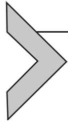

CHAPTER TWO

Receptor control by membrane-tethered ubiquitin ligases in development and tissue homeostasis

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Abstract

Paracrine cell-cell communication is central to all developmental processes, ranging from cell diversification to patterning and morphogenesis. Precise calibration of signaling strength is essential for the fidelity of tissue formation during embryogenesis and tissue maintenance in adults. Membrane-tethered ubiquitin ligases can control the sensitivity of target cells to secreted ligands by regulating the abundance of signaling receptors at the cell surface. We discuss two examples of this emerging concept in signaling: (1) the transmembrane ubiquitin ligases ZNRF3 and RNF43 that regulate WNT and bone morphogenetic protein receptor abundance in response to R-spondin ligands and (2) the membrane-recruited ubiquitin ligase MGRN1 that controls Hedgehog and melanocortin receptor abundance. We focus on the mechanistic logic of these systems, illustrated by structural and protein interaction models enabled by AlphaFold. We suggest that membrane-tethered ubiquitin ligases play a widespread role in remodeling the cell surface proteome to control responses to extracellular ligands in diverse biological processes.

Abbreviations

AGRP agouti-related protein
AML acute myeloid leukemia
ASGR asialoglycoprotein receptor
ASP agouti signaling protein
ATRN attractin
BMP bone morphogenetic protein
BMPR1A bone morphogenetic protein receptor type-1A
BR basic region
CLD cyclophilin-like domain
CRD Cys-rich domain
CUB complement C1r/C1s, uegf, bmp1
DEP dishevelled, egl-10, pleckstrin
DIR dishevelled-interaction region
DIX dishevelled, axin
DUB deubiquitylating enzyme
DVL dishevelled
E1 ubiquitin activating enzyme
E2 ubiquitin conjugating enzyme
E3 ubiquitin ligase enzyme
ECD extracellular domain
EGFL epidermal growth factor-like
ER endoplasmic reticulum
ERAD ER-associated degradation
ESCORT-I endosomal sorting complex required for transport-I
FU furin-like repeat
FZD frizzled
GDU1 glutamine dumper-1
GPC glypican
GPCR G protein-coupled receptor
GPI glycosylphosphatidylinositol

GRAIL	gene related to anergy in lymphocytes
HECT	homologous to the E6-AP carboxyl terminus
Hh	Hedgehog
HRR	His-rich region
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
ICD	intracellular domain
KO	knock-out
KSHV	Kaposi's sarcoma herpesvirus
LDLR	low-density lipoprotein receptor
LGR	Leu-rich repeat G protein-coupled receptor
LRP	low-density lipoprotein receptor-related protein
LRR	Leu-rich repeat
MARCH	membrane-associated RING-CH
MEGF8	multiple EGFL domains protein 8
MGRN1	mahogunin RING finger 1
MHC-I	class I major histocompatibility complex
MIR2	modulator of immune recognition 2
MMM	MGRN1, MEGF8 and MOSMO
PA	protease associated
PD-L1	programmed death-ligand 1
PDZ	postsynaptic density 95, discs large, zona occludens-1
PE	β-propeller/epidermal growth factor-like
PROTAC	proteolysis-targeting chimera
PRR	Pro-rich region
PTCH1	patched 1
RBR	RING-between-RING
RING	really interesting new gene
RSPO	R-spondin
S. pombe	*Saccharomyces pombe*
scFv	single-chain variable fragment
SDC	syndecan
SHH	Sonic Hedgehog
SMO	smoothened
SNARE	SNAP receptor
SRR	Ser-rich region
SUFU	suppressor of fused
TETAMS	tetra-amelia with lung hypo/aplasia syndrome
TFR1	transferrin receptor 1
TM	transmembrane
TMD	TM domain
TSG101	tumor suppressor gene 101
TSP	thrombospondin type 1
Ub	ubiquitin
WNT/PCP	WNT/planar cell polarity
WNT/STOP	WNT-dependent stabilization of proteins
WT	wild type

1. Introduction

The fates of signaling receptors and other membrane proteins are regulated by ubiquitylation during all stages of their life cycle: protein quality control in the endoplasmic reticulum (ER), sorting, trafficking and expulsion into exosomes, endocytic clearance, and lysosomal degradation (Foot, Henshall, & Kumar, 2017). The attachment of ubiquitin (Ub) and polyubiquitin chains to Lys residues on a target protein is carried out by the intricate interplay between three conserved families of enzymes, whose structures and molecular mechanisms have been revealed by various approaches (Cappadocia & Lima, 2018; Hershko, Ciechanover, & Varshavsky, 2000). First, Ub is activated by its attachment to a ubiquitin activating enzyme (E1) through a thioester linkage. Second, the activated Ub is transferred to a Cys on a ubiquitin conjugating enzyme (E2). Finally, ubiquitin ligase enzymes (E3s) facilitate the transfer of Ub from the E2 to specific Lys residues on a substrate protein or to a previously conjugated Ub in a growing polyubiquitin chain. Given the presence of seven Lys residues that decorate the Ub surface, linear or branched chains containing various combinations of Ub linkages can be attached to the substrate, and this topologically diverse "Ub code" can drive different outcomes (Komander & Rape, 2012; Kwon & Ciechanover, 2017). E3s provide the crucial substrate specificity to the ubiquitylation reaction, and sometimes this recognition event requires the assembly of large multiprotein complexes (Harper & Schulman, 2021; Morreale & Walden, 2016; Zheng & Shabek, 2017). The really interesting new gene (RING) E3s comprise the largest family (~600 members), characterized by the presence of a compact RING domain nucleated by two bound Zn^{2+} ions (Deshaies & Joazeiro, 2009). RING domains recruit a Ub-charged E2 and position it optimally for transfer of Ub to a substrate that is captured by a separate recognition module (Metzger, Pruneda, Klevit, & Weissman, 2014). Homologous to the E6-AP carboxyl terminus (HECT) E3s (~29 members) and RING-between-RING (RBR) E3s (13 members) mediate Ub transfer through a two-step process involving a thioester intermediate between Ub and a catalytic Cys on the E3 itself prior to the transfer of Ub onto the substrate (Zheng & Shabek, 2017). An additional level of regulation is afforded by ~100 deubiquitylating enzymes (DUBs) that remove Ub from proteins (Clague, Urbé, & Komander, 2019).

In this chapter we focus on Ub modifications performed by RING E3s that have recognizable transmembrane (TM) helices. Approximately 50 of the ∼600 annotated RING E3s fall into this class (Fenech et al., 2020; Li et al., 2008; Neutzner et al., 2011). However, the actual number of membrane-tethered E3s may be significantly larger, since cytoplasmic E3s can be recruited to the plasma membrane by stable association with a TM co-receptor, and such complexes are difficult to predict by sequence analysis alone. We use the term "membrane-tethered" to refer to both classes of E3s—those that are anchored to the membrane by an intrinsic TM domain and those that are recruited by non-covalent association with a TM protein. Much of the research in this area has been on TM E3s that function in the ER as part of the ER-associated degradation (ERAD) system and other protein quality control pathways (Fenech et al., 2020; Foot et al., 2017; Sardana & Emr, 2021). For example, TM E3s such as the prototype yeast protein Hrd1 ubiquitylate misfolded ER proteins that are retro-translocated through a pore-like assembly to the cytoplasm, tagging them for proteasomal degradation (Phillips & Miller, 2021). However, a growing number of structurally distinct membrane-tethered E3s have been shown to function outside of the ER to regulate the abundance of signaling receptors at the cell surface, and consequently the sensitivity of cells to signaling ligands. We will describe two such systems that function in developmental signaling pathways to control tissue patterning and morphogenesis, as well as in stem cell self-renewal, tissue homeostasis and regeneration. We anticipate that regulation of signaling strength in target cells—the cells exposed to signaling ligands—by membrane-tethered E3s will emerge as a general control mechanism in signaling pathways beyond those discussed in this chapter.

The recognition mechanisms that these membrane-tethered E3s employ to bind their targets and position their RING domains for effective Ub transfer to the cytoplasmic chains of substrate receptors remain largely unknown. We take advantage of the recent advances in the prediction of protein folds and protein-protein interactions by deep learning-based programs like AlphaFold and RoseTTaFold (Baek et al., 2021; Bryant, Pozzati, & Elofsson, 2021; Evans et al., 2021; Jumper et al., 2021; Tunyasuvunakool et al., 2021) to create models of multimodular TM E3 complexes. These methods are useful to generate hypotheses for how E3s recognize substrates through extracellular, TM and intracellular contacts, and how they may themselves be regulated by ligands. *We note that all the structures shown in the figures represent AlphaFold models unless indicated otherwise.*

2. Classification of membrane-tethered E3s

Excluding the E3s involved in protein quality control pathways in the ER, membrane-tethered E3s fall into three broad architectural classes (Fig. 1).

2.1 MARCH family TM E3s

Homologs of the membrane-associated RING-CH (MARCH) proteins were first identified as gene products that allow viruses to evade the host immune response by downregulating class I major histocompatibility complex (MHC-I) proteins (reviewed in Bauer, Bakke, & Preben Morth, 2017). MARCH proteins have been implicated in regulating the cell surface expression and trafficking of many single-pass TM proteins that play a role in T-cell activation: class I and II MHC proteins (antigen presentation), ICAM-1 (cell-cell adhesion), CD4 (T-cell co-receptor), CD86 (co-stimulatory signal), and cytokine receptors. Eleven MARCH family members have been recognized by the close similarity of their distinctive RING domains. Seven of these (MAR1–4, 8–9 and 11) contain a tight hairpin composed of two TM helices that follows an N-terminal RING module, two of them (MAR5–6) have more complex arrangements of multiple angled TM stretches, and two outliers (MAR7 and 10) have a single C-terminal TM helix (Figs. 1A and 2A). The compact RING-TM-TM portion of the major group of MARCH TM E3s is predicted to form their only structured part, although their cytoplasmic chains, composed largely of long disordered segments at both the N- and C-termini, likely carry cryptic modification sites and short interaction motifs. This conserved, $\sim$160 residue-long RING-TM-TM module is capable of both recruiting a Ub-charged E2 via its juxtamembrane RING domain and recognizing the substrate to catalyze Ub transfer. Therefore, substrate recognition likely involves intra-membrane binding of one or multiple TM helices in the substrate to the MARCH TM hairpin motif.

Modeling of the shared RING-TM-TM module reveals that the MARCH RING domain is bipartite, built primarily by the canonical Zn^{2+}-binding motif located just before the TM hairpin, but completed by a conserved β-strand that immediately follows the second TM helix (Fig. 2A). As a result, the MARCH RING domain is closely juxtaposed to the hairpin TM structure at the level of the inner leaflet of the plasma membrane, and may be uniquely responsive to structural rearrangements within the TM hairpin motif upon substrate recognition inside the plasma membrane (Trenker et al., 2021). The more complex TM architectures of MAR5 and MAR6 still display this bipartite RING domain structure, but with some

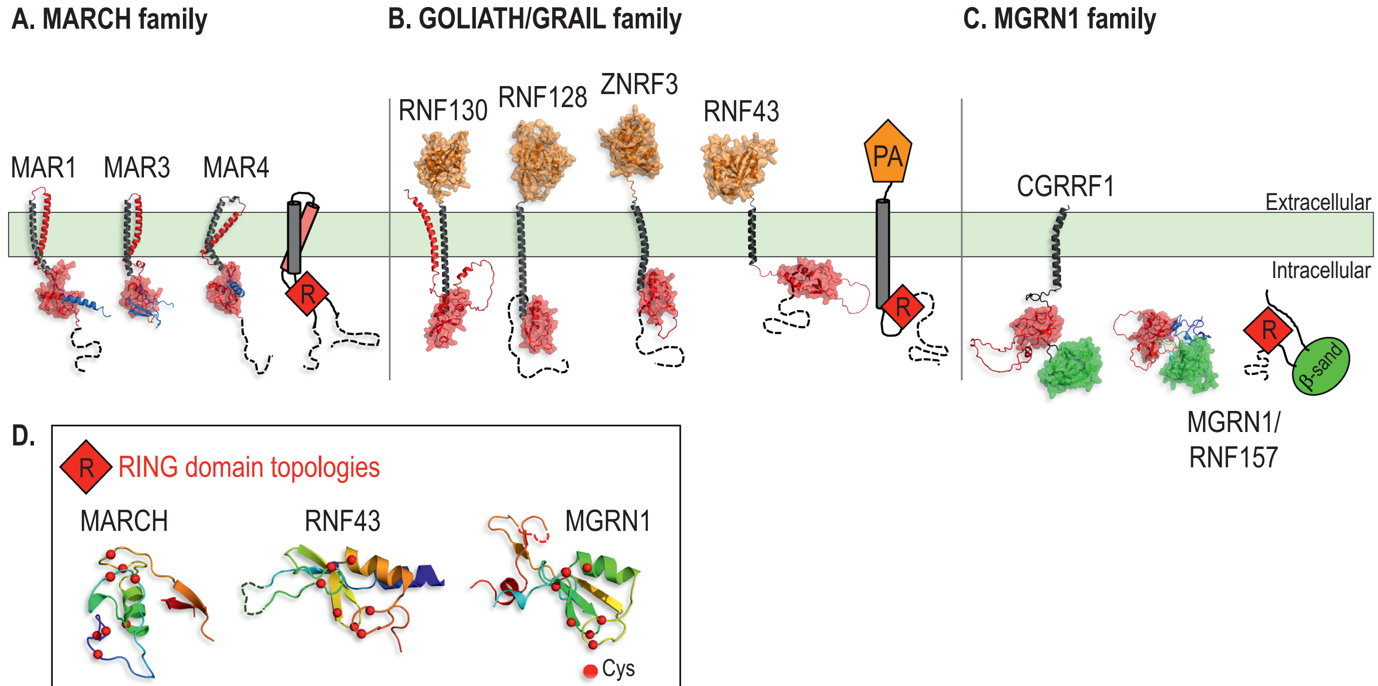


Fig. 1 Structural models of the three main classes of membrane-tethered E3s. (A–C) AlphaFold models of representative members of the MARCH (A), GOLIATH/GRAIL (B) and MGRN1 (C) E3 families, with cartoons used throughout the figures to represent each family. (A) In the MARCH family, substrate recognition is accomplished by two closely linked TM helices (gray and red) folded as a hairpin, and Ub transfer is catalyzed by a tightly associated RING domain. (B) Members of the GOLIATH/GRAIL family contain an extracellular PA domain that can bind to ligands and serve in substrate recognition. (C) The MGRN1 family is characterized by a RING domain juxtaposed to a putative substrate-binding β-sandwich domain (β-sand, green). MGRN1 and RNF157 lack TM helices, but are recruited to the membrane by interactions with single pass TM proteins (see Figs. 6 and 7), while CGRRF1 is tethered to the membrane by a single TM helix. (D) Topologies of the RING domains in one representative member of each of the three E3 families shown. In this and all subsequent figures, the RING domain is shown as a red space-filling model in the structural representations and as a red diamond labeled "R" in the cartoons. All the structures shown in the figures represent AlphaFold models, unless indicated otherwise with a Protein Data Bank (PDB) ID shown in italics. All structures are drawn to the same scale within each figure, except for structures shown in boxes. Dotted lines denote unstructured segments of the proteins for which folds could not be predicted. Molecular graphics were generated with PyMOL (www.pymol.org).

