eISSN 2005-5447
International Journal of Stem Cells Vol. 16, No. 4, 2023 https://doi.org/10.15283/ijsc23070

REVIEW ARTICLE

RNF43 and ZNRF3 in Wnt Signaling - A Master Regulator at the Membrane

Fiona Farnhammer[1,2], Gabriele Colozza[1], Jihoon Kim[3,4]

[1]*Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna Biocenter (VBC), Vienna, Austria*
[2]*Division of Oncology and Children's Research Center, University Children's Hospital Zurich, University of Zurich, Zurich, Switzerland*
[3]*Department of Medical and Biological Sciences, The Catholic University of Korea, Bucheon, Korea*
[4]*Center for Genome Engineering, Institute for Basic Science, Daejeon, Korea*

The Wnt β-catenin signaling pathway is a highly conserved mechanism that plays a critical role from embryonic development and adult stem cell homeostasis. However, dysregulation of the Wnt pathway has been implicated in various diseases, including cancer. Therefore, multiple layers of regulatory mechanisms tightly control the activation and suppression of the Wnt signal. The E3 ubiquitin ligases RNF43 and ZNRF3, which are known negative regulators of the Wnt pathway, are critical component of Wnt signaling regulation. These E3 ubiquitin ligases control Wnt signaling by targeting the Wnt receptor Frizzled to induce ubiquitination-mediated endo-lysosomal degradation, thus controlling the activation of the Wnt signaling pathway. We also discuss the regulatory mechanisms, interactors, and evolution of RNF43 and ZNRF3. This review article summarizes recent findings on RNF43 and ZNRF3 and their potential implications for the development of therapeutic strategies to target the Wnt signaling pathway in various diseases, including cancer.

Keywords: Wnt signaling, RNF43, ZNRF3, Stem cell

Introduction

The Wnt signalling pathway is an evolutionarily highly conserved mechanism that plays crucial roles in embryonic development and tissue homeostasis across metazoan species (1). The pathway is comprised of a family of secreted glycoproteins, first discovered in Drosophila melanogaster during a mutagenesis screening (2, 3). The Wnt ligand - wingless (wg) - was identified as a key regulator of wing and haltere development, with genetic studies revealing its involvement in diverse patterning processes during development. Subsequent identification of other Wnt pathway components, such as armadillo (β-catenin), dishevelled (Dvl), and porcupine (Porc), further confirmed their essential roles in embryonic development which is showing similar phenotype upon gene ableation (4-6). In case of vertebrate, the abnormal Wnt singlaling lead to irregular digit or interdigit development, and disruption in organogenesis and limb development. Injection of Wnt1 mRNA into early Xenopus embryos confirmed their conserved roles in vertebrates and their importance in axis specification (7).

In addition to their function in developmental processes, the Wnt pathway also plays a pivotal role in adult tissue homeostasis by controlling stem cell maintenance in many organs (8). However, dysregulation of the pathway can lead to disease development such as cancer (9).

Received: May 24, 2023, Accepted: July 10, 2023,
Published online: August 30, 2023
Correspondence to **Jihoon Kim**
Department of Medical and Biological Sciences, The Catholic University of Korea, 43 Jibong-ro, Bucheon 14662, Korea
E-mail: jhkim@catholic.ac.kr

Aberrant Wnt signalling has been frequently observed in diverse types of cancers, with mutations in several Wnt signalling components identified to be involved in cancer development. For instance, mutations in the adenomatous polyposis coli (APC) gene occur in about 85% of colorectal cancer (CRC) patients, as well as in hepatoblastoma, pancreatic, stomach cancer, etc. (10, 11). Mutations in CNNTB1, the gene encoding for β-catenin, are also common in various cancers, resulting in a more stable and active form (9). Furthermore, alterations in AXIN are found in a subset of colorectal cancer cases (8). These findings emphasize the crucial role of the Wnt signalling pathway in cancer development and highlight its potential as a therapeutic target.

Due to the highly conserved Wnt signalling pathway plays important roles in embryonic development and adult tissue homeostasis, dysregulation of this pathway has been linked to diseases including cancer and making it an attractive target for therapeutics (12). A number of treatments have been developed to target the Wnt pathway, including porcupine inhibitors and anti-Wnt antibodies, and drugs preventing Wnt binding to its receptors. For example, LGK974, a porcupine inhibitor that prevents Wnt secretion, is currently in phase I clinical trial for multiple solid tumors including colorectal cancer (13). Another potential treatment option targeted directly against Wnts is the usage of anti-Wnt antibodies, such as OMP-54F28 and OMP-18R5 (14). However, these treatments have so far been directed towards a limited number of well-characterized components of the pathway.

In recent years, studies have identified novel Wnt regulators, shedding light on new molecular mechanisms and offering new opportunities for targeted therapies. One promising example is the E3 ubiquitin ligases RNF43 and ZNRF3, which function as tumour suppressors in stem cell homeostasis by downregulating Wnt receptors (15). However, mutations in RNF43 and ZNRF3 can transform them into oncogenes, and mutations in these genes have been found in a number of cancers, including colorectal, endometrial, and stomach cancers, as well as intraductal papillary mucinous neoplasm of the pancreas (16).

Given their importance in modulating Wnt signalling and maintaining proper stem cell activity, this review article will revisit fundamental aspects of RNF43/ZNRF3 function and discuss new findings on their regulatory mechanisms. This will provide insights into the potential of RNF43/ZNRF3 as promising candidates for targeted cancer treatments.

