Unpublished raw data for cell surface binding assay are available upon request.

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