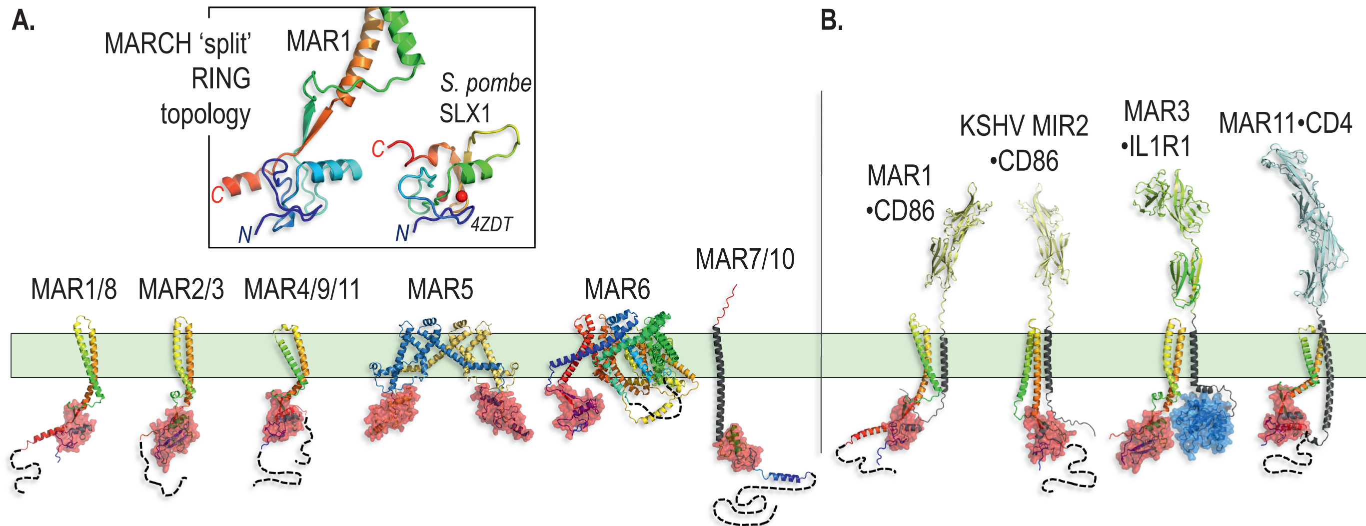


Fig. 2 The MARCH family TM E3s and their substrate recognition mechanisms. (A) AlphaFold models of representatives of the 11 MARCH family members (MAR1/8, MAR2/3, MAR4/9/11 and MAR7/10 have similar structures, so only one of each group is shown in the figure). The unique "split" RING topology is highlighted in the box (see main text for description). For comparison, the bipartite RING domain of MAR1 is shown next to the RING domain of the *Saccharomyces pombe* (*S. pombe*) protein SLX1 (PDB ID 4ZDT) (Lian, Xie, & Qian, 2016). (B) Models of MARCH family members bound to their substrates highlight the importance of interactions between TM helices within the plane of the membrane. CD86 can be targeted by both MAR1 and the viral homolog MIR2 with slightly divergent folds and mechanisms.

variations. MAR5 adopts a predicted dimer fold that completes each of the two RING domains with a polypeptide chain from their respective partner subunits (Fig. 2A). MAR6 reunites the N-terminal portion of the RING domain with a β-strand that follows the C-terminal TM helix, with an intervening 610-residue sequence that crosses the plasma membrane multiple times (Fig. 2A).

The clearest indication that the distinctive MARCH hairpin TM structure is responsible for substrate recognition comes from a comparative study describing the engagement and Ub modification of CD86, a protein that provides co-stimulatory signals to T cells, by two distinct MARCH-class E3s: human MAR1 and modulator of immune recognition 2 (MIR2), a viral MARCH homolog from Kaposi's sarcoma herpesvirus (KSHV) (Fig. 2B) (Trenker et al., 2021).

2.2 GOLIATH/GRAIL family TM E3s

Members of the GOLIATH/GRAIL family of TM E3s have a common domain architecture: an N-terminal extracellular protease associated (PA) domain connected by a linker of varying length to a single TM helix, closely followed by a cytoplasmic RING domain (Figs. 1B and 3A). In the human proteome, we find 12 PA-TM-RING E3s (RNF13, 43, 128, 130, 133, 148–150, 167, 204, 215, and ZNRF3) and two outlier members that lack the PA domain (RNF24 and RNF122). In some of these E3s, the RING domain is predicted to pack against the last two turns of an extended TM helix, restricting their conformational flexibility (Fig. 3A). One point of variability between the members of this family revealed by AlphaFold modeling is the seamless extension of the TM helix into an amphipathic cytoplasmic helix, which forms a rigid scaffold that positions the RING domain at different distances from the plasma membrane. The distance ranges from practically no extension of the TM helix (as in the case of RNF43, in which the RING domain is connected through a linker to a short cytoplasmic extension of the helix) to 5 helical turns (for ZNRF3) or even 8 helical turns (for RNF130, also known as GOLIATH). RNF130 has a second, C-terminally distal TM helix that packs against the canonical TM helix (Fig. 3A) in a manner reminiscent of some MARCH family E3s (Fig. 2).

The best studied of the PA-TM-RING proteins is RNF128, also known as gene related to anergy in lymphocytes (GRAIL) (reviewed in Whiting, Su, Lin, & Garrison Fathman, 2011). RNF128 suppresses T-cell responsiveness and cytokine transcription by ubiquitylating and down-regulating multiple cell surface molecules involved in T-cell activation,

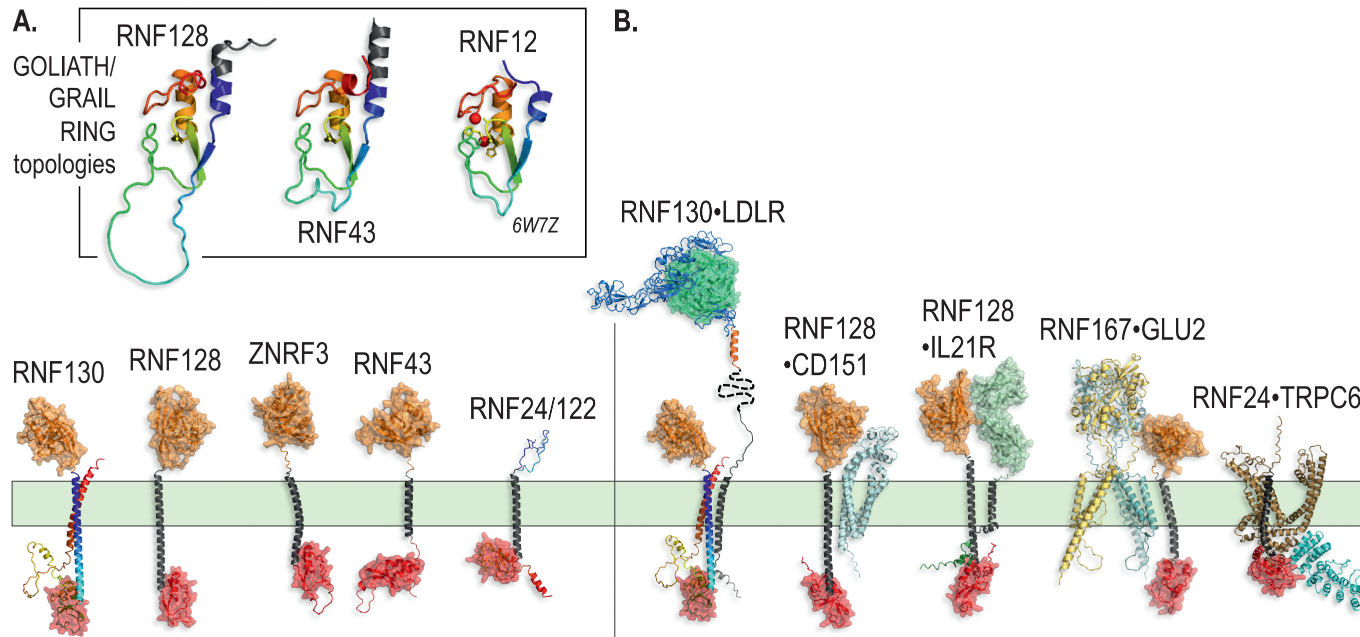


Fig. 3 The GOLIATH/GRAIL family TM E3s and their substrate recognition mechanisms. (A) AlphaFold models of GOLIATH/GRAIL family members (RNF24/122 have similar structures, so only one of them is shown). The RING domain topologies for RNF128 and RNF43 are highlighted in the box. While no structures of the RING domain of GOLIATH/GRAIL family members have been solved, the RING domain most closely resembles that of the crystal structure of RNF12 (PDB ID 6W7Z) (Middleton, Zhu, & Day, 2020), shown for comparison. (B) AlphaFold models of GOLIATH/GRAIL family members interacting with their substrates suggest the importance of recognition events that span extracellular, TM and intracellular domains. The PA domain (orange) of RNF128 binds to the extracellular domains of substrates (Lineberry, Leon, Soares, & Garrison Fathman, 2008).

including CD83, CD81, CD151 and CD40L (Lineberry et al., 2008; Su, Iwai, Lin, & Garrison Fathman, 2009). The *Drosophila* GOLIATH family members have been shown to ubiquitylate the SNAP receptor (SNARE) protein VAMP3: loss-of-function mutations in GOLIATH or GODZILLA in flies result in the accumulation of membrane proteins in Rab5-positive giant endosomes due to defects in recycling endosome trafficking (Yamazaki et al., 2013). ZNRF3 and RNF43, which belong to a distinct branch of the GOLIATH/GRAIL family, regulate the cell surface abundance of WNT and bone morphogenetic protein (BMP) signaling receptors (Hao et al., 2012; Koo et al., 2012; Lee, Seidl, Sun, Glinka, & Niehrs, 2020). PLR-1, a relative of this family in *C. elegans*, also regulates the density of multiple receptors for WNT ligands on the plasma membrane (Moffat, Robinson, Bakoulis, & Clark, 2014). While the mechanism of substrate recognition by these PA-TM-RING proteins remains incompletely understood, studies of RNF128 suggest that the PA domain directly binds to the extracellular domains of substrate TM proteins, recruiting them for ubiquitylation by the cytoplasmic RING domain (Fig. 3B) (Lineberry et al., 2008). Thus, substrate recognition and ubiquitylation are segregated on opposite sides of the plasma membrane. However, ZNRF3 and RNF43 may require a cytoplasmic adaptor protein for substrate recognition, as discussed later (Jiang, Charlat, Zamponi, Yang, & Cong, 2015).

2.3 MGRN1 family membrane-recruited E3s

E3s lacking a TM helix can nevertheless be tightly tethered to the plasma membrane via direct, non-covalent association with an integral membrane protein. Mahogunin RING finger 1 (MGRN1, also known as RNF156) and its vertebrate-specific paralog RNF157 are the only examples of such E3s described to date (Fig. 1C). These E3s are associated with two single-pass TM proteins to regulate Hedgehog and melanocortin receptor signaling (He, Eldridge, Jackson, Gunn, & Barsh, 2003; Kong et al., 2020). Interestingly, MGRN1 and RNF157 are related to CGRRF1 (also known as RNF197), an E3 that is anchored to the membrane by a single N-terminal TM segment but lacks an extracellular domain, and has been implicated in ERAD (Fig. 1C) (Glaeser et al., 2018). It is likely that other cytoplasmic E3s also associate with TM partners to ubiquitylate membrane proteins, but the cytoplasmic sequence motifs or cryptic structural modules in the co-receptors that drive complex formation have not been cataloged.

In the following sections we elaborate in depth on how membrane-tethered E3s control signaling receptors on the cell surface. We focus on

one example of TM E3s—ZNRF3 and RNF43—and one example of a membrane-recruited E3—MGRN1—within the context of the developmental and tissue homeostasis signaling systems in which they have been best characterized.

3. The R-spondin-ZNRF3/RNF43 signaling system tunes WNT and BMP receptor abundance

The ZNRF3 and RNF43 PA-TM-RING E3s have been most extensively studied in the context of the R-spondin (RSPO) system, a signaling module that tunes the abundance of cell surface receptors in the WNT (Hoppler & Moon, 2014) and BMP (Derynck & Miyazono, 2017) pathways by regulated ubiquitylation, endocytosis and lysosomal degradation (see review by Niehrs, 2012 for a timeline of the discovery and initial characterization of the RSPO system). Recent work has also uncovered ZNRF3/RNF43-independent roles for RSPOs as WNT pathway agonists (Carmon, Gong, Yi, Thomas, & Liu, 2014) and antagonists (Reis & Sokol, 2021), and in regulating other signaling pathways including TGFβ (Zhou et al., 2017), ERK/FGF (Reis & Sokol, 2020; Zhang et al., 2017), EGFR (Stevens & Williams, 2021; Yue et al., 2021), MAPK (Zheng et al., 2020) and estrogen receptor regulation via cAMP-PKA signaling (Geng et al., 2020). Since these systems do not use ZNRF3/RNF43, which is the focus of this chapter, we will not discuss them further.

The four members of the RSPO family of secreted glycoproteins (RSPO1–4) were discovered in close succession and immediately linked to activation of WNT/β-catenin signaling or stabilization of β-catenin (Chen et al., 2002; Kamata et al., 2004; Kazanskaya et al., 2004; Kim et al., 2005). The first report describing RSPO2 also suggested it may negatively regulate TGF-β signaling, but it was unclear if this was a secondary consequence of WNT signaling modulation or an independent effect (Kazanskaya et al., 2004). RSPOs were later also linked to regulation of β-catenin-independent WNT signaling, in particular the WNT/planar cell polarity (WNT/PCP) pathway (Ohkawara, Glinka, & Niehrs, 2011). However, the precise mechanism of WNT/β-catenin signaling regulation by RSPOs remained unclear, and their receptors unknown.

Leu-rich repeat G protein-coupled receptor 5 (LGR5) was discovered as a common WNT target gene in normal intestinal crypts and in colon cancer, and was later shown to be an exquisite marker of many types of WNT-driven adult stem cells (reviewed in Barker, Tan, & Clevers, 2013;

de Lau, Peng, Gros, & Clevers, 2014). LGR5 and its close paralogs LGR4 and LGR6 (throughout the chapter, we refer jointly to these three members of the LGR family as "LGRs") were independently identified as RSPO receptors by several groups (Carmon, Gong, Lin, Thomas, & Liu, 2011; de Lau et al., 2011; Glinka et al., 2011; Ruffner et al., 2012). Shortly thereafter, ZNRF3 and RNF43 were described as the effectors of RSPO signaling (Hao et al., 2012; Koo et al., 2012). ZNRF3 and RNF43 target WNT receptors for ubiquitylation and lysosomal degradation, and binding of RSPOs to both LGRs and ZNRF3/RNF43 prevents this process by promoting clearance of ZNRF3/RNF43 from the plasma membrane. Thus, the outcome of RSPO signaling through this mechanism is the accumulation of WNT receptors at the plasma membrane, which results in increased sensitivity of cells to WNT ligands. Additionally, the heparan sulfate proteoglycan (HSPG) syndecan 4 was also identified as an RSPO3 receptor involved in activation of WNT/PCP signaling (Ohkawara et al., 2011).

Experiments in cells and mice lacking LGR4/5/6 then led to the discovery that RSPO2 and RSPO3 can signal independently of LGRs (Lebensohn & Rohatgi, 2018; Szenker-Ravi et al., 2018). Similar findings were reported in 293T cells lacking LGR4 (Park et al., 2018). LGR-independent signaling was shown to be physiologically relevant, since mice lacking LGR4/5/6 did not exhibit many of the phenotypes observed in mice lacking RSPO2 or RSPO3, suggesting that RSPO2 and RSPO3 could still promote signaling in *Lgr4/5/6* triple knock-out (KO) mice (Szenker-Ravi et al., 2018). In the absence of LGRs, RSPOs were shown to use HSPGs such as glypicans (GPCs) and syndecans (SDCs) as alternative receptors to promote potentiation of WNT/β-catenin signaling through a mechanism that still required interactions between RSPOs and ZNRF3/RNF43, as well as internalization of RNF43 (Dubey et al., 2020; Lebensohn & Rohatgi, 2018).

More recently, RSPO2 and RSPO3 were shown to downregulate Type I BMP receptor levels through another LGR-independent mechanism (Lee et al., 2020). In this context, RSPO binding to ZNRF3/RNF43 and to the BMP receptor bone morphogenetic protein receptor type-1A (BMPR1A, also known as ALK3) promoted internalization and degradation of BMPR1A. This mechanism is very different from the way in which RSPOs regulate WNT receptor levels: binding of RSPOs to ZNRF3/RNF43 and BMPR1A directly downregulates BMPR1A levels, whereas binding of RSPOs to ZNRF3/RNF43, LGRs and/or HSPGs indirectly upregulates WNT receptors by preventing ZNRF3/RNF43 from inducing the ubiquitin-dependent internalization and lysosomal degradation of WNT receptors.

In the following sections, we first describe the system architecture of these different RSPO-ZNRF3/RNF43 signaling modalities, including the protein components, their relevant domains and interactions, and some of the post-translational regulation relevant to their signaling properties. We then discuss the mechanisms for each of the three signaling modalities, considering similarities and differences between them. Finally we discuss some physiological and pathological contexts in which these divergent signaling modalities operate, and consider the prospect of leveraging the modular nature of the RSPO-ZNRF3/RNF43 signaling system for therapeutic applications.

3.1 System architecture—Components, domains and interactions

The RSPO-ZNRF3/RNF43 signaling system includes five main interacting components: ligands, engagement receptors, effector receptors, target receptors and adaptors. While some of these components have been previously referred to using these terms (i.e., LGR4/5/6 have been called "engagement receptors" and ZNRF3/RNF43 "effector receptors" for RSPOs (Chen, Chen, Lin, Fang, & He, 2013; Xie et al., 2013)), here we define them as follows. Ligands comprise the four members of the RSPO family that initiate the signaling cascade. Engagement receptors are TM or membrane-tethered cell surface proteins that engage RSPO ligands. They include LGR4/5/6, HSPGs such as GPCs and SDCs, and the type I BMP receptor BMPR1A. Effector receptors are the TM E3s ZNRF3 and RNF43, which also engage RSPO ligands and transduce the signal by directly or indirectly modulating the abundance of cell surface receptors. Target receptors are the WNT receptors frizzled (FZD) and low-density lipoprotein receptor-related protein 6 (LRP6), and the type I BMP receptor BMPR1A. The final outcome of RSPO signaling is to effect changes in the cell surface abundance of target receptors, and in so doing, tune the sensitivity of cells to WNT and BMP ligands. BMPR1A is unique in that it is both an engagement receptor and a target receptor, since it binds RSPOs directly and its abundance on the cell surface is regulated by ZNRF3/RNF43. Finally, adaptors are proteins that mediate the specificity of ZNRF3/RNF43 towards their target receptors. Dishevelled (DVL) is the only such adaptor described so far. In the following sections we describe the domain structure of these components and the interactions relevant to RSPO-ZNRF3/RNF43 signaling (Figs. 4 and 5). We focus on the mammalian proteins, but descriptions of these components in other species can be found in the various reviews cited.