Wnt Signalling

Wnt signalling is classified into two pathways depending on the involvement of β-catenin - canonical and non-canonical signalling. The canonical and non-canonical pathways operate using different sets of proteins to transmit downstream signals and are activated by different ligands (17). Wnt1 class Wnt ligands including Wnt2, Wnt3, Wnt3a, and Wnt8a activates canonical signalling which induces proliferation, differentiation, maturation, etc., whereas Wnt5a type Wnt ligands such as Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, and Wnt11 mediates non-canonical signalling which mediates cellular polarization and migration (18). These pathways are controlled by distinct downstream components, which operate in different cellular processes and environments.

In the canonical Wnt pathway, the activity of the transcriptional co-activator β-catenin is mainly regulated. In the absence of Wnt ligands, a "destruction complex" comprising of AXIN1/2, APC, glycogen synthase 3β (GSK3β), and casein kinase 1 (CK1) promotes β-catenin turnover (19-25). The scaffold proteins APC and AXIN bind β-catenin, which is then sequentially phosphorylated by CK1 and GSK3β at serine and threonine residues, respectively. The phosphorylation events result in the recruitment of the E3 ligase $SCF^{\beta\text{-Trcp}}$, which ubiquitylates Lys-19 and Lys-49 residues of β-catenin, thereby initiating its proteasomal degradation. On the other hand, Wnt signalling is initiated when Wnt ligands bind to a member of the Frizzled (FZD) family of seven-pass transmembrane receptors and low-density lipoprotein-related protein 5 or 6 (LRP5/6), which form a co-receptor complex in a Wnt-dependent manner (26, 27). This interaction triggers the phosphorylation of the intracellular domain of LRP5/6 and subsequently recruits and phosphorylates DVL. Phosphorylated LRP and DVL interact with AXIN in the β-catenin destruction complex, retaining the complex at the cell membrane. Tethering AXIN to the plasma membrane prevents β-catenin degradation, allowing β-catenin to translocate into the nucleus, where it associates with TCF/LEF DNA binding factors and activates Wnt target gene transcription (Fig 1).

Regulation of Wnt Signalling through RNF43/ZNRF3

Due to the importance of the Wnt signalling pathway from embryonic development to the maintenance of adult tissues, dysregulation of Wnt signalling is often associated with various diseases, including cancer (19). RNF43, an

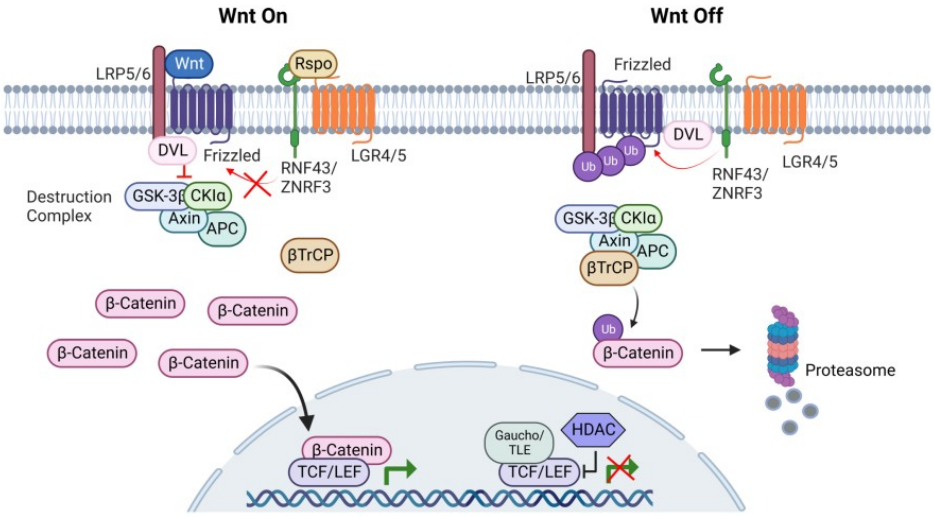

Fig. 1. Overview of Wnt/β-catenin signaling pathway. The absence of Wnt ligands induces the phosphorylation of β-catenin by the destruction complex. The complex comprises the scaffold protein AXIN, APC, and the GSK3β and CK1α. Without Wnt ligands, β-catenin undergoes GSK3β mediated phosphorylation, followed by ubiquitination facilitated by β-TrCP for proteasomal degradation. Therefore, target genes of Wnt signaling are not activated. Activation of the canonical pathway ensues upon the binding of secreted canonical Wnt ligands to FZD receptors and LRP co-receptors. Subsequently, CK1α and GSK3β phosphorylate LRP receptors, facilitating the recruitment of DVL proteins to the plasma membrane. As a result, β-catenin experiences stabilization and accumulation, and subsequently translocates into the nucleus. Within the nucleus, β-catenin forms an active complex with LEF/TCF proteins by displacing TLE/Gaucho complexes and recruiting co-activators involved in histone modification. This transcriptional switch triggers a multitude of alterations in cellular processes.

E3 ubiquitin ligase, was discovered as a gene frequently upregulated in colorectal cancer (28). Other studies reported abnormal expression of RNF43 in other types of cancers including gastric and pancreatic cancers. However, under homeostatic conditions, Rnf43 expression is very limited such as Lgr5 positive crypt stem cells (15). Furthermore, studies have shown that ZNRF3 mutation is also enriched in many cancer types (19).