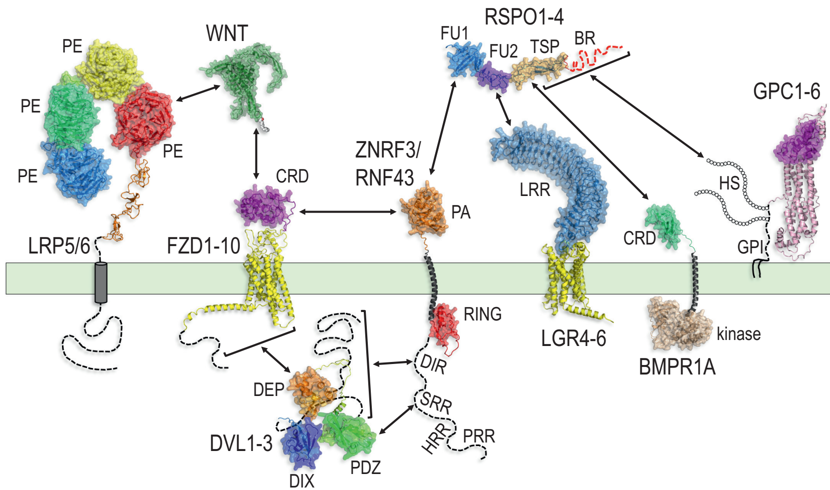


Fig. 4 The RSPO-ZNRF3/RNF43 signaling system: components, domains and interactions. AlphaFold models of the major components of the RSPO-ZNRF3/RNF43 signaling system, indicating the domains and protein-protein interactions (double arrows) relevant for signal transduction. See main text for description. Dotted lines represent parts of the polypeptide chains for which the structure could not be predicted by AlphaFold. The HS chains and GPI anchor of GPC1–6 were drawn to represent their approximate sites of attachment to the polypeptide chain, but are not intended to depict their actual structures or dimensions.

3.1.1 Ligands: RSPOs

RSPO1–4 are the four members of the RSPO subfamily of thrombospondin type 1 (TSP) repeat-containing proteins. All RSPOs contain two N-terminal tandem Cys-rich furin-like repeats connected by a flexible hinge, referred to as furin-like repeat 1 (FU1) and furin-like repeat 2 (FU2), followed by the TSP domain and a C-terminal region rich in basic amino acids (Lys and Arg), referred to as the basic region (BR) (Figs. 4 and 5A). This domain architecture is highly conserved among the four RSPOs (Kim et al., 2008; reviewed in de Lau, Snel, & Clevers, 2012), suggesting common functions. However, the length of the BR varies significantly between family members.

The FU1 domain of RSPOs interacts with the extracellular PA domain of ZNRF3 and RNF43 (Figs. 4 and 5B). Conserved residues in the RSPO FU1 domain and the ZNRF3 or RNF43 extracellular PA domain form an extensive interface comprising a mixture of hydrophobic and complementary charged interactions, as shown by a series of X-ray crystallographic structures (Chen et al., 2013; Peng et al., 2013; Zebisch et al., 2013;

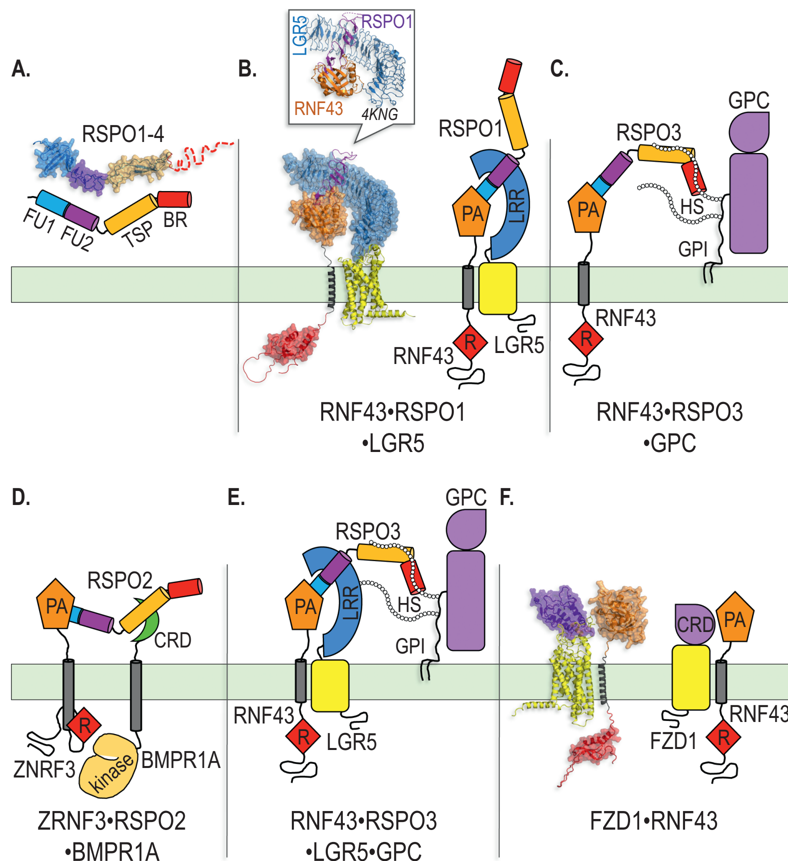


Fig. 5 Protein complexes involved in ZNRF3/RNF43-mediated, LGR- and HSPG-dependent potentiation of WNT signaling, and BMPR1A-dependent inhibition of BMP signaling by RSPOs. (A) AlphaFold model and cartoon representation of RSPO1–4, showing the predicted modular architecture of the FU1, FU2, TSP and BR domains. (B) AlphaFold model and cartoon representation of the RNF43-RSPO1-LGR5 ternary complex that regulates WNT signaling by driving ZNRF3/RNF43 internalization and lysosomal degradation. In the model, a fragment of RSPO1 composed only of the FU1 and FU2 domains is shown, while in the cartoon representation full-length RSPO1 is shown to illustrate that the TSP/BR domains would extend into an open space not occupied by other polypeptides. The box shows the structure, solved by X-ray crystallography (PDB ID 4KNG), of the extracellular LRR domain of LGR5 and the PA domain of RNF43 bound to the RSPO1 FU1-FU2 fragment (Chen et al., 2013). Note that the crystal structure is nearly superimposable with the AlphaFold model. (C) and (D) Cartoon representations of the ternary complexes that mediate HSPG-dependent potentiation of WNT signaling (C) and BMPR1A-dependent inhibition of BMP signaling (D) by RSPOs. (E) Cartoon representation of a hypothetical quaternary complex that could promote simultaneous LGR- and HSPG-dependent potentiation of WNT signaling by RSPOs. While the existence

(Continued)

Zebisch & Yvonne Jones, 2015a). One distinctive feature of the FU1 domain, termed the "Met-finger" because it contains a Met residue at the tip, inserts into a hydrophobic pocket in the ZNRF3/RNF43 PA domain as a key determinant of the RSPO-ZNRF3/RNF43 interaction, and may account for the difference in the binding affinities between the four RSPO family members and ZNRF3/RNF43 (Zebisch et al., 2013). Point mutations in residues R66 and Q71 within the FU1 domain of RSPO1 (and corresponding residues in other RSPOs) abolish the interaction between RSPOs and ZNRF3/RNF43 (Xie et al., 2013; Zebisch et al., 2013), although there is some discrepancy between experiments about the extent to which these mutations impair potentiation of WNT signaling (Lebensohn & Rohatgi, 2018; Xie et al., 2013).

The FU2 domain of RSPOs interacts with the large Leu-rich repeat (LRR) array in the extracellular domain (ECD) of LGRs primarily through hydrophobic interactions, although charged interactions between residues in the FU1 domain and LGRs have also been described (Figs. 4 and 5B) (Chen et al., 2013; Peng et al., 2013; Wang et al., 2013; Xu, Xu, Rajashankar, Robev, & Nikolov, 2013; Zebisch & Yvonne Jones, 2015a). Point mutations in residues F106 and/or F110 within the FU2 domain of RSPO1 (and corresponding residues in other RSPOs) abrogate binding of RSPOs to LGRs and eliminate potentiation of WNT signaling (Peng, de Lau, Forneris, et al., 2013; Xie et al., 2013). RSPO proteins containing point mutations in these FU2 domain residues are therefore useful reagents to study LGR-independent modes of RSPO signaling (Dubey et al., 2020; Lebensohn & Rohatgi, 2018; Park et al., 2018; Szenker-Ravi et al., 2018).

The FU1 and FU2 domains used to be considered the "business end" of the mature RSPO proteins (de Lau et al., 2014), since a fragment comprising

Fig. 5—Cont'd of such a complex has not been confirmed experimentally, it is compatible with the spatial arrangement of the relevant domains in RSPO based on solved crystal structures (B), and is consistent with the ability of the TSP/BR domains of RSPO3, as well as HSPGs, to potentiate WNT/β-catenin signaling beyond the levels promoted by the FU1-FU2 fragment and LGRs alone (Dubey et al., 2020; Lebensohn & Rohatgi, 2018). (F) AlphaFold model and cartoon representation of a FZD1-RNF43 complex. The model suggests that the FZD1 CRD would interact with the PA domain of RNF43 and drive contacts between the TM helix of RNF43 and the 7TM of FZD1, potentially orienting the RING domain for ubiquitin transfer. In (B–F), ZNRF3 or RNF43 are arbitrarily shown for illustrative purposes, but both E3s are thought to mediate all of these signaling modalities. In (B–E) RSPO1, RSPO2 or RSPO3 are arbitrarily shown for illustrative purposes, but other RSPO ligands capable of mediating each of these signaling modalities are indicated in Table 1.

these two domains is necessary and sufficient to potentiate WNT signaling (Kazanskaya et al., 2004; Kim et al., 2008; Li et al., 2009). For this reason, all of the structural studies described were done with this minimal fragment. However, we now know that these two domains make only a partial contribution to the full repertoire of RSPO functions, since they are insufficient to signal through LGR-independent mechanisms (Dubey et al., 2020; Lebensohn & Rohatgi, 2018; Lee et al., 2020) and are significantly less potent than the full-length proteins when signaling through LGRs (Dubey et al., 2020; Lebensohn & Rohatgi, 2018).

The TSP and BR domains interact with heparin—a glycosaminoglycan polymer that mimics heparan sulfate (HS)—and with the HS chains of HSPGs such as GPCs and SDCs (Figs. 4 and 5C) (Bell et al., 2008; Chang et al., 2016; Glinka et al., 2011; Nam, Turcotte, Smith, Choi, & Yoon, 2006; Ohkawara et al., 2011; Ren et al., 2018). While the TSP and BR domains are often described separately, molecular modeling predicts that the positively charged surface of the TSP and BR domains forms a continuous binding interface for heparin (Ayadi, 2008; Dubey et al., 2020). Indeed, the TSP and BR domains can individually mediate binding of RSPOs to heparin (Nam et al., 2006) and RSPO constructs containing either the TSP or BR domain can induce HSPG-dependent potentiation of WNT signaling (Lebensohn & Rohatgi, 2018). The TSP domain is also required for binding of RSPO2 and RSPO3 to BMPR1A during downregulation of BMP signaling (Figs. 4 and 5D) (Lee et al., 2020).

3.1.2 Engagement receptors: LGRs, HSPGs, BMPR1A

LGR4/5/6 were identified and validated as RSPO engagement receptors through various independent approaches (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011; Ruffner et al., 2012). They are classified as the three Class B members of the LGR subgroup of the rhodopsin family of G protein-coupled receptors (GPCRs) (reviewed in Barker et al., 2013; de Lau et al., 2014) and mark stem cells in many embryonic and adult tissues (Kinzel et al., 2014; reviewed in Koo & Clevers, 2014; Leung, Tan, & Barker, 2018). They contain a large ECD consisting of 16–17 LRRs followed by a hinge region and the distinctive 7TM domain of rhodopsin-like GPCRs (Fig. 4). The concave face of the curved structure formed by the LRR array interacts with the FU2 domain of RSPOs (Figs. 4 and 5B) (Chen et al., 2013; Peng, de Lau, Forneris, et al., 2013; Wang et al., 2013; Xu et al., 2013; Zebisch & Yvonne Jones, 2015a). No G protein-coupled signaling activity triggered by binding to RSPO

ligands has been reported (Carmon et al., 2011; de Lau et al., 2011), supporting the notion that LGRs transduce RSPO signals through other mechanisms discussed later.

HSPGs have also been implicated as engagement receptors for RSPOs (Dubey et al., 2020; Lebensohn et al., 2016; Lebensohn & Rohatgi, 2018; Ohkawara et al., 2011; Ren et al., 2018). HSPGs are a diverse class of cell surface and extracellular matrix glycoproteins decorated with HS glycosaminoglycan polysaccharide chains (reviewed in Christianson & Belting, 2014; Sarrazin, Lamanna, & Esko, 2011). Abundant carboxyl and sulfate groups on the HS chains make them polyanionic, promoting interactions with polybasic domains on proteins. While HSPGs broadly include the cell surface-associated GPCs and SDCs, the secreted extracellular matrix HSPGs (agrin, perlecan and type XVIII collagen) and the secretory vesicle proteoglycan serglycin, only GPCs and SDCs have been implicated as RSPO receptors. In mammals, there are six GPCs (GPCs1–6) and four SDCs (SDC1–4). GPCs are tethered to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor (Fig. 4), while SDCs are single-pass TM proteins. HS chains are attached to the protein core of GPCs close to the plasma membrane, and to the protein core of SDCs at more peripheral sites. Ligands can bind either to the protein core or to the HS chains of HSPGs. RSPOs interact with the HS chains of HSPGs through the TSP and BR domains (Figs. 4 and 5C) (Bell et al., 2008; Chang et al., 2016; Nam et al., 2006) and this interaction is required for RSPOs to potentiate WNT/β-catenin signaling (Dubey et al., 2020; Lebensohn & Rohatgi, 2018; Ren et al., 2018) as well as WNT/PCP signaling (Ohkawara et al., 2011).

The cell surface abundance of the type I BMP receptor BMPR1A can also be regulated by RSPOs (Lee et al., 2020). In this case, BMPR1A appears to be both the engagement and target receptor, since its own internalization is triggered when RSPO2 or RSPO3 bind to it and cross-link it with the effector receptors ZNRF3/RNF43. BMPR1A is one of seven type I TGFβ receptors in humans. It contains a small extracellular Cys-rich domain (CRD), a TM domain, and an intracellular juxtamembrane domain rich in Gly and Ser residues followed by a Ser kinase domain (Fig. 4) (reviewed in Heldin & Moustakas, 2016). The BMPR1A CRD binds with high affinity to the TSP domains of RSPO2 and RSPO3 (Figs. 4 and 5D) (Lee et al., 2020). The mechanism driving BMPR1A internalization following engagement of RSPOs is unknown (see Section 3.2.6).

3.1.3 Effector receptors: ZNRF3, RNF43

The closely related ZNRF3 and RNF43 proteins are members of the GOLIATH/GRAIL family of PA-TM-RING E3s (Fig. 3) (reviewed in de Lau et al., 2014; Hao, Jiang, & Cong, 2016; Zebisch & Yvonne Jones, 2015b). They were discovered as WNT/β-catenin target genes whose expression was correlated with that of *AXIN2* mRNA in primary tissue microarray data (Hao et al., 2012), or with LGR5-GFP abundance in LGR5+ small intestinal crypt stem cells (Koo et al., 2012). Both ZNRF3 and RNF43 contribute to a negative feedback mechanism that downregulates WNT receptor levels (Hao et al., 2012; Koo et al., 2012). A genome-wide, forward genetic screen in haploid human cells designed to find attenuators of WNT/β-catenin signaling—genes that when deleted enhanced signaling in the presence of a low dose WNT ligand—later uncovered *ZNRF3* as the top hit (Lebensohn et al., 2016). Since the HAP1 cells in which this screen was conducted do not express *RNF43* mRNA (Lebensohn et al., 2016), these unbiased screen results suggest that *ZNRF3* is the most potent attenuator of WNT signaling in the genome, at least in haploid human cells.