In vitro functional analysis of the role of RNF43 by Sugiura et al. (29) has demonstrated that RNF43 has a ubiquitin ligase function since RNF43 ubiquitylates nuclear protein HAP95. Additionally, Koo et al. (15) have reported that Rnf43 and Znrf3 works as a E3 ubiquitin ligase which control Wnt signaling by performing conditional ablation of Rnf43 and its paralogue Znrf33 from the mouse small intestine induces the formation of adenomas due to hyperactive Wnt signalling. These findings indicate that Rnf43 and Znrf3 acts as a negative regulator of Fzd, reducing its cell surface concentration via ubiquitylation mediated protein degradation. In addition, Hao et al. (30) also found ZNRF3 enriched in colon cancer with hyperactive β-catenin signalling. Co-immunoprecipitation experiments have revealed that RNF43/ZNRF3 form a complex with FZD and LRP. Cell-surface biotinylation assays of Myc-FZD with cells treated with siRNA of ZNRF3 and ZNRF3⊿RING mutant resulted in an upregulation of FZD, while cells with ZNRF3 over expression showed a decreased FZD level. The mode of action of ZNRF3 occurs through FZD ubiquitylation, as a FZD KO mutant, entire intracellular Lys residues are mutated to Ala, cannot be ubiquitylated by ZNRF3 overexpression so the FZD KO expression remains at higher level than wild type FZD. These data confirm that RNF43/ZNRF3 function as negative regulators of Wnt receptors by decreasing their cell surface levels.

The Regulation of RNF43 and ZNRF3

RNF43 and ZNRF3 are crucial genes that play a significant role in Wnt signalling activation (8). Therefore, its expression is tightly regulated at both transcriptional and post-transcriptional levels (31, 32). The activation of Wnt/β-catenin promotes RNF43/ZNRF3 expression whose, in turn, downregulate Wnt signalling by reducing receptor stability, thereby forming a negative feedback loop. Tight regulation of RNF43/ZNRF3 at the transcriptional level is essential for maintaining steady-state levels of Wnt activity.

It is noteworthy that RNF43 is a Wnt target gene that is not expressed in all cells under active β-catenin signalling. While Wnt stimulation in MEFs and MCF7 cell lines induces RNF43 expression, it was not detected in Wnt-stimulated ES cells (33). This observation suggests

context-dependent or cell-type-specific expression of RNF43. At the protein level, RNF43 and ZNRF3 are downregulated through their interaction with LGR and RSPO via auto-ubiquitylation dependent degradation (15, 16, 30). This finding further demonstrated the importance of regulating RNF43/ZNRF3 expression to maintain appropriate levels of Wnt activity in various cells and tissues.

Regulatory Mechanism via LGR/R-Spondin

The LGRs, LGR 4/5/6, are receptors for a class of secreted Wnt agonists, known as RSPO, and a key stem cell marker in several adult tissues exhibiting active Wnt signalling, such as the crypt base columnar (CBC) cells in the small intestine (34-40). In humans, there are four different RSPO capable of binding to the extracellular domains of LGR4/5 and RNF43/ZNRF3 to form a trimeric complex that regulates the Wnt pathway (30, 35, 41). Addition of exogenous human RSPO1 to mouse colonic crypt by injection stabilizes β-catenin protein levels, leading to upregulation of the Wnt signal (42). Furthermore, Rspo2 expression positively correlates with Wnt signalling and functions as a secreted activator in Xenopus (43).

In 2011, two groups, Liu group and Clevers group, demonstrated that LGR4/5/6 directly interact with RSPO1～4 by *in vitro* pull-dwon assay, leading to potentiated Wnt/β-catenin signalling and internalization of the trimeric complex (34, 35). A year later, Hao et al. (30) further elucidated this regulatory mechanism, showing that the interaction between RSPO/LGR/ZNRF3 potentiated Wnt signalling via downregulation of ZNRF3. Following studies confirmed that RSPO, LGR, and RNF43/ZNRF3 interaction is necessary for Wnt signal potentiation (44-47).

Although RSPO1 and 4 require LGR for their interaction with RNR43/ZNRF3, Lebensohn and Rohatgi (48) demonstrated that Rspo2 and 3 can downregulate RNF43/ZNRF3 even without LGRs. In mouse embryos, the expression pattern of Lgr4/5/6 only partially overlaps with Znrf3, while Rspo2, which controls limb development with Rspo3 during embryogenesis, has the same expression pattern as Znrf3 (49). In Lgr4/5/6 triple knockout cells, RSPO2 or RSPO3 still upregulate Wnt signalling, indicating that Lgr receptors are dispensable for specific R-spondins. This Lgr-independent antagonistic interaction between Rspo2/3 and Rnf43/Znrf3 is critical for limb development.

DVL Mediates the Interaction between RNF43/ZNRF3 and FZD

The crucial role of RNF43/ZNRF3 in downregulating FZD receptor is well-established, however, the underlying molecular mechanism remains unclear. Recent study have identified DVL as a key regulator of RNF43 activity (47). In particular, DVL1/2/3 triple knockout (TKO) in HEK293 cells resulted in a phenotype similar to RNF43/ZNRF3 depletion which is increased cell surface ZFD and LRP6 level, indicating the essential role of DVL in regulating FZD receptor levels. Further analysis revealed that DVL interacts with RNF43 and mediates RNF43-dependent FZD ubiquitylation. These findings suggest that DVL plays a critical role in regulating the RNF43/ZNRF3-FZD axis, and provide important insights into the molecular mechanism underlying this process.

The G-protein-coupled seven-transmembrane receptors FZD are integral membrane proteins that play a key role in the Wnt signaling pathway (50). The Drosophila frizzled-2 (Dfz2) was discovered as the first frizzled family gene which respond to the addition of Wg therefore inducing Wnt signaling activation (51). The FZD receptor contains a signal peptide sequence, a N-terminal cysteine-rich domain (CRD) that binds its ligand Wnt, seven hydrophobic transmembrane (TM) domains, and linker region between CRD and TM. The regions between TM I and II, III and IV, and V and VI form intracellular loops (iLoops), with two binding motifs within the third loop, iLoop3, and a highly conserved KTXXXW motif in the Fzd C-terminal region mediating the interaction with Dvl (52, 53). Recent study demonstrated that the linker region of FZD contribute to selective interaction with Wnt molecules. Ko et al. (54) identified that linker region swapped FZD4 mutant with non-canonical Wnt signaling receptor FZD6 linker region has defect on binding with WNT3A and failed to interact with LRP6 and DVL.

DVL is a family of phosphoproteins that interact with Fzd iLoop3 binding motifs I and II via its C-terminus, and with the binding motif III on the C-terminal domain of Fzd via its DEP domain (DEP-C), which is important for Wnt signaling (52). Tauriello and colleagues demonstrated that the binding of DEP-C domain of Dvl interact with Fzd to the DEP domain of Dvl has been confirmed, and a Dishevelled-interacting region on RNF43/ZNRF3 has been mapped (47, 52). However, the identification of a particular region of DVL that interacts with RNF43/ZNRF3 has not yet been identified, indicating the possibility of the existence of multiple binding sites or the presence of a mediator that facilitates the interaction. Moreover, this regulatory mechanism can be context-dependent since canonical and noncanonical Wnt pathways use different interaction and motifs. These findings provide important insights into the complex regulation of the Wnt signaling

pathway and have important implications for stem cell biology.

Phospho-Dependant RNF43 and ZNRF3 Regulation

The activity of RNF43 in regulating Wnt signaling is known to be regulated in a phospho-dependent manner through CK1 by two independent groups in 2020 (55, 56). Tsukiyama et al. has demonstrated that CK1 directly interacts with two regions in the cytoplasmic portion of RNF43, independently of other components of the β-catenin destruction complex (56). Mutations in the CK1 binding site of RNF43 disrupt its function, demonstrating the requirement of CK1 for RNF43-mediated regulation of Wnt signaling. In HEK293T cells, treatment with Wnt3a leads to decreased phosphorylation of the C-terminal region of RNF43, while R-spondin1 treatment increases it. Recent studies by Tsukiyama et al. (56) have identified S474, S475, and S476 as the residues on RNF43 that are phosphorylated by CK1. Substituting these serine residues with phosphomimetic residues leads to sustained activity of RNF43, while anti-phospho mutations prevent RNF43-mediated downregulation of FZD from the cell surface. The phosphorylation status of these key residues may depend on prior phosphorylation of S478, as a S478D mutation hinders RNF43-mediated downregulation of FZD, while the phosphomimetic S478A mutation does not interfere with the function of RNF43.

Similar to RNF43, ZNRF3 also regulated by phosphorylation by CK1. Ci et al. (57) identified that ZNRF3 is targeted by the Cullin based E3 ubiquitin ligase complex $SCF^{\beta TrCP}$ for ubiquitylation mediated degradation. The authors demonstrated that CK1 is required for the $SCF^{\beta TrCP}$ mediated ZNRF3 ubiquitylation since ZNRF3 R474A phosphodegron mutant was more stable than wild-type and CK1 promotes ZNRF3 ubiquitylation followed by degradation. Moreover, two studies from the same lab identified phosphorylation mediated ZNRF3 regulation. Chang et al. (58) identified that Protein tyrosine phosphatase receptor-type kappa (PTPRK) maintains ZNRF3 '4Y' endocytic signal as an unphosphorylated status to induce Wnt receptor depletion and depletion of Ptprk lead to reduction of Spemann organizer effector gene expression and promoting head and axial defects in Xenopus embryo. Additional study by Kim et al. (59) demonstrated that mesenchymal-epithelial transition factor (MET) is the kinase responsible for the phosphorylation of 4Y motif. The authors confirmed that MET bind to ZNRF3 which is induced by MET ligand HGF (hepatocyte growth factor, scatter factor) for the enhancement of Wnt signaling.

Evolutionary Conservation of RNF43 and ZNRF3

The Wnt pathway is highly conserved across metazoans, with key components of the canonical pathway ubiquitous throughout the animal kingdom (60, 61) (Table 1). While no protozoan possesses a complete Wnt signalling pathway, some of its components are present in protozoan or-

Table 1. Conservation of Wnt pathway components in different model organisms. Except otherwise indicated, data was obtained from flybase for *D. melanogaster*, HGNC for *H. sapiens*, Xenbase for *X. laevis*, and wormbook for *C. elegans*

H. sapiens	*X. laevis*	*D. melanogaster*	*C. elegans*
DVL1,2,3	dvl1,2,3	dsh	mig-5; dsh-1; dsh-2
WNT1, 2, 2B, 3, 3A, 4, 5A, 5B, 6, 7A, 7B, 8A, 8B, 9A, 9B, 10A, 10B, 11, 16	wnt1, 2, 2b, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 7c, 8a, 8b, 9a, 9b, 10a, 10b, 11, 11b, 16	wg; wnt2, 4, 5, 6, 10	mom-2; lin-44; egl-20; cwn-1; cwn-2
β-Catenin	β-Catenin	arm	wrm-1; hmp-1; bar-1; sys-1
FZD1, 2, 3, 4, 5, 6, 7, 8, 9, 10	fzd1, 2, 3, 4, 5, 6, 7, 8, 9, 10	fz, fz2, fz3, fz4	mom-5; lin-17; mig-1; cfz-2
LEF1; TCF7 (TCF-1); TCF7L1 (TCF3); TCF7L2 (TCF4)	lef1, tcf7, tcf7l1, tcf7l2	pan	pop-1
RNF43; ZNRF3	rnf43; znrf3	-	plr-1
R-spondin	r-spondin	-	-
Lgr	lgr	-	-
LRP5, 6	lrp5, 6	arr	-
APC, APC2	apc, apc2	apc, apc2	apr-1
AXIN1, 2	axin1, 2	axn	pry-1, axl-1
CK1α	ck1α	ck1α	kin-19
GSK3A, GSK3B	gsk3a, gsk3b	sgg	gsk-3
PP2A	pp2a	pp2a	-
β-Trcp	btrcp	slmb	lin-23

ganisms such as Gska (GSK3), CK1, Aardvark (β-catenin), and Wnt-related receptors found in *Dictyostelium discoideum*, indicating that the origin of this pathway dates back to a pre-metazoan period (62-64).