The extracellular PA domain of ZNRF3 and RNF43 interacts with the FU1 domain of RSPOs (Figs. 4 and 5B) as discussed earlier. Comparison of ZNRF3 ECD structures in isolation and in complex with RSPO ligands did not reveal major conformational differences (Zebisch et al., 2013), suggesting that signal transduction upon binding of RSPOs is unlikely to be an autonomous property of the ZNRF3/RNF43 proteins, instead requiring other components of the system. The PA domain of the GOLIATH/GRAIL family member RNF128 (Fig. 3) interacts with transmembrane receptors such as CD40L and CD83 and targets them for ubiquitylation (Lineberry et al., 2008), suggesting that the PA domain of ZNRF3/RNF43 may do the same for the FZD family of WNT receptors. However, data regarding an interaction between the PA domain of ZNRF3/RNF43 and the ECD of FZD is conflicting, as we discuss later. The catalytic RING domain (Fig. 4) is required for ubiquitylation of the WNT receptor FZD, which leads to its internalization and lysosomal degradation, resulting in decreased sensitivity to WNT ligands (Hao et al., 2012; Koo et al., 2012). The RING domain also appears to be required for membrane clearance of BMPR1A (Lee et al., 2020). In addition to their defining PA, TM and RING domains, ZNRF3 and RNF43 have disordered cytoplasmic extensions containing a dishevelled-interaction region (DIR)

followed by Ser-, His-, and Pro-rich regions (SRR, HRR and PRR, respectively) (Fig. 4). The DIRs of ZNRF3 and RNF43 interact with the C-terminal two thirds of DVL (Fig. 4) (Jiang et al., 2015). Another region of the RNF43 intracellular domain (ICD) located C-terminal to the DIR has also been postulated to interact with DVL2 (Fig. 4) (Tsukiyama et al., 2015). Phosphorylation/dephosphorylation of the ICD also regulates RNF43-mediated FZD ubiquitylation (Tsukiyama et al., 2020), as well as ZNRF3/RNF43 internalization (Chang, Kim, Glinka, Reinhard, & Niehrs, 2020; Kim, Reinhard, & Niehrs, 2021).

3.1.4 Target receptors: FZDs, LRP6, BMPR1A

FZD proteins (reviewed in Huang & Klein, 2004; MacDonald & He, 2012; Niehrs, 2012; Wang, Chang, Rattner, & Nathans, 2016) were the first WNT receptors to be identified (Bhanot et al., 1996). The 10 FZDs in humans (FZD1–10) are Class F members of the GPCR superfamily (reviewed in Malbon, 2004; Schulte & Bryja, 2007; Schulte & Wright, 2018). FZDs transduce both β-catenin-dependent and β-catenin-independent WNT signals, including those in the WNT/PCP, WNT/calcium (reviewed in Niehrs, 2012) and WNT-dependent stabilization of proteins (WNT/STOP) pathways (Acebron, Karaulanov, Berger, Huang, & Niehrs, 2014). FZDs contain an extracellular CRD followed by a linker region, a 7TM domain, and an ICD of variable length (Fig. 4). The FZD CRD interacts directly with WNT ligands (Fig. 4) via contacts at two opposing faces of the globular CRD, with the principal interaction involving the palmitate group of WNT docking into a hydrophobic groove in the CRD (Janda, Waghray, Levin, Thomas, & Christopher Garcia, 2012). Replacement of several conserved Lys residues throughout the intracellular loops of the FZD 7TM and the ICD with Arg residues abrogated changes in FZD levels in response to ZNRF3/RNF43 over-expression or depletion (Hao et al., 2012; Koo et al., 2012), suggesting that these Lys residues may be ubiquitylated by ZNRF3/RNF43. However, the relative contributions of these potential ubiquitylation sites to regulation of FZD levels by ZNRF3/RNF43 have not been determined.

LRP5 and LRP6, and the Drosophila ortholog Arrow, are WNT co-receptors required for WNT/β-catenin signaling but not for β-catenin-independent WNT/PCP signaling (reviewed in He, Semenov, Tamai, & Zeng, 2004; MacDonald & He, 2012). LRP5/6 are large (>1600 amino acids) single-pass TM proteins with an ECD formed by a closely packed

set of four tandem β-propeller/epidermal growth factor-like (PE) repeats, followed by three low-density lipoprotein receptor (LDLR) type A repeats (Fig. 4). The LRP6 ECD interacts with WNT ligands in a manner that allows WNTs to simultaneously bind the FZD CRD (Fig. 4), bridging them into a ternary receptor complex that triggers cytoplasmic WNT signaling (Bourhis, Tam, Franke, Bazan, & Ernst, 2010; Chu et al., 2013; Hirai, Matoba, Mihara, Arimori, & Takagi, 2019; Tamai et al., 2000). While LRP6 internalization and degradation is regulated by ZNRF3/RNF43 (Chang et al., 2020; Giebel et al., 2021; Hao et al., 2012; Kim et al., 2021), the elements in LRP6 required for this regulation remain unknown.

The type I BMP receptor BMPR1A, discussed earlier, is unique among the targets of RSPO-ZNRF3/RNF43-dependent regulation in that it also engages RSPOs directly (Figs. 4 and 5D) (Lee et al., 2020), and can therefore be considered both a target and an engagement receptor. However, unlike in the case of WNT receptors, it is unclear whether regulation of cell surface BMPR1A abundance by RSPO-ZNRF3/RNF43-mediated endocytosis and lysosomal degradation involves BMPR1A ubiquitylation.

3.1.5 Adaptors: DVL

The three DVL proteins (DVL1–3 in humans) are crucial intracellular components of both β-catenin-dependent and β-catenin-independent WNT signaling pathways (reviewed in Gao & Chen, 2010; MacDonald & He, 2012; Sharma, Castro-Piedras, Simmons Jr, & Pruitt, 2018). They bind the cytoplasmic segments of FZD receptors and route WNT signals to the WNT/β-catenin or WNT/PCP pathways by forming distinct signaling complexes (reviewed in Gammons & Bienz, 2018; Mlodzik, 2016). DVLs interact with a diverse array of proteins through three highly conserved modules connected by flexible linkers that mediate their molecular functions: an N-terminal dishevelled, axin (DIX) domain, a central postsynaptic density 95, discs large, zona occludens-1 (PDZ) domain, and a C-terminal dishevelled, egl-10, pleckstrin (DEP) domain (Fig. 4). The DIX domain undergoes dynamic head-to-tail homo-polymerization (Kishida et al., 1999; Schwarz-Romond et al., 2007), which leads to formation of DVL assemblies (Schwarz-Romond, Metcalfe, & Bienz, 2007), and can also undergo hetero-polymerization with the related DAX domain of AXIN (Fiedler, Mendoza-Topaz, Rutherford, Mieszczanek, & Bienz, 2011; Kishida et al., 1999). The PDZ domain interacts with many proteins that mediate both WNT/β-catenin and WNT/PCP signaling, and may be

involved in distinguishing between these two pathways (reviewed in Sharma et al., 2018). The interaction between the PDZ domain and a KTXXXW motif in the intracellular C-terminal tail of FZD recruits DVL to the WNT receptor complex and is crucial for transduction of WNT signals (Umbhauer et al., 2000; Wong et al., 2003). The DEP domain also targets DVL to the plasma membrane (reviewed in Consonni, Maurice, & Bos, 2014). It has a positively charged surface that likely interacts with phospholipids (Simons et al., 2009; Wong et al., 2000), and the DEP domain together with the C-terminal region of DVL interacts with a discontinuous motif in the FZD ICD (Tauriello et al., 2012).

DVL has been postulated as an adaptor required for recognition of FZD by ZNRF3/RNF43, a prerequisite step in promoting FZD degradation (Jiang et al., 2015). The three-way physical interaction between ZNRF3/RNF43, DVL and FZD is essential for the WNT/β-catenin inhibitory activity of ZNRF3/RNF43. This interaction is mediated by binding of the DVL DEP domain to FZD (Fig. 4), and by contacts between segments in the C-terminal two thirds of DVL (notably excluding the DIX, PDZ, and DEP domains) and the DIR of ZNRF3/RNF43 (Fig. 4). Accordingly, the DEP domain, but not the DIX or PDZ domains, are required for ZNRF3/RNF43-dependent FZD downregulation, and fusion of the DEP domain to ZNRF3/RNF43 eliminates the requirement of DVL to downregulate FZD levels (Jiang et al., 2015). An interaction between the PDZ domain of DVL and a region of the RNF43 ICD located C-terminal to the DIR is essential for inhibition of β-catenin-independent signaling through an undefined mechanism (Tsukiyama et al., 2015). This inhibition does not require the ubiquitin ligase activity of RNF43, or interactions between RNF43 and FZD, and does not result in downregulation of cell surface FZD.

3.2 RSPO-ZNRF3/RNF43 signaling mechanisms

In this section we describe three modalities of RSPO signaling mediated by ZNRF3/RNF43 that regulate the abundance of cell surface receptors, we contrast their salient features, and we discuss their plausible underlying molecular mechanisms. Other mechanisms through which ZNRF3/RNF43 control WNT/β-catenin signaling that do not impinge on the regulation of cell surface receptor levels will not be addressed here, but we refer the reader to the primary literature (Loregger et al., 2015; Spit et al., 2020).

3.2.1 LGR-dependent, ZNRF3/RNF43-mediated potentiation of WNT/β-catenin signaling by RSPO1-4

The first full picture of a mechanism driving potentiation of WNT/β-catenin signaling by RSPOs emerged with the discovery that ZNRF3 and RNF43 promote ubiquitylation-dependent internalization and lysosomal degradation of the WNT receptors FZD and LRP6 (Hao et al., 2012; Koo et al., 2012). Following internalization, RNF43 and FZD co-localize in RAB5+ early endosomes, and the final fate of FZD is lysosomal rather than proteasomal degradation, as surmised from the fact that the process can be inhibited by the lysosomal V-ATPase inhibitor bafilomycin A but not the proteasome inhibitor MG132 (Koo et al., 2012). Subsequent studies showed that at least three conditions contribute to ZNRF3/RNF43-mediated internalization and degradation of WNT receptors: (1) interaction of the adaptor protein DVL with both FZD and ZNRF3/RNF43 (Jiang et al., 2015), (2) phosphorylation of Ser residues in the SRR of ZNRF3/RNF43 (Tsukiyama et al., 2020) and (3) dephosphorylation of a 4Tyr motif in the DIR of ZNRF3 (Chang et al., 2020; Kim et al., 2021). Therefore, in the absence of RSPOs, clearance of FZD and LRP6 from the plasma membrane results in decreased sensitivity of cells to WNT ligands.

In the presence of RSPO ligands, binding of the FU1 domain of RSPO to the PA domain of ZNRF3/RNF43 and of the FU2 domain of RSPO to the LRRs of LGR4/5/6 results in formation of a ternary complex (Figs. 4 and 5B) (Chen et al., 2013; Moad & Pioszak, 2013; Xie et al., 2013; Zebisch & Yvonne Jones, 2015a). This molecular assembly triggers internalization of ZNRF3/RNF43, followed by lysosomal degradation, through a poorly understood process that requires the catalytic RING domain of ZNRF3/RNF43 (Hao et al., 2012) and can be counteracted through deubiquitylation of ZNRF3/RNF43 by the DUB USP42 (Giebel et al., 2021). As a consequence of ZNRF3/RNF43 clearance from the plasma membrane, ubiquitylation-dependent internalization and lysosomal degradation of FZD and LRP6 is diminished, leading to the accumulation of these WNT co-receptors on the cell surface (Hao et al., 2012). Therefore, the outcome of this RSPO signaling modality is to increase the sensitivity of cells to WNT ligands.

Because ZNRF3/RNF43 and LGRs do not interact directly with each other, the secreted RSPOs must engage both of them simultaneously through the adjacent FU1 and FU2 domains, respectively, acting as molecular cross-linkers (Fig. 5B) (Zebisch & Yvonne Jones, 2015b). The TSP

and BR domains of RSPOs would appear to be dispensable for this mode of signaling, since they escape contact with either ZNRF3/RNF43 or LGRs. This is partially borne out by the fact that a fragment comprising only the FU1 and FU2 domains of RSPOs is sufficient to promote WNT/β-catenin signaling in cells and support the growth of small intestinal organoids (Kazanskaya et al., 2004; Kim et al., 2008; Li et al., 2009; Peng, de Lau, Forneris, et al., 2013). However, while this FU1-FU2 construct displays full signaling efficacy at sufficiently high concentrations, it is much less potent than the full-length protein containing the TSP/BR domains both in cells and in small intestinal organoids (Dubey et al., 2020; Kim et al., 2008; Lebensohn & Rohatgi, 2018), demonstrating that the TSP/BR domains contribute to signaling even in the presence of LGRs (Fig. 5E).

3.2.2 HSPG-dependent, ZNRF3/RNF43-mediated potentiation of WNT/β-catenin signaling by RSPO2/3

Unexpectedly, RSPO2 and RSPO3, but not RSPO1 or RSPO4, are capable of potentiating WNT/β-catenin signaling in cells and mice lacking LGRs, albeit with lower potency and efficacy than in cells containing LGRs (Lebensohn & Rohatgi, 2018; Park et al., 2018; Szenker-Ravi et al., 2018). Furthermore, full length RSPO3 containing inactivating mutations in the LGR-interacting FU2 domain could still promote WNT/β-catenin signaling in haploid human cells, again with lower potency and efficacy than the wild type (WT) counterpart (Lebensohn & Rohatgi, 2018). Similarly, RSPO2 and RSPO3 constructs lacking the BR domain and containing inactivating mutations in the LGR-interacting FU2 domain could also potentiate WNT/β-catenin signaling in HEK293 cells (Szenker-Ravi et al., 2018). These experiments, in which potentiation of WNT/β-catenin signaling was partially retained following perturbations of either the LGR receptors or the LGR-binding FU2 domain on the RSPO ligands themselves, conclusively demonstrated the capacity of RSPOs to signal independently of LGRs. This begged an urgent question: is there an alternative engagement receptor for RSPOs?

To answer that question, we mapped the domains in RSPO3 required for signaling in the absence of LGRs through mutagenesis, domain deletion and domain swapping experiments (Lebensohn & Rohatgi, 2018). The ZNRF3/RNF43-interacting FU1 domain, and the HS-interacting TSP and/or BR domains of RSPO3 were required (constructs lacking either

the TSP or the BR domain, but not both, could support signaling) (Figs. 4 and 5C). Furthermore, the precise amino acid composition of the TSP/BR domains is not a critical determinant for signaling in the absence of LGRs: replacing the TSP/BR domains of RSPO3 with those of RSPO1, which cannot signal without LGRs, did not impair signaling (Lebensohn & Rohatgi, 2018) even though the TSP/BR domains of RSPO3 and RSPO1 exhibit very low sequence conservation (21% identical amino acids in the human proteins (de Lau et al., 2012)). These results suggested that LGR-independent signaling may be mediated by electrostatic interactions between the TSP and/or BR domains and the HS chains of HSPGs. Modeling of the TSP/BR domains of RSPO3 predicted two positively charged grooves lined by basic Lys and Arg residues that could potentially dock HS chains like those present in HSPGs (Dubey et al., 2020). Indeed, signaling by RSPO3 in cells lacking all LGRs was nearly completely abolished by three different manipulations that disrupted the interaction between the TSP/BR domains and the HS chains of HSPGs: 1. mutation of some of the Lys/Arg residues in the TSP/BR domains to charge-reversing Glu residues; 2. addition of heparin, which competes for binding to the HS chains of HSPGs; and 3. disruption of the gene encoding EXTL3, a glycosyltransferase specifically required for HSPG biosynthesis but dispensable for the synthesis of other glycosaminoglycans and proteoglycans (Dubey et al., 2020; Lebensohn & Rohatgi, 2018).

The conclusive demonstration that interactions between the TSP and/or BR domains of RSPOs and the HS chains of HSPGs mediate LGR-independent signaling (Fig. 5C) came from ligand engineering experiments (Dubey et al., 2020). A synthetic RSPO3 construct in which the entire TSP and BR domains were replaced with a single-chain variable fragment (scFv) that specifically binds to the HS chains of GPCs potentiated WNT/β-catenin signaling with the same potency and efficacy as WT RSPO3 in cells lacking LGRs. Experiments in which individual or entire families of HSPGs (including all GPCs or all SDCs) were eliminated in haploid human cells demonstrated that RSPO3 can signal in a redundant manner via either GPCs, SDCs or potentially another HSPG by engaging their HS chains rather than their protein cores (Dubey et al., 2020). Furthermore, genome-wide screens in haploid human cells lacking LGR4/5/6 did not reveal additional receptors required for potentiation of WNT signaling by RSPO3, making HSPGs the most likely engagement receptors for RSPOs in the absence of LGRs (Dubey et al., 2020).