In model organisms, the core elements of the Wnt pathway are well conserved, emphasizing the importance of the pathway. Regulators of the Wnt pathway, including RNF43 and ZNRF3, are highly conserved among animal species, differing primarily in their mode of protein-protein interaction via their ectodomain. Zebisch et al. (46) reported structural study of RNF43 and ZNRF3 in complex with other Wnt signaling component including Furin-like (FU) domain of Rspo2. In that study, the authors identified that non-ligand bound ZNRF3 ectodomain ($ZNRF3_{ecto}$) could form dimer and $ZNRF3_{ecto}$ dimer become less flexible when it bound with FU domain of Rspo2 ($Rspo2_{FU}$) so that 2 : 2 form of $ZNRF3_{ecto}$-$Rspo2_{FU}$ could comprised. Interestingly, dimerization of RNF43 with Rspo was not observed. However, the binding interface between $Rspo_{FU}$ and RNF43 or ZNRF3 was essentially the same. Although ZNRF3 ectodomain share high structural similarity with RNF43 ectodomain, Peng et al. (45) demonstrated that RNF43 and ZNRF3 also have structural difference. While ZNRF3 N-terminal strands β1 and β2 form an extended β-hairpin flap, RNF43 has a flexible loop region in its N-terminal region. However, both regulators are structurally similar and belong to the PA-TM-RING family of ubiquitin ligases. This family is characterized by three domains: a N-terminal protease-associated (PA) domain, really interesting new gene (RING) domain, and C-terminal TM domain (65).

The human PA-TM-RING family comprises 12 proteins, all of which possess these domains (65) (Fig 2). The PA domain itself is a conserved sequence motif among proteases and predicted to function as a protein-protein interaction domain. The RING domain is a catalytic E3 ligase domains, and all PA-TM-RING proteins have the ability to ubiquitylate their substrates. The RING, PA, and TM domains are highly conserved among homologous proteins. The PA-TM-RING family gene GRAIL (gene related to anergy in lymphocytes) was initially identified by Anandasabapathy et al. (66) as a key regulator of T cell anergy. Following studies have demonstrated the role of PA-TM-RING family genes as a E3 ubiquitin ligase including plant homologue RMR (receptor homology-transmembranedomain-RING-H2 motif protein) in addition to RNF43 related PA-TM-RING family genes (67, 68).

In mice and humans, Wnt pathway components share a high degree of homology, including RNF43 and ZNRF3, and human PA-TM-RING family proteins or their orthologues are also conserved in mice. *Xenopus* Rnf128 and *Drosophila melanogaster's* Godzilla and Goliath belong to the same protein family and are known to be required for the trafficking of recycling endosomes. Godzilla is also implicated in the Wnt pathway, particularly in the regulation of Wingless transcytosis. Similarly, *Caenorhabditis elegans* has an orthologue of RNF43/ZNRF3, named PLR-1, which downregulates Wnt signalling by ubiquitylating *C. elegans* Fzd Wnt receptors Cfz-2, Lin-17, Mig-1, and Mom-5, thereby reducing the receptors' cell-surface levels. As neither R-spondin nor LGR4/5 are conserved in *C. elegans*, PLR-1 may have alternative regulatory mechanisms, potentially at the transcriptional level. The highly conserved nature of Wnt pathway components across species highlights its importance in animal development and evolution.

Conclusion

The Wnt signalling pathway plays a crucial role in development and the maintenance of homeostasis in adult tissues and RNF43 and its paralogue ZNRF3 plays critical role on the regulation of Wnt pathway. However, alterations in its components have been associated with various malignancies. Recent research has led to a better understanding of the Wnt signalling pathway, but there is still a need for a more profound insight into the regulators of this pathway for the development of targeted therapies for Wnt dysregulation-related malignancies.

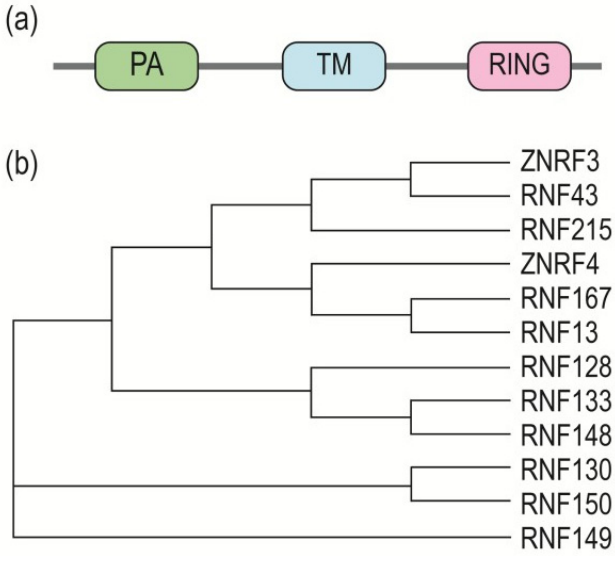

Fig. 2. PA-TM-RING family genes. (a) Domain architecture of PA-TM-RING family genes. (b) Amino acid sequence alignment of human PA-TM-RING family.