The ZNRF3/RNF43-binding FU1 domain is also required for LGR-independent signaling (Fig. 5C) (Lebensohn & Rohatgi, 2018; Park et al., 2018; Szenker-Ravi et al., 2018). In fact, it is the FU1 domain, rather than the HSPG-interacting TSP/BR domains, that determines whether a given RSPO family protein can signal in the absence of LGRs. This was demonstrated by domain-swapping experiments in which the FU1 domain of RSPO3 conferred on RSPO1 the ability to signal without LGRs, and conversely an RSPO3 chimera containing the FU1 domain of RSPO1 lost its ability to signal without LGRs (Lebensohn & Rohatgi, 2018). Because the affinities of the FU1 domains from RSPO3 ($K_D \sim 60\,nM$) and from RSPO1 ($K_D \sim 6.8\,\mu M$) towards ZNRF3 are markedly different (Zebisch et al., 2013), this difference may determine the requirement for LGRs (Lebensohn & Rohatgi, 2018). We speculate that in the presence of a high-affinity interaction between the FU1 domain of RSPO2 ($K_D \sim 25\,nM$) or RSPO3 ($K_D \sim 60\,nM$) and ZNRF3 (Zebisch et al., 2013), the interaction between the FU2 domain and LGRs can be functionally replaced by the interaction between the TSP/BR domains and HSPGs. However, the lower-affinity interaction between the FU1 domain of RSPO1 ($K_D \sim 6.8\,\mu M$) or RSPO4 ($K_D \sim 300\,\mu M$) and ZNRF3 (Zebisch et al., 2013) would require the high-affinity interaction between the FU2 domain and LGRs ($K_D \sim 2$–$3\,nM$) (de Lau et al., 2011; Glinka et al., 2011; Zebisch et al., 2013) in order to signal.

While the TSP/BR domains are not required for signaling in the presence of LGRs, they substantially increase the potency of signaling by RSPOs in cells and small intestinal organoids (Dubey et al., 2020; Kim et al., 2008; Lebensohn & Rohatgi, 2018). In fact, at lower concentrations of RSPO3, the interaction of the FU2 domain with LGRs is not sufficient to drive efficient endocytosis of RNF43, and HSPG binding mediated by the TSP/BR domains is also required even in the presence of LGRs (Dubey et al., 2020). Furthermore, at limiting concentrations, RSPO3 was significantly more potent than RSPO1 in supporting the growth of intestinal organoids (Greicius et al., 2018), consistent with the ability of RSPO3 but not RSPO1 to signal through both LGR-dependent and LGR-independent mechanisms (Dubey et al., 2020; Kim et al., 2008; Lebensohn & Rohatgi, 2018). Although none of the structural studies discussed earlier included the TSP/BR domains of RSPOs, one of the structural models of the LGR5-RSPO1-RNF43 ternary complex suggested that the TSP/BR domains would extend into an open space not occupied by other polypeptides (Fig. 5B) (Chen et al., 2013), and would therefore be

available to interact with other molecules such as HSPGs. This would allow RSPOs to bind two engagement receptors—LGRs and HSPGs—and an effector receptor—ZNRF3 or RNF43—simultaneously (Figs. 4 and 5E), consistent with the ability of HSPGs to potentiate LGR-dependent signaling (Dubey et al., 2020). Therefore, HSPGs may enhance the potency of RSPO signaling by trapping RSPOs near the cell surface, increasing their local concentration and promoting binding to LGRs. In support of this model, depletion of HS chains or removal of the TSP/BR domains reduces binding of RSPOs to the cell surface, while depletion of LGR4 does not (Ren et al., 2018).

We and others initially referred to the modality of RSPO signaling that takes place in the absence of LGRs as "LGR-independent" (Lebensohn & Rohatgi, 2018; Park et al., 2018; Szenker-Ravi et al., 2018), but LGR-independent signaling has since been shown to happen in more than one way (see Section 3.2.3). Therefore, in the context of the WNT/β-catenin pathway, where in the absence of LGRs RSPO signaling is mediated by HSPGs (Dubey et al., 2020; Lebensohn & Rohatgi, 2018), we will henceforth refer to this modality as "HSPG-dependent" RSPO signaling.

3.2.3 BMPR1A-dependent, ZNRF3/RNF43-mediated inhibition of BMP signaling by RSPO2/3

A third mechanism of signaling by RSPOs, also independent of LGRs but mediated by ZNRF3/RNF43, has recently been described (Lee et al., 2020). In this case, RSPO2 and RSPO3, but not RSPO1 or RSPO4, antagonize BMP signaling in a process that is independent of WNT/β-catenin and WNT/PCP signaling. RSPO2 and RSPO3 interact directly with ZNRF3 and the type I BMP receptor BMPR1A (Figs. 4 and 5D), triggering internalization and lysosomal degradation of BMPR1A. This results in decreased sensitivity of target cells to BMP ligands.

Domain analysis revealed that the FU1 and TSP domains of RSPO2 are required to antagonize BMP signaling (Fig. 5D) (Lee et al., 2020). RSPO2 interacts with the BMPR1A ECD with high affinity ($K_D \sim 4.8$ nM), comparable to that of the FU2-mediated RSPO-LGR interaction ($K_D \sim$2–3 nM). The TSP domain of RSPO2 and RSPO3, but not the FU1, FU2 or BR domains, is required for binding to the BMPR1A ECD. Furthermore, domain-swapping experiments revealed that the capacity to downregulate BMP receptor levels resides in the TSP domain: while WT RSPO1 did not antagonize BMP signaling, an RSPO1 chimera

containing the TSP domain of RSPO2 bound to BMPR1A and antagonized BMP signaling. siRNA-mediated knock-down of ZNRF3/RNF43 or overexpression of a dominant negative ZNRF3 lacking the RING domain prevented RSPO2-induced destabilization of BMPR1A and inhibition of BMP signaling. On the other hand, siRNA-mediated knock-down of LGR4/5 did not affect inhibition of BMP signaling by RSPO2. These results suggest that BMP antagonism by RSPO2 requires ZNRF3/RNF43 but not LGRs. Consistent with these requirements, the ZNRF3/RNF43-binding FU1 domain of RSPO2, but not the LGR-binding FU2 domain, was required to antagonize BMP receptor signaling (Lee et al., 2020).

RSPO2 triggers BMPR1A clearance from the cell surface by acting as a cross-linking ligand between BMPR1A and ZNRF3 (Fig. 5D) (Lee et al., 2020). *In vitro* binding assays and co-localization experiments demonstrated that ZNRF3 interacted with BMPR1A in the presence of RSPO2, and formation of a ZNRF3-RSPO2-BMPR1A ternary complex depended on the FU1 and TSP domains of RSPO2. In cells that produce RSPO2, BMPR1A was absent from the plasma membrane but colocalized with ZNRF3 in cytoplasmic vesicles, as well as with the early endosome marker EEA1 and the lysosome marker Lamp1. Knock-down of RSPO2 abolished endosomal and lysosomal localization, and resulted in accumulation of BMPR1A at the plasma membrane. Therefore, RSPO2 bridges ZNRF3 and BMPR1A, and routes the ternary complex for lysosomal degradation, antagonizing BMP signaling. The authors proposed that a similar mechanism applies to RSPO3, but not RSPO1 or RSPO4 (Lee et al., 2020).

3.2.4 Comparing different modalities of RSPO-ZNRF3/RNF43 signaling

The three different modalities of ZNRF3/RNF43-mediated RSPO signaling described so far, LGR-dependent potentiation of WNT/β-catenin signaling, HSPG-dependent potentiation of WNT/β-catenin signaling, and BMPR1A-dependent antagonism of BMP signaling, illustrate the versatile modularity of the RSPO-ZNRF3/RNF43 signaling system (Table 1). These signaling modes are defined by a "combinatorial code" in which the FU1, FU2, TSP and/or BR domains of RSPOs interact with different combinations of engagement, effector and target receptors to modulate the WNT/β-catenin or BMP pathways (Figs. 4 and 5 and Table 1). Furthermore, differences in the extent to which individual domains of distinct RSPO ligands interact with these receptors, presumably determined by their binding affinities, dictates the modalities through which each RSPO

Table 1 Summary of the three RSPO signaling modalities regulated by ZNRF3/RNF43.

Signaling modality	RSPO ligands	Required domains in RSPO	Engagement receptors	Effector receptors	Target receptors	Adapter	Direct/indirect effect of RSPO binding on target receptor levels	Up/down-regulation of target receptor by RSPO
LGR-dependent potentiation of WNT/β-catenin signaling	RSPO1–4	FU1, FU2	LGR4–6	ZNRF3/ RNF43	FZD, LRP6	DVL	Indirect	Up-regulation
HSPG-dependent potentiation of WNT/β-catenin signaling	RSPO2/3	FU1, TSP/BR	HSPGs (GPCs, SDCs)	ZNRF3/ RNF43	FZD, LRP6?	DVL?	Indirect	Up-regulation
BMPR1A-dependent inhibition of BMP signaling	RSPO2/3	FU1, TSP	BMPR1A	ZNRF3/ RNF43	BMPR1A	?	Direct	Down-regulation

See main text for description.

ligand can signal (Table 1). Finally, depending on whether RSPOs engage target receptors and directly promote their membrane clearance, as in the case of BMPR1A, or indirectly effect changes in target receptor levels by modulating ZNRF3/RNF43 internalization and potentially ubiquitin ligase activity, as in the case of the WNT receptors FZD and LRP6, the functional outcome is either down- or up-regulation of the signaling pathway, respectively (Table 1).

In accordance with the opposite ways in which ZNRF3/RNF43 function during regulation of the WNT receptors FZD and LRP6 versus regulation of the BMP receptor BMPR1A (Table 1), the molecular mechanisms leading to internalization and lysosomal degradation of target receptors are different between the two pathways. Furthermore, the molecular mechanisms leading to ZNRF3/RNF43 membrane clearance and lysosomal degradation, triggered by binding of RSPOs to ZNRF3/RNF43 and to the different engagement receptors, have not been fully elucidated. In Sections 3.2.5 and 3.2.6, we discuss potential molecular mechanisms controlling ZNRF3/RNF43-dependent membrane clearance and degradation of target receptors in the WNT and BMP pathways, as well as those controlling RSPO-dependent membrane clearance of ZNRF3/RNF43 and engagement receptors. In the case of BMPR1A-dependent signaling, these mechanisms are one and the same.

3.2.5 Molecular mechanisms controlling internalization and degradation of target receptors in the WNT pathway

In the context of WNT signaling, ZNRF3/RNF43-dependent ubiquitylation of the target receptor FZD on Lys residues within the cytoplasmic loops of the 7TM domain and/or the C-terminal tail targets FZD to RAB5+ early endosomes and CD63+ lysosomes (Hao et al., 2012; Koo et al., 2012). This results in FZD internalization and lysosomal degradation, leading to decreased sensitivity to WNT ligands. ZNRF3 and RNF43 are most likely co-internalized with FZD—RNF43 co-localized with FZD5 in internal vesicles (Koo et al., 2012)—and this endocytic process is regulated by phosphorylation/dephosphorylation of a conserved 4Tyr motif within the DIR of ZNRF3 (Chang et al., 2020; Kim et al., 2021).

Several lines of evidence demonstrate that the ubiquitin ligase activity of ZNRF3/RNF43 is required for FZD ubiquitylation, internalization and degradation. Overexpression of WT ZNRF3 or RNF43 increased ubiquitylation of FZD, decreased cell surface FZD levels and reduced WNT-induced pathway activity (Hao et al., 2012; Koo et al., 2012).

Conversely, overexpression of ZNRF3 or RNF43 mutants containing inactivating point mutations in, or altogether lacking the catalytic RING domain suppressed ubiquitylation, increased the plasma membrane expression and extended the half-life of FZD, abolishing the inhibitory effect of ZNRF3/RNF43 on WNT signaling (Hao et al., 2012; Koo et al., 2012). Inactivating mutations in, or deletion of the RING domain of ZNRF3/RNF43 also enhanced WNT-induced pathway activity by acting in a dominant-negative fashion (Hao et al., 2012; Koo et al., 2012). Furthermore, FZD variants in which all conserved cytoplasmic Lys residues were mutated to Arg were not internalized upon expression of RNF43 (Koo et al., 2012), and the membrane levels of these FZD mutants did not increase upon depletion of ZNRF3 (Hao et al., 2012). Ubiquitylation of FZD was reduced in cells lacking the WNT pathway scaffold protein DVL, which as discussed earlier may serve as an adaptor that targets ZNRF3/RNF43 to FZD (Jiang et al., 2015). Finally, ZNRF3 and RNF43 could be co-immunoprecipitated with FZD (Hao et al., 2012; Koo et al., 2012). This compilation of experiments strongly supports a model in which ZNRF3/RNF43 directly ubiquitylate FZD, but we note that FZD ubiquitylation by ZNRF3/RNF43 has not been reconstituted *in vitro* with purified components.

A recently described "phospho-switch" also modulates the ability of ZNRF3/RNF43 to regulate WNT receptor levels (Tsukiyama et al., 2020). Phosphorylation by casein kinase 1 of 3 Ser residues located in the SRR of RNF43 (also conserved in ZNRF3) was required for down-regulation of cell surface FZD and for suppression of WNT/β-catenin signaling. Phosphorylation of RNF43 at these residues promoted ubiquitylation of FZD, and in turn its endocytosis and lysosomal degradation. The precise mechanism underlying regulation of FZD ubiquitylation by this phospho-switch remains unknown, but does not appear to involve changes in the protein-protein interactions (including binding to the E2 UbcH5C), oligomerization state or subcellular localization of RNF43 (Tsukiyama et al., 2020).

How do ZNRF3/RNF43 recognize FZD for ubiquitylation? As discussed earlier, it has been proposed that DVL, which binds both the DIR of ZNRF3 and the ICD of FZD (Fig. 4), acts as a substrate adaptor that targets ZNRF3/RNF43 to FZD (Jiang et al., 2015). Furthermore, direct binding of the ZNRF3/RNF43 PA domain to the CRD of FZD (Fig. 4) has also been proposed as a recognition mechanism

(Tsukiyama et al., 2015), but this subject is still debated (Radaszkiewicz & Bryja, 2020 and reviewed by Tsukiyama, Koo, & Hatakeyama, 2021). One study detected an interaction between the RNF43 PA domain and the FZD CRD (Tsukiyama et al., 2015), while others did not (Jiang et al., 2015; Peng et al., 2013). Several studies showed that deletion or replacement of the PA domain prevented ZNRF3/RNF43 from promoting FZD internalization and suppressing WNT/β-catenin signaling (Koo et al., 2012; Moffat et al., 2014; Spit et al., 2020; Tsukiyama et al., 2015), while another study found that deletion of the PA domain had none of these effects (Radaszkiewicz & Bryja, 2020). AlphaFold modeling suggests that the FZD1 CRD is well positioned to interact with the PA domain of RNF43, which could drive contacts between the TM helix of RNF43 and the 7TM of FZD, and orient the RING domain for ubiquitin transfer (Fig. 5F). So while the question of how FZD is recognized as a substrate by ZNRF3/RNF43 is still unresolved, one possibility is that extracellular contacts between the ZNRF3/RNF43 PA domain and the FZD CRD, intramembrane packing of ZNRF3/RNF43 and FZD TM helices, and intracellular interactions mediated by DVL all play a role in substrate recognition.

LRP6 internalization and degradation is also regulated by ZNRF3/RNF43 (Chang et al., 2020; Giebel et al., 2021; Hao et al., 2012; Kim et al., 2021) and while ZNRF3 could be co-immunoprecipitated with LRP6 (Hao et al., 2012), no single domain or motif in LRP6 has been identified as a target of ubiquitylation or regulation by ZNRF3/RNF43. Therefore the mechanism of LRP6 receptor regulation by the RSPO-ZNRF3/RNF43 system has not been determined. Some possibilities include direct ubiquitylation of LRP6 by ZNRF3/RNF43—although this has not been demonstrated experimentally—or co-internalization of LRP6 with FZD, mediated by WNT ligands or other mutual binding partners.

3.2.6 Molecular mechanisms controlling membrane clearance of ZNRF3/RNF43 and engagement receptors

Importantly, ubiquitylation and internalization of WNT receptors is not regulated directly by interactions between RSPOs and these target receptors, but is instead prevented indirectly as a result of RSPOs binding to and downregulating ZNRF3/RNF43 through LGR-dependent and/or HSPG-dependent mechanisms (Table 1). On the other hand, downregulation of BMPR1A is the direct result of RSPOs interacting with and promoting the internalization of ZNRF3/RNF43 (Table 1). Therefore, the mechanisms

controlling membrane clearance of ZNRF3/RNF43 are crucial to the regulation of target receptors in both the WNT and BMP pathways.

During LGR-dependent signaling, binding of RSPOs to both ZNRF3/RNF43 and LGRs is required for internalization of the ternary complex, since mutation of key residues in the ZNRF3/RNF43-interacting FU1 domain or the LGR-interacting FU2 domain of RSPOs abolishes potentiation of WNT/β-catenin signaling (Peng, de Lau, Forneris, et al., 2013; Xie et al., 2013; Zebisch et al., 2013). However, the precise molecular mechanism whereby formation of this ternary complex drives its internalization is not fully understood. One model is that RSPO acts as a cross-linking ligand that couples ZNRF3/RNF43 to LGRs, and since LGR5 undergoes constitutive clathrin-mediated endocytosis (Snyder, Rochelle, Lyerly, Caron, & Barak, 2013; Snyder et al., 2017), mere coupling could result in the co-internalization of ZNRF3/RNF43. This is consistent with the finding that RSPO-dependent potentiation of WNT/β-catenin signaling requires clathrin-mediated endocytosis (Glinka et al., 2011). Further support of this model comes from the fact that synthetic RSPO ligands that cross-link ZNRF3/RNF43 to constitutively endocytosed receptors can promote ZNRF3/RNF43 internalization and upregulate WNT signaling. Engineered "surrogate RSPO" bispecific ligands comprising a ZNRF3- or RNF43-specific scFv fused to the immune cytokine IL-2, which binds to the constitutively internalized IL-2 receptor CD25, leads to co-internalization of ZNRF3 and stimulation of WNT signaling in CD25+ cells (Luca et al., 2020). Additionally, synthetic RSPO2 ligands retaining only the ability to bind ZNRF3/RNF43 through the FU1 domain and fused to scFvs targeting them to the liver-specific asialoglycoprotein receptor (ASGR), which is predominantly expressed on hepatocytes and undergoes rapid endocytosis, increased cell surface FZD and enhanced WNT signaling specifically in cells that express ASGRs (Zhang et al., 2020). Similar results were obtained when these synthetic RSPO2 ligands were fused to scFvs targeting them to a ubiquitously expressed cell surface receptor, transferrin receptor 1 (TFR1), which undergoes continuous endocytosis (Zhang et al., 2020). Finally, the need for RSPOs can be bypassed altogether as long as their cross-linking functionality is provided: appending DmrA and DmrC heterodimerization domains to the C-termini of ZNRF3 and LGR4, respectively, enabled the membrane clearance of ZNRF3 in response to addition of an A/C dimerizer (Hao et al., 2012).

These disparate systems demonstrate that cross-linking ZNRF3/RNF43 to a constitutively endocytosed cell surface receptor, whether it be through

RSPOs themselves or other artificial cross-linkers, can clear ZNRF3/RNF43 from the plasma membrane and promote upregulation of WNT receptors. However, there is evidence that cross-linking of ZNRF3/RNF43 to the engagement receptors is not sufficient in all physiological contexts, and ubiquitylation/deubiquitylation of ZNRF3/RNF43, or potentially engagement receptors, is also involved in regulating their internalization. First, the RING domain of ZNRF3 and RNF43 is required for RSPO1 (or for the A/C dimerizer discussed earlier) to reduce the membrane level of ZNRF3 (Hao et al., 2012), suggesting that membrane clearance requires the ubiquitin ligase activity of ZNRF3/RNF43. Furthermore, the intracellular portion of ZNRF3 and the full-length protein purified by immunoprecipitation exhibit RING domain-dependent auto-ubiquitylation in *in vitro* ubiquitylation assays (Chang et al., 2020; Hao et al., 2012). Therefore, one possibility is that auto-ubiquitylation of ZNRF3 is required for internalization, although this mechanism has not been directly demonstrated. Second, deubiquitylation of ZNRF3/RNF43 by the DUB USP42 stabilizes ZNRF3/RNF43 at the plasma membrane and "stalls" the LGR4-RSPO-ZNRF3/RNF43 ternary complex, preventing its clearance from the cell surface (Giebel et al., 2021). In this way USP42 antagonizes RSPOs by protecting ZNRF3/RNF43 from RSPO- and ubiquitin-dependent internalization, thereby increasing the ubiquitylation and turnover of FZD and LRP6 receptors, and inhibiting WNT signaling. Since ubiquitylation of membrane proteins can drive their internalization (reviewed in MacGurn, Hsu, & Emr, 2012), auto-ubiquitylation of ZNRF3/RNF43 in response to RSPOs may therefore be a second mechanism promoting membrane clearance of ZNRF3/RNF43.

Alternatively or in addition to auto-ubiquitylation, ubiquitylation of another substrate by ZNRF3/RNF43, for instance the engagement receptors themselves, may promote endocytosis of the receptors and associated ZNRF3/RNF43 molecules. This hypothesis is supported by the fact that bringing RNF43 in close proximity to transmembrane proteins, including a synthetic GFP-TM-NanoLuc construct as well as the endogenous immune checkpoint protein programmed death-ligand 1 (PD-L1), can promote their internalization and lysosomal degradation (Cotton, Nguyen, Gramespacher, Seiple, & Wells, 2021). In the case of PD-L1, a synthetic bispecific IgG, or "abTAC," that bound to the ECDs of both RNF43 and PD-L1 was used to recruit RNF43 to PD-L1. Since neither of these two proteins are internalized or degraded constitutively, this experiment

showed that recruitment of RNF43 and a target TM protein in close proximity is sufficient to induce internalization and lysosomal degradation of the target protein, independently of RSPOs. Therefore, simultaneous binding of RSPO ligands to ZNRF3/RNF43 and engagement receptors, which would bring them in close proximity, may be sufficient to promote ubiquitylation of the engagement receptors and internalization of the ternary complex. However, whether RSPOs actively regulate the ubiquitin ligase activity of ZNRF3/RNF43, and therefore affect the endocytic efficiency of this process, remains an unanswered question.

In the case of HSPG-dependent signaling, we surmise that RSPO2/3-mediated cross-linking of ZNRF3/RNF43 and HSPGs (Fig. 5C) promotes ternary complex co-internalization driven by endocytosis of HSPGs. HSPGs are autonomous endocytosis receptors that can mediate the internalization of growth factors and morphogens among other ligands (reviewed in Christianson & Belting, 2014). They can undergo constitutive or ligand-induced endocytosis, followed in some cases by lysosomal degradation (Burbach, Friedl, Mundhenke, & Rapraeger, 2003; Fuki et al., 1997; Fuki, Meyer, & Williams, 2000; Wittrup et al., 2009). During HSPG-dependent potentiation of WNT/β-catenin signaling by RSPO3, RNF43 is internalized in a process that requires the interaction of the TSP/BR domains with HSPGs (Dubey et al., 2020). Since GPCs are tethered to the plasma membrane through a GPI anchor (Fig. 4) and do not have a cytoplasmic domain that can be ubiquitylated by ZNRF3/RNF43, in this signaling modality ternary complex internalization cannot be driven by ubiquitylation of the engagement receptor.

In contrast to the indirect regulation of WNT receptor internalization by RSPOs, BMPR1A clearance from the plasma membrane is driven by direct binding of RSPOs to both BMPR1A and ZNRF3/RNF43 (Table 1 and Fig. 5D), which promotes internalization and lysosomal degradation of the ternary complex (Lee et al., 2020). The molecular mechanism through which the ZNRF3/RNF43-RSPO2/3-BMPR1A complex is internalized has not been defined. In this case, internalization of BMPR1A is the step being regulated rather than being a constitutive process like the endocytosis of LGRs or HSPGs. Therefore, the mere cross-linking of BMPR1A and ZNRF3/RNF43 by RSPOs would not be sufficient to drive internalization of either receptor. We surmise that ubiquitylation of either ZNRF3/RNF43 or BMPR1A, induced by binding of RSPO2 or RSPO3, is likely the main mechanism driving internalization of the ternary complex.

In summary, we described two molecular mechanisms that could drive ZNRF3/RNF43 internalization and lysosomal degradation: (1) co-internalization of ZNRF3/RNF43 promoted by RSPO-mediated cross-linking to a constitutively endocytosed engagement receptor, and (2) endocytosis driven by ubiquitylation of ZNRF3/RNF43, engagement receptors or both, promoted by RSPO-mediated ternary complex formation. The latter could be driven by regulated auto-ubiquitylation of ZNRF3/RNF43 or by trans-ubiquitylation of the engagement receptors.

3.3 Physiological, pathological and therapeutic implications of distinct RSPO-ZNRF3/RNF43 signaling modalities

The RSPO-ZNRF3/RNF43 system has important functions during embryonic development and in adult tissue homeostasis. Aberrant regulation caused by mutations in ZNRF3/RNF43 or by RSPO fusions that cause elevated expression can lead to cancer. We refer the reader to some excellent reviews on the physiology and pathology of the RSPO-ZNRF3/RNF43 system (Bugter, Fenderico, & Maurice, 2021; de Lau et al., 2012; Hao et al., 2016; Jin & Yoon, 2012; Nagano, 2019; Raslan & Yoon, 2019; Ter Steege, ter Steege, & Bakke, 2021). Here we describe the principal phenotypes caused by disruption of different components of the system, and discuss how the discovery of the three RSPO signaling modalities presented earlier compels us to re-interpret some of these phenotypes. We also posit that the modular nature of RSPO proteins presents a unique opportunity to manipulate the RSPO-ZNRF3/RNF43 system for therapeutic benefit.

To the best of our knowledge, the comprehensive phenotype of the ubiquitous *Znrf3/Rnf43* double KO mouse has not been published, but would be predicted to result in early embryonic lethality. However, conditional *Znrf3/Rnf43* double KO in the intestinal epithelium (driven by *Cyp1a1-cre* or *Villin-creERT2*) resulted in marked expansion of the proliferative compartment (with hyperproliferative cells containing high levels of β-catenin), upregulation of WNT target genes, and increased numbers of intestinal stem and Paneth cells (Koo et al., 2012). Clonal deletion of *Znrf3/Rnf43* in the intestinal epithelium or in intestinal stem cells (driven by *Lgr5-creERT2*) resulted in adenoma formation, with continuous expansion of stem cells and generation of Paneth cells but no other differentiated cell types (Koo et al., 2012). Intestinal organoids derived from *Znrf3/Rnf43* double KO mice grew faster than controls and lost the dependence on RSPO1 supplementation, but not on secreted WNT3A, consistent with

the role of ZNRF3/RNF43 in mediating RSPO-dependent potentiation of WNT signaling (Koo et al., 2012). The WNT/β-catenin pathway is also a major regulator of liver metabolic zonation, development and regeneration (reviewed in Hu & Monga, 2021; Monga, 2014; Yang et al., 2014). Inducible, systemic combined deletion of *Znrf3* and *Rnf43* (driven by *Rosa26-creERT2*) in mice induced hepatocyte proliferation and extended metabolic zonation, measured as a marked increase in the expression, as well as zonal expansion, of the liver-specific WNT/β-catenin target proteins GS and CYP2E1 (Planas-Paz et al., 2016). Furthermore, deletion of *Znrf3* and *Rnf43* specifically in hepatocytes (driven by Ad5cre virus) led to the formation of multiple liver tumors, primarily classified as hepatocellular carcinomas (Sun et al., 2021). Thus, ZNRF3/RNF43 control the hepatic WNT/β-catenin signaling gradient and metabolic liver zonation, and prevent liver tumor formation. Simultaneous disruption of *znrf3* and *rnf43* by injection of TALENs into two-cell stage *Xenopus tropicalis* embryos resulted in development of ectopic limbs, ranging from diplopodia (duplication of digits) to complete polymelia (presence of supernumerary limbs), including quadruplication of forelimbs in extreme cases (Szenker-Ravi et al., 2018). These three examples, and many others not discussed here, demonstrate that loss of ZNRF3 and/or RNF43 results in elevated WNT/β-catenin signaling in various tissues. However, given that ZNRF3/RNF43 regulate all three RSPO signaling modalities discussed earlier, the phenotypes caused by their disruption do not distinguish the specific physiological functions of LGR-dependent, HSPG-dependent and BMPR1A-dependent RSPO signaling.

The expression patterns of the four RSPOs in mice are distinct (Nam, Turcotte, & Yoon, 2007) and, not surprisingly, so are the phenotypes associated with their disruption, illustrating the pleiotropic roles of RSPOs during embryogenesis (reviewed in de Lau et al., 2012; Jin & Yoon, 2012; Nagano, 2019). Mutations in *RSPO1* cause a rare human syndrome characterized by XX male sex reversal, palmoplantar hyperkeratosis (abnormal thickening of the palms and soles) and predisposition to squamous cell carcinoma of the skin (Parma et al., 2006). Loss of *Rspo1* in mice confirmed that the absence of RSPO1 at the gonadal differentiation stage causes partial sex reversal (Tomizuka et al., 2008). Human mutations in *RSPO2* cause tetra-amelia with lung hypo/aplasia syndrome (TETAMS), a severe condition characterized by amelia (the complete absence of limbs), lung hypo/aplasia, cleft lip-palate, and labioscrotal fold aplasia

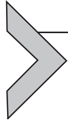
(Szenker-Ravi et al., 2018). Consistently, loss of *Rspo2* in mice causes limb malformations or amelia, severe malformations of laryngeal-tracheal cartilages, lung hypoplasia, and palate malformations (Aoki, Kiyonari, Nakamura, & Okamoto, 2008; Bell et al., 2008; Nam et al., 2007; Yamada et al., 2009). Loss of *Rspo3* in mice results in severe placental vascular defects, causing death of the mutant mice around embryonic day (E)10 (Aoki et al., 2007; Kazanskaya et al., 2008). Mutations in human *RSPO4* were found in individuals affected with anonychia, a rare autosomal recessive congenital syndrome characterized by partial or complete absence of fingernails and toenails (Bergmann et al., 2006; Blaydon et al., 2006; Brüchle et al., 2008; Ishii et al., 2008).

While previously it may have been tempting to attribute these different phenotypes to the distinct expression patterns of RSPO1–4, an additional explanation must be considered in light of the multiple RSPO signaling modalities discussed in this chapter. Differences in *RSPO* KO phenotypes may also be explained by the capacity of different RSPOs to signal through LGR-dependent, HSPG-dependent and BMPR1A-dependent mechanisms. This possibility is supported by the finding that ubiquitous *Lgr4/5/6* triple KO mice do not exhibit many of the phenotypes observed in *Rspo2* KO or *Rspo3* KO mice (Szenker-Ravi et al., 2018). *Lgr4/5/6* triple KO mice die around E14.5–18.5, but the embryos undergo normal development of the limbs and lungs, as well as normal placental vascularization, suggesting RSPO2 and RSPO3 signaling is largely unaffected. From these experiments it can be surmised that certain developmental processes governed by RSPO2 and RSPO3 occur independently of LGRs. However, other processes that are also regulated by RSPO2, such as palate and tongue development, rely on LGRs, since both *Rspo2* KO and *Lgr4/5/6* triple KO mice exhibit cleft palate and ankyloglossia (tongue-tie).

Comparison of the phenotypes caused by *Lgr4/5/6* KO and by *RSPO1–4* loss-of-function mutations in humans and mice can help distinguish between LGR-dependent and LGR-independent effects of RSPOs, but whether these effects are driven by potentiation of WNT signaling through HSPGs or by downregulation of BMP signaling through BMPR1A (or by yet other pathways regulated by RSPOs) is less clear. Little is known about the physiological contexts in which HSPG-dependent and BMPR1A-dependent RSPO signaling operate. During nephrogenesis, strong RSPO-dependent activation of WNT/β-catenin signaling is essential for nephron progenitors to differentiate and undergo mesenchymal to

epithelial transition, and this process occurs largely in an LGR-independent manner (Vidal et al., 2020), suggesting the possibility that nephrogenesis is driven in part by HSPG-dependent RSPO signaling. In multiple myeloma cells, RSPO binds to SDC1 in a HS-dependent manner, and this event is required for optimal stimulation of WNT/β-catenin signaling (Ren et al., 2018). In *Xenopus*, RSPO2 cooperates with Spemann organizer effectors to inhibit BMP signaling during embryonic axis formation (Lee et al., 2020), and BMP signaling inhibition by RSPO2 maintains autocrine self-renewal in acute myeloid leukemia (AML) (Sun et al., 2021). Elucidating the complete repertoire of biological and pathological processes controlled by RSPOs through different signaling modalities will require a combination of approaches. Disrupting entire families of engagement receptors, as was done with the LGRs in mice (Szenker-Ravi et al., 2018), could provide additional insights, but this is a challenging prospect for HSPGs, since RSPO3 (and presumably RSPO2) can signal redundantly through GPCs, of which there are six members in mammals, and SDCs, of which there are an additional four (Dubey et al., 2020; Lebensohn & Rohatgi, 2018).