RNF43 and ZNRF3 are members of the PA-TM-RING protein family and known negative regulators of Wnt signalling. They play a significant role in the regulation of cell-surface levels of Fzd, which is a shared component of both the canonical and noncanonical pathways. Despite the recent studies, their functions in noncanonical signalling are not yet fully understood and further investigation into the impact of mutations in RNF43/ZNRF3 on malignancies will also be crucial for developing specialized treatments and predicting cancer outcomes.

Overall, a more profound understanding of the Wnt signalling pathway and its regulators will provide an opportunity for the development of more targeted therapies for malignancies caused by Wnt dysregulation. Further research on the role of RNF43/ZNRF3 in noncanonical signalling and their regulatory mechanisms is necessary to develop specialized treatments and to predict cancer outcomes.

Potential Conflict of Interest

The authors have no conflicting financial interest.

References

1. Holstein TW. The evolution of the Wnt pathway. Cold Spring Harb Perspect Biol 2012;4:a007922
2. Nüsslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. Nature 1980; 287:795-801
3. Sharma RP, Chopra VL. Effect of the wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. Dev Biol 1976;48:461-465
4. Noordermeer J, Klingensmith J, Perrimon N, Nusse R. Dishevelled and armadillo act in the Wingless signalling pathway in Drosophila. Nature 1994;367:80-83
5. Peifer M, Sweeton D, Casey M, Wieschaus E. Wingless signal and zeste-white 3 kinase trigger opposing changes in the intracellular distribution of armadillo. Development 1994;120:369-380
6. Siegfried E, Chou TB, Perrimon N. Wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. Cell 1992;71:1167-1179
7. Sokol S, Christian JL, Moon RT, Melton DA. Injected Wnt RNA induces a complete body axis in Xenopus embryos. Cell 1991;67:741-752
8. Steinhart Z, Angers S. Wnt signaling in development and tissue homeostasis. Development 2018;145:dev146589
9. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene 2017;36:1461-1473
10. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: molecular basis of colorectal cancer. N Engl J Med 2009;361:2449-2460
11. Groenewald W, Lund AH, Gay DM. The role of WNT pathway mutations in cancer development and an overview of therapeutic options. Cells 2023;12:990
12. Jung YS, Park JI. Wnt signaling in cancer: therapeutic targeting of Wnt signaling beyond β-catenin and the destruction complex. Exp Mol Med 2020;52:183-191
13. ClinicalTrials.gov. A study of LGK974 in patients with malignancies dependent on Wnt ligands [Internet]. Bethesda: U.S. National Library of Medicine; Year Mon date [cited 2023 May 22]. Available from: https://clinicaltrials.gov/ct2/show/NCT01351103
14. Krishnamurthy N, Kurzrock R. Targeting the Wnt/beta-catenin pathway in cancer: update on effectors and inhibitors. Cancer Treat Rev 2018;62:50-60
15. Koo BK, Spit M, Jordens I, Low TY, Stange DE, van de Wetering M, van Es JH, Mohammed S, Heck AJ, Maurice MM, Clevers H. Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. Nature 2012;488:665-669
16. Hao HX, Jiang X, Cong F. Control of Wnt receptor turnover by R-spondin-ZNRF3/RNF43 signaling module and its dysregulation in cancer. Cancers (Basel) 2016;8:54
17. Qin K, Yu M, Fan J, Wang H, Zhao P, Zhao G, Zeng W, Chen C, Wang Y, Wang A, Schwartz Z, Hong J, Song L, Wagstaff W, Haydon RC, Luu HH, Ho SH, Strelzow J, Reid RR, He TC, Shi LL. Canonical and noncanonical Wnt signaling: multilayered mediators, signaling mechanisms and major signaling crosstalk. Genes Dis 2023 doi: 10.1016/j.gendis.2023.01.030 [Epub ahead of print]
18. Chae WJ, Bothwell ALM. Canonical and non-canonical Wnt signaling in immune cells. Trends Immunol 2018;39: 830-847
19. Bugter JM, Fenderico N, Maurice MM. Mutations and mechanisms of Wnt pathway tumour suppressors in cancer. Nat Rev Cancer 2021;21:5-21
20. Munemitsu S, Albert I, Souza B, Rubinfeld B, Polakis P. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc Natl Acad Sci U S A 1995;92:3046-50
21. Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P. Binding of GSK3β to the APC-β-catenin complex and regulation of complex assembly. Science 1996;272: 1023-1026
22. Rubinfeld B, Souza B, Albert I, Müller O, Chamberlain SH, Masiarz FR, Munemitsu S, Polakis P. Association of the APC gene product with β-catenin. Science 1993;262:1731-1734
23. Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X. Control of β-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 2002;108: 837-847
24. Hart M, Concordet JP, Lassot I, Albert I, del los Santos R, Durand H, Perret C, Rubinfeld B, Margottin F, Benarous R, Polakis P. The F-box protein β-TrCP associates with phosphorylated β-catenin and regulates its activity in the cell. Curr Biol 1999;9:207-210
25. Amit S, Hatzubai A, Birman Y, Andersen JS, Ben-Shushan