Given the modular structure of RSPO proteins (Fig. 4) and the different domain requirements for distinct signaling modalities (Table 1), "modality-specific" engineered RSPO ligands—ligands that can signal exclusively through a single signaling modality—could yield further insights. This concept was demonstrated by experiments with RSPO chimeras in which domains from RSPOs capable of signaling through distinct modalities were swapped, rendering the chimeras competent or incompetent to signal through a different modality (Lebensohn & Rohatgi, 2018; Lee et al., 2020). In other experiments, a domain required for one signaling modality was mutated, deleted or replaced by synthetic scFvs, leaving only the domains necessary to target RSPOs to engagement receptors that mediate a different signaling modality (Dubey et al., 2020; Lebensohn & Rohatgi, 2018; Lee et al., 2020). Furthermore, the modularity of the RSPO-ZNRF3/RNF43 signaling system should enable the use of such modality-specific ligands for therapeutic or regenerative applications. The ability to target RSPOs to desired tissues through engagement of tissue-specific receptors has been demonstrated (Luca et al., 2020; Zhang et al., 2020) and could potentially be combined with modality-specific mutations to selectively target a single signaling pathway specifically in an affected tissue.



4. Regulation of Hedgehog and melanocortin receptor abundance by the membrane-recruited E3 MGRN1

MGRN1 and its paralog RNF157 (Fig. 1C) belong to a unique class of E3s that do not contain a TM domain but are recruited to the plasma membrane through interactions with other single-pass TM proteins. The TM partners of MGRN1 regulate its substrate specificity, much like substrate adaptors do in multi-subunit RING class E3s (Metzger et al., 2014). We describe two such systems in which association with two related single-pass TM proteins directs MGRN1 to ubiquitylate different GPCRs. We predict that the recruitment of cytoplasmic E3s to the plasma membrane is a mechanism used more broadly to regulate the cell surface abundance of membrane receptors, and consequently to regulate signaling sensitivity.

4.1 The MGRN1-MEGF8-MOSMO complex, an attenuator of Hedgehog signaling strength

Mgrn1 encodes an eponymous RING family E3 (also known as RNF156, Figs. 1C and 6A), and was identified as the mutated gene at the *mahoganoid* locus in mice (He et al., 2003; Phan, Lin, LeDuc, Chung, & Leibel, 2002). *Mgrn1* has been studied because of its effects on coat color determination and spongiform neurodegeneration. However, ~25% of *Mgrn1*$^{-/-}$ embryos die during gestation with heterotaxy (defects in left-right patterning of organs) and complex cardiac anomalies, suggesting an additional role for *Mgrn1* during development (Cota et al., 2006).

MEGF8, the gene encoding multiple epidermal growth factor-like (EGFL) domains protein 8 (MEGF8, Fig. 6B–D) was among those compiled by a bioinformatics screen for genes that encode uncommonly large (>1000 amino acids) membrane-embedded proteins that contain multiple EGFL domains (Nakayama et al., 1998). This effort was motivated by the observation that these characteristics—large size, a TM domain and multiple EGFL domains—were seen in proteins that play important roles in cell-cell or cell-extracellular matrix interactions, such as AGRIN or receptors and ligands of the NOTCH family. MEGF8 was subsequently noted to have homology to a single-pass TM protein commonly referred to as attractin (ATRN, Fig. 7A), which was identified by positional cloning of a mouse gene from the *mahogany* locus implicated in both body weight and coat color (discussed in Section 4.2) (Gunn et al., 1999; Nagle et al., 1999).

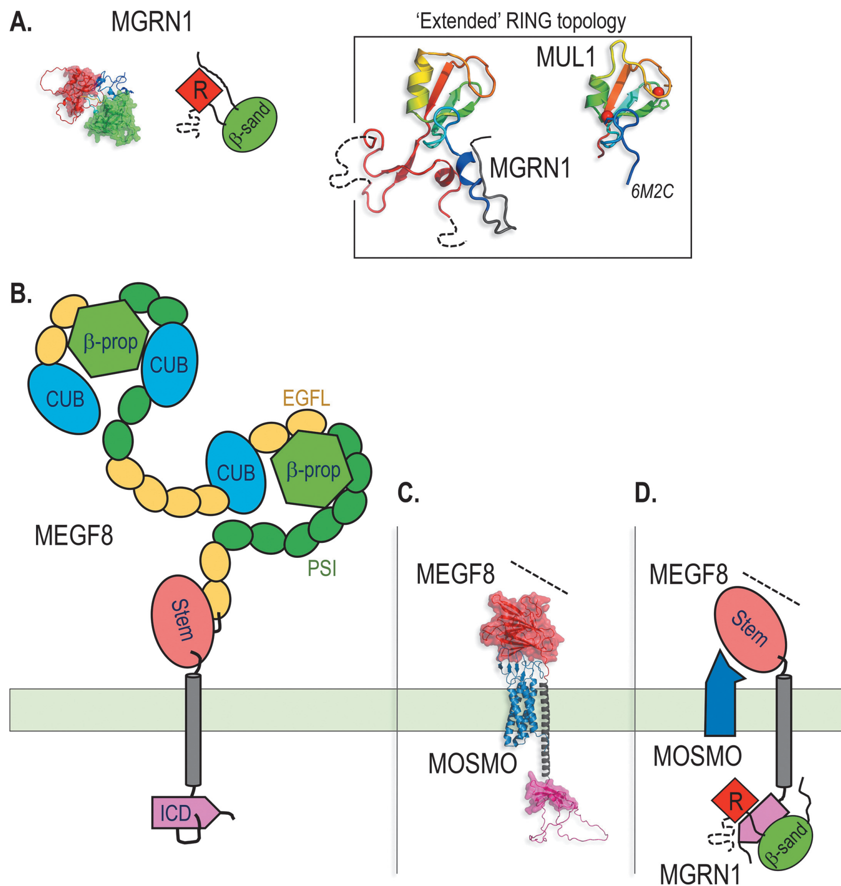


Fig. 6 Architecture of the MMM complex, an attenuator of Hh signaling. (A) MGRN1 is a cytoplasmic E3 containing a RING domain and a β-sandwich domain. An AlphaFold structural model is shown on the left and a cartoon representation on the right. The box shows the AlphaFold prediction of the MGRN1 "extended" RING domain, which most closely resembles the structure of the RING domain from MUL1 (PDB ID 6M2C). (B) MEGF8 contains a massive ECD with a pseudo-repeat architecture. A central spine composed of multiple EGFL and PSI domains is decorated with two 6-blade β-propellers and three complement C1r/C1s, uegf, bmp1 (CUB) domains. The extracellular domain is perched on a juxta-membrane, extracellular Stem domain, followed by a TM helix that extends into the cytoplasm and connects to an ICD. (C) AlphaFold model of the 4-pass TM protein MOSMO (related to the Claudin family of 4TM proteins) complexed with a fragment of MEGF8 containing the Stem, TM and ICD. The Stem stacks on top of the extracellular β-sheet of MOSMO, promoting the "zippering" of the 4TM bundle of MOSMO with the single TM helix of MEGF8. (D) Cartoon depicting the assembly of the MGRN1-MEGF8-MOSMO complex, excluding the large pseudo-repeat ECD of MEGF8 (shown in (B)).

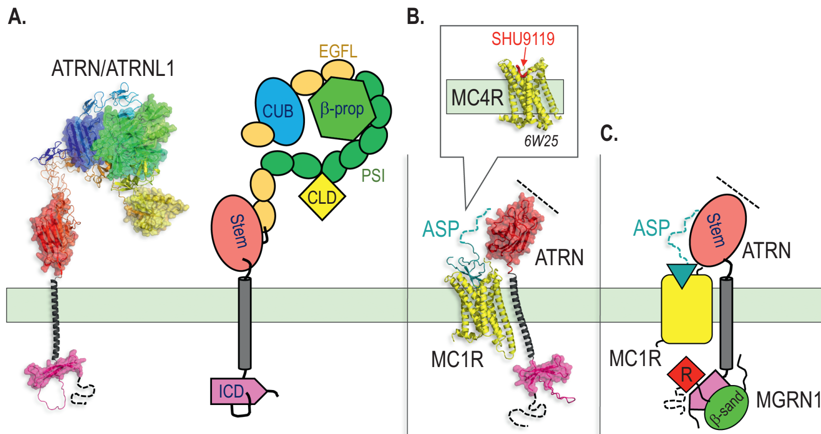


Fig. 7 The MGRN1-ATRN complex, an attenuator of melanocortin receptor signaling. (A) AlphaFold model and cartoon representation of ATRN/ATRNL1 (ATRN and ATRNL1 are two closely related proteins, so only one of them is shown). Note the similarities between ATRN and MEGF8 (Fig. 6B). The ECD of ATRN has only one of the two repeats present in MEGF8. ATRN has a cyclophilin-like domain (CLD) not found in MEGF8, but shares a 6-blade β-propeller and a CUB domain. The domain coloring in the cartoon is matched to the structural model. (B) and (C) AlphaFold model (B) and cartoon representation (C) of how the ligand ASP could cross-link MC1R to ATRN. The C-terminus of ASP forms a β-hairpin that occupies a putative ligand binding site in MC1R. The box in (B) shows the solved structure of MC4R in complex with the antagonist SHU9119 (PDB ID 6W25) (Yu et al., 2020), which occupies the same site predicted to interact with ASP by AlphaFold. The N-terminus of ASP (dotted aqua line) is well positioned to interact with the Stem domain of ATRN.

Insights into the biological function of MEGF8 came from mouse embryos carrying loss-of-function mutations in *Megf8*, as well as from human patients with a recessively inherited birth defect syndrome called Carpenter's Syndrome (Engelhard et al., 2013; Twigg et al., 2012; Zhang et al., 2009). In both cases, loss of MEGF8 resulted in heterotaxy, severe congenital heart defects, pre-axial polydactyly, and skeletal and craniofacial defects. While *Megf8* clearly plays a widespread role in the development of multiple tissues, the underlying mechanisms remained unclear. MEGF8 was proposed to be a modifier of BMP and nodal signaling due to its role in left-right patterning and peripheral axon guidance (Engelhard et al., 2013; Zhang et al., 2009). In addition, a possible connection to Hedgehog (Hh) signaling was suggested by the observations that (1) Carpenter's Syndrome can also be caused by mutations in *RAB23*, a negative regulator of Hh signaling and that (2) Carpenter's Syndrome phenotypes resembled

those of a related syndrome caused by mutations in the Hh transcription factor GLI3 (Eggenschwiler, Espinoza, & Anderson, 2001; Twigg et al., 2012). However, the molecular mechanism of action of both MGRN1 and MEGF8 remained unknown.

MGRN1, MEGF8 and MOSMO, a completely uncharacterized 4-pass TM protein of the Claudin family (Fig. 6C–D), had never been linked to each other until they were all identified in a genome-wide, loss-of-function CRISPR screen designed to uncover attenuators of Hh signaling strength (Pusapati et al., 2018). This screen was performed by exposing cells to a sub-saturating concentration of the Hh ligand Sonic Hedgehog (SHH) and selecting cells containing mutations that enhanced the activity of a Hh transcriptional reporter. Biochemical analyses demonstrated that all three proteins form a complex, which we named the MMM complex. MGRN1 stably associates with the C-terminal, cytoplasmic tail of MEGF8, and MOSMO associates with this MEGF8-MGRN1 subcomplex (Kong et al., 2020, 2021) (Fig. 6D). The MMM complex represents a new architecture for E3 complexes: a TM protein with a massive extracellular domain that stably associates with a cytoplasmic RING E3 through a short cytoplasmic tail.

4.1.1 Biochemical and cellular functions of the MMM complex

While there is little homology between the MMM proteins and ZNRF3/RNF43 (except for the presence of a RING domain in MGRN1), there are conceptual similarities in their mechanisms of action. The MMM complex regulates the abundance of the Hh signal transducer smoothened (SMO) at the cell surface by ubiquitylation (Kong et al., 2020), similar to the way in which ZNRF3/RNF43 regulate the abundance of FZD receptors. SMO transmits the Hh signal across the membrane and is the closest relative of the FZD receptors in the Class F family of GPCRs. Loss of MGRN1, MEGF8 or MOSMO markedly reduced SMO endocytosis and degradation, leading to SMO accumulation on the cell surface and the membrane of the primary cilium (Kong et al., 2020, 2021; Pusapati et al., 2018). Primary cilia function as compartments for Hh signaling, and increased SMO accumulation in primary cilia enhances Hh signaling strength (Huangfu & Anderson, 2005; Huangfu et al., 2003). Consequently, the concentration of SHH required to induce target genes is reduced by nearly an order of magnitude when any of the MMM proteins are lost (Pusapati et al., 2018). In summary, just as FZDs are target receptors for ZNRF3/RNF43, SMO is the target receptor for the MMM complex.

Both the integrity of the RING domain of MGRN1 and the physical interaction between MGRN1 and the MEGF8 ICD are required for ubiquitylation of SMO by the MMM complex (Kong et al., 2020). The MGRN1-MEGF8 interaction requires the amino acid sequence MASRPFA, a motif that is conserved in proteins of the MEGF8/ATRN family across evolution (Gunn et al., 1999; Nagle et al., 1999). Direct binding of SMO to MMM complex components has not been reported, so the mechanism of SMO recognition by the MMM complex remains to be elucidated. The large ECD of MEGF8 is dispensable for SMO recognition. A truncated protein containing only the TM domain (TMD) and ICD of MEGF8 (which also cannot bind to MOSMO) is nonetheless sufficient to mediate SMO ubiquitylation, reduce its levels at the cell surface and dampen Hh signaling (Kong et al., 2020). However, it is not known whether this TMD-ICD segment of MEGF8 recognizes SMO directly or through an adaptor protein. Importantly, mere recruitment of MGRN1 to the plasma membrane is not sufficient to drive SMO ubiquitylation. The MEGF8 TMD, or the precise arrangement of the TMD relative to the ICD, is likely to be important because replacement of the MEGF8 TMD with the TMD of a different single-pass TM protein abolished SMO ubiquitylation, despite the fact that MGRN1 was still recruited to the membrane and retained ubiquitin ligase activity (Kong et al., 2020). Therefore, MEGF8 likely functions as a substrate adaptor that recruits SMO, and perhaps other substrates, for ubiquitylation by MGRN1.

The function of the 4TM protein MOSMO remains to be fully elucidated. MOSMO is a Claudin family protein whose distinctive extracellular loop structure, which folds into a compact, disulfide-locked β-sheet, forms a cell surface interaction platform for a previously uncharacterized extracellular juxtamembrane domain in MEGF8, which we named the Stem domain (Fig. 6C–D) (Kong et al., 2021). Loss of MOSMO partially reduces MEGF8 levels at the cell surface, suggesting a role in trafficking (Kong et al., 2021). While MOSMO is not absolutely required for SMO ubiquitylation by the MEGF8-MGRN1 subcomplex in an overexpression system, the severe phenotypes of *Mosmo*$^{-/-}$ mice, which are similar to those of *Megf8*$^{-/-}$ mice, suggest that the association of MOSMO with MEGF8 and MGRN1 may in fact play a critical role in activation of the MMM complex.

A key unanswered question in MMM complex function is the role of the large MEGF8 ECD (Fig. 6B). Just like ZNRF3/RNF43 are regulated by RSPO ligands, it is likely that the MMM complex is also regulated by interactions of the MEGF8 ECD with a soluble extracellular protein,

a component of the extracellular matrix or another membrane protein on the same or an adjacent cell. The unique architecture of the MMM complex suggests a regulatory mechanism whereby the TM topology of the MEGF8 substrate adaptor allows extracellular signals to directly regulate the selection and ubiquitylation of specific substrates by MGRN1 in the cytoplasm.

In both cultured cells and mouse embryos, loss of MMM complex components in target cells receiving Hh signals leads to elevated sensitivity to Hh ligands (Kong et al., 2020, 2021). The pattern of elevated Hh signaling in embryos lacking MMM components is distinct in a very specific way from what is observed in embryos lacking patched 1 (PTCH1) or suppressor of fused (SUFU), two negative regulators of Hh signaling. Loss of PTCH1 or SUFU leads to the ectopic activation of Hh signaling in multiple tissues, showing that these proteins suppress basal signaling activity even in the absence of Hh ligands (Cooper et al., 2005; Goodrich, Milenkovic, Higgins, & Scott, 1997; Svard et al., 2006). In contrast, Hh signaling in MMM mutant mice remains dependent on Hh ligands: Hh target gene expression is localized correctly in the embryo, but the strength of signaling is elevated (Kong et al., 2021). Thus, like ZNRF3 and RNF43, the MMM complex proteins are best characterized as "attenuators" of signaling rather than negative regulators, because their effects remain dependent on the presence of WNT or Hh ligands, respectively. In summary, the MMM complex forms a signaling module that calibrates how the Hh ligand gradient is decoded by target cells.