E, Mann M, Ben-Neriah Y, Alkalay I. Axin-mediated CKI phosphorylation of β-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev 2002;16:1066-1076
26. Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP, He X. LDL-receptor-related proteins in Wnt signal transduction. Nature 2000;407:530-535
27. Nusse R, Clevers H. Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell 2017;169:985-999
28. Giannakis M, Hodis E, Jasmine Mu X, Yamauchi M, Rosenbluh J, Cibulskis K, Saksena G, Lawrence MS, Qian ZR, Nishihara R, Van Allen EM, Hahn WC, Gabriel SB, Lander ES, Getz G, Ogino S, Fuchs CS, Garraway LA. RNF43 is frequently mutated in colorectal and endometrial cancers. Nat Genet 2014;46:1264-1266
29. Sugiura T, Yamaguchi A, Miyamoto K. A cancer-associated RING finger protein, RNF43, is a ubiquitin ligase that interacts with a nuclear protein, HAP95. Exp Cell Res 2008; 314:1519-1528
30. Hao HX, Xie Y, Zhang Y, Charlat O, Oster E, Avello M, Lei H, Mickanin C, Liu D, Ruffner H, Mao X, Ma Q, Zamponi R, Bouwmeester T, Finan PM, Kirschner MW, Porter JA, Serluca FC, Cong F. ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. Nature 2012;485:195-200
31. Bian J, Dannappel M, Wan C, Firestein R. Transcriptional regulation of Wnt/β-catenin pathway in colorectal cancer. Cells 2020;9:2125
32. Tsukiyama T, Koo BK, Hatakeyama S. Post-translational Wnt receptor regulation: is the fog slowly clearing?: the molecular mechanism of RNF43/ZNRF3 ubiquitin ligases is not yet fully elucidated and still controversial. Bioessays 2021; 43:e2000297
33. Tsukiyama T, Fukui A, Terai S, Fujioka Y, Shinada K, Takahashi H, Yamaguchi TP, Ohba Y, Hatakeyama S. Molecular role of RNF43 in canonical and noncanonical Wnt signaling. Mol Cell Biol 2015;35:2007-2023
34. Carmon KS, Gong X, Lin Q, Thomas A, Liu Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling. Proc Natl Acad Sci U S A 2011;108:11452-11457
35. de Lau W, Barker N, Low TY, Koo BK, Li VS, Teunissen H, Kujala P, Haegebarth A, Peters PJ, van de Wetering M, Stange DE, van Es JE, Guardavaccaro D, Schasfoort RB, Mohri Y, Nishimori K, Mohammed S, Heck AJ, Clevers H. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature 2011;476:293-297
36. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 2007;449: 1003-1007
37. Barker N, Rookmaaker MB, Kujala P, Ng A, Leushacke M, Snippert H, van de Wetering M, Tan S, Van Es JH, Huch M, Poulsom R, Verhaar MC, Peters PJ, Clevers H. Lgr5(+ ve) stem/progenitor cells contribute to nephron formation during kidney development. Cell Rep 2012;2:540-552
38. de Visser KE, Ciampricotti M, Michalak EM, Tan DW, Speksnijder EN, Hau CS, Clevers H, Barker N, Jonkers J. Developmental stage-specific contribution of LGR5(+) cells to basal and luminal epithelial lineages in the postnatal mammary gland. J Pathol 2012;228:300-309
39. Snippert HJ, Haegebarth A, Kasper M, Jaks V, van Es JH, Barker N, van de Wetering M, van den Born M, Begthel H, Vries RG, Stange DE, Toftgård R, Clevers H. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science 2010;327:1385-1389
40. Wang Y, Dong J, Li D, Lai L, Siwko S, Li Y, Liu M. Lgr4 regulates mammary gland development and stem cell activity through the pluripotency transcription factor Sox2. Stem Cells 2013;31:1921-1931
41. Xie Y, Zamponi R, Charlat O, Ramones M, Swalley S, Jiang X, Rivera D, Tschantz W, Lu B, Quinn L, Dimitri C, Parker J, Jeffery D, Wilcox SK, Watrobka M, LeMotte P, Granda B, Porter JA, Myer VE, Loew A, Cong F. Interaction with both ZNRF3 and LGR4 is required for the signalling activity of R-spondin. EMBO Rep 2013;14:1120-1126
42. Kim KA, Kakitani M, Zhao J, Oshima T, Tang T, Binnerts M, Liu Y, Boyle B, Park E, Emtage P, Funk WD, Tomizuka K. Mitogenic influence of human R-spondin1 on the intestinal epithelium. Science 2005;309:1256-1259
43. Kazanskaya O, Glinka A, del Barco Barrantes I, Stannek P, Niehrs C, Wu W. R-Spondin2 is a secreted activator of Wnt/β-catenin signaling and is required for Xenopus myogenesis. Dev Cell 2004;7:525-534
44. de Lau W, Peng WC, Gros P, Clevers H. The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. Genes Dev 2014;28:305-316
45. Peng WC, de Lau W, Madoori PK, Forneris F, Granneman JC, Clevers H, Gros P. Structures of Wnt-antagonist ZNRF3 and its complex with R-spondin 1 and implications for signaling. PLoS One 2013;8:e83110
46. Zebisch M, Xu Y, Krastev C, MacDonald BT, Chen M, Gilbert RJ, He X, Jones EY. Structural and molecular basis of ZNRF3/RNF43 transmembrane ubiquitin ligase inhibition by the Wnt agonist R-spondin. Nat Commun 2013; 4:2787
47. Jiang X, Charlat O, Zamponi R, Yang Y, Cong F. Dishevelled promotes Wnt receptor degradation through recruitment of ZNRF3/RNF43 E3 ubiquitin ligases. Mol Cell 2015;58:522-533
48. Lebensohn AM, Rohatgi R. R-spondins can potentiate WNT signaling without LGRs. Elife 2018;7:e33126
49. Szenker-Ravi E, Altunoglu U, Leushacke M, Bosso-Lefèvre C, Khatoo M, Thi Tran H, Naert T, Noelanders R, Hajamohideen A, Beneteau C, de Sousa SB, Karaman B, Latypova X, Başaran S, Yücel EB, Tan TT, Vlaminck L, Nayak SS, Shukla A, Girisha KM, Le Caignec C, Soshnikova N, Uyguner ZO, Vleminckx K, Barker N, Kayserili H, Reversade B. RSPO2 inhibition of RNF43 and ZNRF3 governs limb development independently of LGR4/5/6. Nature 2018;557:564-569

50. Schulte G, Bryja V. The frizzled family of unconventional G-protein-coupled receptors. Trends Pharmacol Sci 2007;28:518-525
51. Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R. A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature 1996;382:225-230
52. Tauriello DV, Jordens I, Kirchner K, Slootstra JW, Kruitwagen T, Bouwman BA, Noutsou M, Rüdiger SG, Schwamborn K, Schambony A, Maurice MM. Wnt/β-catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. Proc Natl Acad Sci U S A 2012;109:E812-E820
53. Umbhauer M, Djiane A, Goisset C, Penzo-Méndez A, Riou JF, Boucaut JC, Shi DL. The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/beta-catenin signalling. EMBO J 2000;19:4944-4954
54. Ko SB, Mihara E, Park Y, Roh K, Kang C, Takagi J, Bang I, Choi HJ. Functional role of the frizzled linker domain in the Wnt signaling pathway. Commun Biol 2022;5:421
55. Spit M, Fenderico N, Jordens I, Radaszkiewicz T, Lindeboom RG, Bugter JM, Cristobal A, Ootes L, van Osch M, Janssen E, Boonekamp KE, Hanakova K, Potesil D, Zdrahal Z, Boj SF, Medema JP, Bryja V, Koo BK, Vermeulen M, Maurice MM. RNF43 truncations trap CK1 to drive niche-independent self-renewal in cancer. EMBO J 2020;39:e103932
56. Tsukiyama T, Zou J, Kim J, Ogamino S, Shino Y, Masuda T, Merenda A, Matsumoto M, Fujioka Y, Hirose T, Terai S, Takahashi H, Ishitani T, Nakayama KI, Ohba Y, Koo BK, Hatakeyama S. A phospho-switch controls RNF43-mediated degradation of Wnt receptors to suppress tumorigenesis. Nat Commun 2020;11:4586
57. Ci Y, Li X, Chen M, Zhong J, North BJ, Inuzuka H, He X, Li Y, Guo J, Dai X. SCFβ-TRCP E3 ubiquitin ligase targets the tumor suppressor ZNRF3 for ubiquitination and degradation. Protein Cell 2018;9:879-889
58. Chang LS, Kim M, Glinka A, Reinhard C, Niehrs C. The tumor suppressor PTPRK promotes ZNRF3 internalization and is required for Wnt inhibition in the Spemann organizer. Elife 2020;9:e51248
59. Kim M, Reinhard C, Niehrs C. A MET-PTPRK kinase-phosphatase rheostat controls ZNRF3 and Wnt signaling. Elife 2021;10:e70885
60. Martin BL, Kimelman D. Wnt signaling and the evolution of embryonic posterior development. Curr Biol 2009;19:R215-R219
61. Loh KM, van Amerongen R, Nusse R. Generating cellular diversity and spatial form: Wnt signaling and the evolution of multicellular animals. Dev Cell 2016;38:643-655
62. Eichinger L, Pachebat JA, Glöckner G, Rajandream MA, Sucgang R, Berriman M, Song J, Olsen R, Szafranski K, Xu Q, Tunggal B, Kummerfeld S, Madera M, Konfortov BA, Rivero F, Bankier AT, Lehmann R, Hamlin N, Davies R, Gaudet P, Fey P, Pilcher K, Chen G, Saunders D, Sodergren E, Davis P, Kerhornou A, Nie X, Hall N, Anjard C, Hemphill L, Bason N, Farbrother P, Desany B, Just E, Morio T, Rost R, Churcher C, Cooper J, Haydock S, van Driessche N, Cronin A, Goodhead I, Muzny D, Mourier T, Pain A, Lu M, Harper D, Lindsay R, Hauser H, James K, Quiles M, Madan Babu M, Saito T, Buchrieser C, Wardroper A, Felder M, Thangavelu M, Johnson D, Knights A, Loulseged H, Mungall K, Oliver K, Price C, Quail MA, Urushihara H, Hernandez J, Rabbinowitsch E, Steffen D, Sanders M, Ma J, Kohara Y, Sharp S, Simmonds M, Spiegler S, Tivey A, Sugano S, White B, Walker D, Woodward J, Winckler T, Tanaka Y, Shaulsky G, Schleicher M, Weinstock G, Rosenthal A, Cox EC, Chisholm RL, Gibbs R, Loomis WF, Platzer M, Kay RR, Williams J, Dear PH, Noegel AA, Barrell B, Kuspa A. The genome of the social amoeba Dictyostelium discoideum. Nature 2005;435:43-57
63. Prabhu Y, Eichinger L. The Dictyostelium repertoire of seven transmembrane domain receptors. Eur J Cell Biol 2006;85:937-946
64. Harwood AJ. Dictyostelium development: a prototypic Wnt pathway? Methods Mol Biol 2008;469:21-32
65. Cai C, Tang YD, Zhai J, Zheng C. The RING finger protein family in health and disease. Signal Transduct Target Ther 2022;7:300
66. Anandasabapathy N, Ford GS, Bloom D, Holness C, Paragas V, Seroogy C, Skrenta H, Hollenhorst M, Fathman CG, Soares L. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. Immunity 2003;18:535-547
67. Wang H, Rogers JC, Jiang L. Plant RMR proteins: unique vacuolar sorting receptors that couple ligand sorting with membrane internalization. FEBS J 2011;278:59-68
68. Zebisch M, Jones EY. ZNRF3/RNF43--a direct linkage of extracellular recognition and E3 ligase activity to modulate cell surface signalling. Prog Biophys Mol Biol 2015;118:112-118