4.1.2 Developmental roles of the MMM complex

The common function of the three proteins in the MMM complex that is suggested by biochemical analyses is also supported by mouse genetic studies (Cota et al., 2006; Kong et al., 2020, 2021; Zhang et al., 2009). *Mosmo*$^{-/-}$ and *Megf8*$^{-/-}$ mouse embryos exhibit similar developmental defects, also shared by Carpenter's Syndrome patients: heterotaxy, severe congenital heart defects, pre-axial digit duplication, skeletal defects, craniofacial defects and neurodevelopmental abnormalities. In addition, SMO abundance in the ciliary membrane is markedly elevated in nearly all *Mosmo*$^{-/-}$ and *Megf8*$^{-/-}$ embryonic tissues, consistent with observations in cultured cells (Kong et al., 2020, 2021). Some of these embryonic phenotypes were initially not observed in *Mgrn1*$^{-/-}$ embryos because MGRN1 is partially redundant with RNF157 (Cota et al., 2006; Kong et al., 2020). However, *Mgrn1*$^{-/-}$; *Rnf157*$^{-/-}$ embryos exhibit a constellation of defects very similar to those seen in *Mosmo*$^{-/-}$ and *Megf8*$^{-/-}$ embryos. Beyond common phenotypes,

strong genetic interactions and gene dosage effects between mutant alleles of *Megf8*, *Mgrn1* and *Rnf157* support the conclusion that MGRN1/RNF157 and MEGF8 function together to regulate a common set of developmental processes (Kong et al., 2020).

The widespread nature of the defects observed in mouse embryos carrying mutations in the MMM genes points to a central role of the MMM complex in the regulation of cell-cell communication. MMM complex components have been linked to nodal, BMP and Hh signaling, but a direct role in regulation of signaling components has only been established for SMO in the Hh pathway (Engelhard et al., 2013; Pusapati et al., 2018). The expression patterns of *Megf8*, *Mgrn1* and *Mosmo* do not provide many clues to the developmental functions of the MMM complex: they are ubiquitously expressed in all three germ layers and in all major cardiac populations (Cota et al., 2006; Kong et al., 2021; Wang et al., 2020; Zhang et al., 2009). Thus, control of MMM complex activity, perhaps by a ligand or by post-translational modifications, is likely to be the key regulated step that explains its tissue-specific roles.

An unresolved question is whether all of the embryonic defects seen in MMM mutant mice are caused by upregulation of SMO and elevated Hh signaling, or whether some are caused by regulation of other unidentified substrates that function in other pathways. The pre-axial digit duplication seen in MMM mutant embryos is likely to be driven by elevated Hh signaling, since this phenotype can be completely reversed by administration of vismodegib, a placenta-permeable small molecule inhibitor of SMO (Kong et al., 2020, 2021). However, vismodegib only partially rescues the congenital heart defects observed in MMM mutant embryos, which could be due to a suboptimal schedule of *in utero* vismodegib administration, or could also indicate that other pathways are regulated by the MMM complex. Further research will be necessary to comprehensively identify substrates of the MMM complex other than SMO and to test for possible roles of the MMM complex in other developmental signaling pathways.

Heterotaxy is a prominent feature of MMM mutant mouse embryos and Carpenter's Syndrome patients, and may be the root cause of the severe congenital heart defects observed in both (Cota et al., 2006; Kong et al., 2020, 2021; Zhang et al., 2009; Twigg et al., 2012). The MMM complex regulates left-right patterning at an early stage in embryogenesis. In both *Megf8*$^{-/-}$ and *Mgrn1*$^{-/-}$ embryos, abnormal expression of all three canonical left-expressed genes (*Nodal*, *Lefty*, *Pitx2*) was observed (Cota et al., 2006; Zhang et al., 2009). Conditional disruption of *Megf8* in various cardiac

lineages using a panel of cre drivers (*cTnt-cre*, *Wt1-cre*, *Tie2-cre*, *Wnt1-cre*, *Mesp1-cre*) did not reproduce the heart defects seen in the global *Megf8* KO. Timed global deletion of *Megf8* at E7.5 also did not reproduce the cardiac defects (Wang et al., 2020). These data suggest that *Megf8* is required for cardiac development at a time point earlier than cardiac organogenesis and supports the hypothesis that the heart defects seen in MMM mutant mice are a consequence of disrupted left-right patterning early in development.

Current models suggest that Hh signaling plays a permissive role in left-right patterning of the mouse embryo: reduced Hh signaling caused by loss of SMO leads to a midline heart tube that fails to loop and an embryo that fails to turn (Levin, Johnson, Stern, Kuehn, & Tabin, 1995; Tsiairis & McMahon, 2009; Zhang, Ramalho-Santos, & McMahon, 2001). While this model does not readily explain how the elevated Hh signaling seen in MMM mutant embryos leads to left-right patterning defects, loss-of-function mutations in the Hh negative regulator *Sufu* do cause abnormalities in embryo turning, heart looping and expression of the left-expressed gene *Pitx2* (Cooper et al., 2005). Thus, normal left-right patterning may depend on a just-right, or "goldilocks," level of Hh signaling strength calibrated by the MMM complex. Alternatively, the MMM complex may regulate another pathway involved in left-right patterning, such as nodal or BMP signaling. Elucidating how the MMM complex regulates left-right patterning is critical to understanding its developmental roles and, consequently, the etiology of birth defects caused by mutations in MMM genes.

4.2 The MGRN1-ATRN system regulates melanocortin receptors

Regulation of Hh signaling by the MMM complex shares many themes with another membrane-tethered E3 complex, formed by MGRN1 and the MEGF8-related protein ATRN, that functions in a paracrine fashion to regulate melanocortin receptor signaling (reviewed in He, Eldridge, et al., 2003). The four melanocortin receptors, MC1R, MC2R, MC3R and MC4R, are GPCRs that bind to peptide agonists, including α-MSH and ACTH, and regulate diverse physiological processes in vertebrates. We focus here on the regulation of mouse coat color, which serves as a useful paradigm for paracrine cell–cell interactions that orchestrate both tissue patterning during development and tissue homeostasis in adults.

Melanocortin receptor activity is controlled by agonists, like α-MSH, and inverse agonists. Agouti signaling protein (ASP) and agouti-related protein (AGRP) are both inverse agonists of the MC1R receptors in the skin and the MC4R receptors in the hypothalamus. They reduce basal and α-MSH-stimulated receptor activity (Lu et al., 1994).

Hair follicle melanocytes switch between producing the pigments eumelanin (dark) and pheomelanin (light). In mice, the presence of a subapical light band in an otherwise dark hair leads to the agouti coat color. The dark eumelanic hair pigmentation is driven by MC1R signaling in melanocytes. The light pheomelanic band in each hair is generated by a paracrine signaling interaction between dermal cells at the base of each hair follicle and neighboring melanocytes. Transient secretion of ASP from the dermal cells inhibits MC1R signaling, causing a switch to pheomelanin synthesis and the generation of the light band on each hair. Ubiquitous and constitutive expression of ASP results in a light coat as well as hyperphagia and obesity, caused by inhibition of MC4R in the hypothalamus. This short-range dermal-melanocyte signaling circuit is similar to how RSPOs secreted by stromal cells influence WNT signaling in epithelial stem cells of the intestinal crypts (Greicius et al., 2018).

Mgrn1 and *Atrn* were identified as genes required for the inhibitory effect of ASP on melanocortin receptor signaling (Gunn et al., 1999; He, Eldridge, et al., 2003; He, Lu, et al., 2003; Nagle et al., 1999; Phan et al., 2002). The pigmentation changes caused by ectopic ASP expression can be suppressed by mutations in *Mgrn1* and *Atrn*. Epistasis analyses have placed *Mgrn1* and *Atrn* downstream of ASP but upstream of MC1R. Strikingly, genetic analyses show that *Mgrn1* and *Atrn* are required for ASP signaling, despite the fact that purified ASP alone is a high-affinity antagonist of MC1R in biochemical assays (Willard et al., 1995). These genetic studies suggested that MGRN1 and ATRN are required for the inhibitory effects of ASP on MC1R in target melanocytes.

4.2.1 Cellular and biochemical models for the regulation of melanocortin receptors by ASP, MGRN1 and ATRN

ATRN is a single pass TM protein related to MEGF8. The massive extracellular domain of MEGF8 is composed of two tandem repeats (Fig. 6B). ATRN is more compact and has only one of these repeats, but shares the single TM helix and short intracellular tail found in MEGF8 (Fig. 7A). While the C-terminal domain of ASP binds to MC1R with high affinity

($K_D \sim 1$ nM), the N-terminal chain of ASP binds to the ECD of ATRN with ~500-fold lower affinity (Fig. 7B-C) (He et al., 2001; Willard et al., 1995). These genetic and biochemical studies are most consistent with a model in which the binding of ASP to ATRN transmits a signal to MGRN1 in the cytoplasm that ultimately leads to the downregulation of melanocortin receptors.

There are striking similarities between the ASP-MGRN1-ATRN module and the MMM complex: ATRN and MEGF8 are related proteins, MGRN1 is shared, and both systems regulate GPCRs. The simplest model that emerges from this comparison is that the MGRN1-ATRN complex ubiquitylates and downregulates MC1R and MC4R at the cell surface in response to binding of ASP. In this model, ASP acts as a ligand that cross-links melanocortin receptors to ATRN (Fig. 7B-C), analogous to how RSPO2 and RSPO3 cross-link BMPR1A to ZNRF3/RNF43 (Fig. 5D). AlphaFold modeling suggests that ASP can simultaneously bind to MC1R through its C-terminus and to the Stem domain in ATRN through its N-terminus, thereby positioning the RING domain of ATRN for Ub transfer to the cytoplasmic surface of MC1R (Fig. 7B-C).

There is some evidence to support this model. ASP promotes trafficking of MC4R to the lysosome and its subsequent degradation in a manner that depends on both MGRN1 and ATRN (Kim, Olzmann, Barsh, Chin, & Li, 2007; Overton & Leibel, 2011). Loss of ATRN leads to elevated MC4R levels at the cell surface (Overton & Leibel, 2011), analogous to how loss of MEGF8 leads to elevated SMO levels at the cell surface (Pusapati et al., 2018). However, a physical interaction between the C-terminal tail of ATRN and MGRN1, analogous to that between MEGF8 and MGRN1, has not been demonstrated, and neither has a role for such an interaction in melanocortin receptor downregulation. Notably, the MASRPFA motif in the MEGF8 cytoplasmic tail that is required for binding to MGRN1 is conserved in ATRN (Kong et al., 2020; Nagle et al., 1999), and this motif in the *Drosophila* ATRN ortholog is required for association with *Drosophila* MGRN1 (Nawaratne, Kudumala, Kakad, & Godenschwege, 2021). Ubiquitylation of MC1R by an MGRN1-ATRN complex also remains to be established. MGRN1 was shown to ubiquitylate MC2R, but it is not clear whether this reaction required ATRN (Cooray, Guasti, & Clark, 2011). MGRN1 also ubiquitylates tumor suppressor gene 101 (TSG101), a component of the endosomal sorting complex required for transport-I (ESCORT-I) complex that mediates the trafficking of ubiquitylated cell surface receptors from the plasma membrane to the

lysosome for degradation (Jiao et al., 2009; Kim et al., 2007). Based on this finding, a different model has been proposed in which the effect of MGRN1 on melanocortin receptor trafficking is an indirect consequence of its regulation of TSG101. Further experiments will therefore be required to elucidate the biochemical function of the MGRN1-ATRN complex.

4.3 Evolutionary insights into MGRN1 and the MEGF8/ATRN protein family

MEGF8 and ATRN family proteins are conserved across metazoans and in their closest living relatives, the choanoflagellates (Pusapati et al., 2018). The *Drosophila* ortholog of *Megf8* is also required for early embryonic development (Lloyd, Toegel, Fulga, & Wilkie, 2018). However, MGRN1 is more widely distributed throughout evolution, found in all major eukaryotic lineages (Pusapati et al., 2018). The *Arabidopsis thaliana* ortholog of MGRN1, called LOG2, can be functionally replaced by human MGRN1 (Guerra, Pratelli, Kraft, Callis, & Pilot, 2013). LOG2 is recruited to the plasma membrane by binding to the cytoplasmic tail of the single-pass TM protein glutamine dumper-1 (GDU1) (Guerra et al., 2017). The LOG2-GDU1 complex has been implicated in amino acid transport. These observations suggest that members of the MGRN1 family of RING E3s have evolved to associate with multiple single-pass TM proteins across eukaryotes to regulate the activity of diverse membrane receptors and transporters. In this scheme, MGRN1 provides the ubiquitin ligase function while the single-pass TM protein functions as a substrate adaptor to select targets for ubiquitylation.

This ancient membrane-tethered E3 system has likely been adapted to regulate signaling pathways at multiple times during evolution. While MGRN1 is found in all eukaryotes, Hh signaling is only present in a subset of the metazoan lineages where MEGF8 is found. The MGRN1-ATRN system seems to have been co-opted to regulate melanocortin receptor signaling much later in evolution, since ASP and melanocortin receptors are only found in vertebrates.

5. Conclusions

Membrane-tethered E3s can tune the sensitivity of cells to ligands by promoting the internalization and degradation of specific signaling receptors. They can target substrates constitutively, like ZNRF3/RNF43

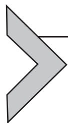
target FZDs, or they can be regulated by secreted ligands that direct them to specific substrates, like RSPOs direct ZNRF3/RNF43 to BMPR1A or ASP directs MGRN1-ATRN to melanocortin receptors. Thus, membrane-tethered E3s enable extracellular ligands to directly control the ubiquitylation of substrates in the cytoplasm. We propose that regulation of membrane receptors by membrane-tethered E3s plays a widespread and understudied role in tuning cell sensitivity to paracrine signals that control embryonic development and tissue homeostasis, as exemplified by the two conceptually analogous systems described in this chapter: (1) the RSPO-ZNRF3/RNF43 module that regulates WNT and BMP receptors, and (2) the MMM and MGRN1-ATRN modules that regulate Hh and melanocortin receptors, respectively.

Compared to cytoplasmic or nuclear E3s, membrane-tethered E3s face unique challenges in substrate recognition and modification because of a physical barrier, the plasma membrane, that creates three discrete zones for protein-protein interactions: the intracellular and extracellular spaces, and the plane of the membrane. These three regions likely create composite binding surfaces that promote the assembly of protein complexes in which the RING domain of the membrane-tethered E3 is optimally positioned in the cytoplasm to transfer Ub to the substrate. In this respect, there are architectural and mechanistic parallels between the assembly of membrane-tethered E3-substrate complexes and of cytokine-nucleated signaling receptor assemblies, which are driven by the "zippering" together of the full complex, from binding of ligands to receptors, to ECD and TM contacts between receptors, and finally cytoplasmic domain interactions that may also recruit downstream signaling proteins (Spangler, Moraga, Mendoza, & Christopher Garcia, 2015). These aggregate contacts contribute to the stability, lifetime and signaling strength of the receptor complex, and can be sites for therapeutic modulation or for engineering of tunable receptors (Rosenbaum, Clemmensen, Bredt, Bettler, & Strømgaard, 2020).

For cytoplasmic and nuclear E3s, proteolysis-targeting chimeras (PROTACs) have emerged as a therapeutic modality that enables target degradation driven by small molecules (Bond & Crews, 2021; Schneider et al., 2021). PROTACs function as bivalent linkers that direct E3s to specific substrates for ubiquitylation and degradation. Recently, a strategy conceptually analogous to PROTACs has been applied to induce the degradation of PD-L1, a TM immune checkpoint ligand, by RNF43 (Cotton et al., 2021). Targeting membrane-tethered E3s to heterologous signaling receptors using small molecules, surrogate ligands or bivalent nanobody

or antibody constructs is a promising strategy to modulate signaling strength for therapeutic purposes.

Molecular models and protein-protein interaction predictions enabled by AlphaFold and RoseTTaFold, like those presented in this chapter, will enable the rapid generation of hypotheses about how membrane-tethered E3s fold, assemble and recognize substrates. The ability of these new deep learning-based algorithms to predict protein folds and interactions relies in good part on their capacity to tease out faint signals of *co*-evolutionary linkage between amino acid positions on the same and on partner polypeptide chains by mining sequence databases. These algorithmic approaches should work well with the evolutionary pairings that underlie membrane-tethered E3-substrate systems. We expect that the characterization of other membrane-tethered E3-receptor systems will unravel new regulatory layers in many signaling pathways.



Note added in proof

While the manuscript was being processed for publication, two groups reported on the regulation of additional cell surface proteins by ZNRF3/RNF43. Zhu and colleagues described the regulation of Hulula (Hwa), a determinant of the Spemann organizer and dorsal body axis formation in *Xenopus laevis*, by ZNRF3 (Zhu et al., 2021). ZNRF3 binds and ubiquitylates Hwa, thereby regulating its lysosomal trafficking and degradation. Radaszkiewicz and colleagues described the regulation of β-catenin-independent, WNT5A-induced signaling by RNF43 in normal physiology and during melanoma invasion (Radaszkiewicz et al., 2021). RNF43 interacts with components of the WNT5A signaling pathway, including the receptors ROR1 and ROR2, and the signal transducers VANGL1 and VANGL2. RNF43 induces VANGL2 ubiquitylation and proteasomal degradation, promotes ROR1 internalization, and inhibits ROR2 activation. We presume that some of the mechanisms described in this chapter may apply to the regulation of ZNRF3/RNF43 in these contexts.

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