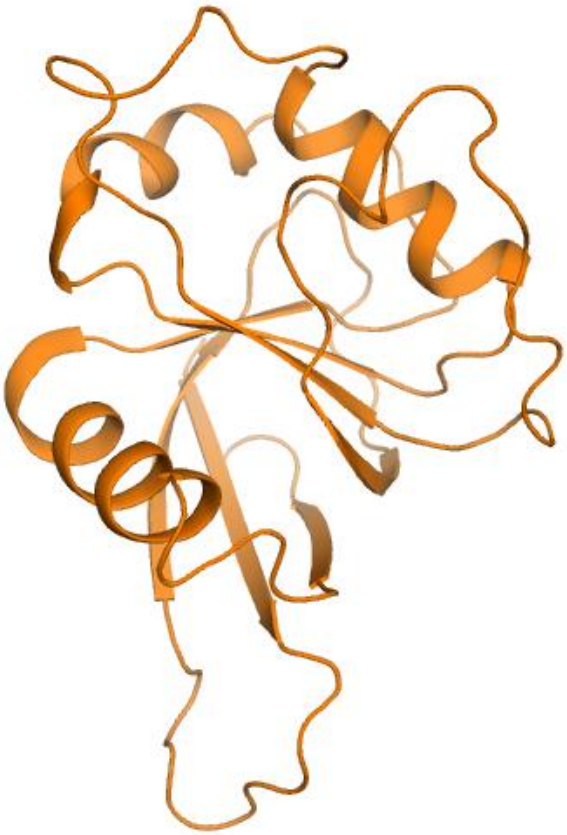
The role of receptor-associated E3 ligases in WNT signaling and disease

By Lotte Bruens



Master Thesis

December 2nd, 2013 – February 13th, 2014

Hubrecht Institute
Developmental Biology and Stem Cell Research

Cancer, Stem cells & Developmental biology

Universiteit Utrecht

Front picture: Crystal structure of extracellular domain of RNF43.

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Index

Abstract

The WNT target gene LGR5 was initially identified as a marker of adult intestinal stem cells. Later, it was found to also mark stem cells in other organs, including the pancreas, stomach, mammary gland, liver, and kidney. LGR5 and its homologues LGR4 and LGR6 are receptors for R-spondins. Their WNT signaling-enhancing ability is an essential requirement for WNT-driven adult stem cells. The LGR5/R-spondin complexes function through neutralizing RNF43 and ZNRF3, two transmembrane RING E3 ubiquitin ligases expressed in stem cells that target the WNT receptor Frizzled for degradation. RNF43 and ZNRF3 themselves are also WNT target genes and thus constitute a negative feedback loop. For RNF43 and ZNRF3 to function, they most likely should be able to interact with both R-spondin and the WNT/Frizzled complex. The binding to R-spondin, mediated by their extracellular PA domain has been described in great detail. RNF43/ZNRF3 binding to Frizzled, however, has not been fully studied. Studies performed with PLR-1, the ortholog of RNF43 and ZNRF3 in *C. elegans*, strongly indicate an involvement of the PA domains of these E3 ligases in Frizzled recognition. Studies performed with lymphocyte-expressed GRAIL, an E3 ligase with similar domain composition, also suggest that the PA domain is required to direct the substrate specificity of the ubiquitin ligase modification. In the case of RNF43 and ZNRF3 this may suggest a competitive binding model for the PA domain, either binding to R-spondin or to Frizzled. Mapping of somatic mutations in the *RNF43* gene, found in the COSMIC cancer database, reveals a surprisingly high number within the PA domain. Mutations affecting the level of RNF43 protein or the presumed ability of its PA domain to interact with the WNT/Frizzled complex may result in hyper activated WNT signaling and initiation of cancer. Such phenomena were reported for mouse experiments upon deletion of both E3 ligases. Aberrant WNT signaling due to mutated APC or ß-catenin drives tumorigenesis in almost 90% of the colon cancer patients. RNF43 and or ZNRF3 may function as tumor suppressors and mutated versions may contribute to colon cancer. The same may apply to other tissues, since a high incidence of RNF43 mutations can be detected in tumors of the pancreas, biliary tract, ovary and endometrium.

Introduction

The WNT signaling pathway is one of the major instructive players during animal development. Over the last two decades we have begun to appreciate that signals derived from this signal transduction system also drive stem cells in various tissues at the adult stage. Most of this knowledge was obtained in the study of the regenerative capacity of the intestinal epithelial layer. Mutations, moreover, in the genes encoding components of this canonical WNT pathway appeared responsible for the initiation of tumors in this tissue and in other WNT-driven regenerative stem cell-based systems. This thesis will first provide a general description of the cell renewal activity in the epithelial layer. The second part will focus on two RING domain-type E3 ligases: RNF43 and ZNRF3. Recent reports have uncovered these plasma membrane receptors as components of a delicate negative feed-back loop of the WNT pathway. Mutated versions of these proteins, affecting their WNT-regulatory role, seem involved in the biology of tumors of various tissues.

Chapter 1.

Self-renewal of the intestinal crypt

In 1982, the *WNT1* gene, then named *Int1*, was first identified in virally induced mouse breast tumors, since the gene was activated by the integration of proviral DNA of the mouse mammary tumor virus[1]. A little later it was shown that *WNT1* is a homolog of the *Wingless* (*Wg*) gene in *Drosophila*[2]. Because it had already been shown that Wg is involved segment polarity and body axis formation during larval development[3], it was thought that WNT1 would also be involved in embryonic development. A study injecting mouse WNT1 mRNA in early frog embryos indeed confirmed this role, since this injection led to a duplication of the body axis[4]. The information obtained by using this assay in combination with the studies in *Drosophila*, revealed that the WNT pathway is a highly conserved signaling cascade involved in development, commonly called the canonical WNT pathway. The following years, Frizzleds were identified as WNT receptors[5], in combination with LRPs/Arrows as co-receptors[6], and TCF/LEF transcription factors were identified as WNT nuclear effectors[7,8]. See **Figure 1** for the canonical WNT pathway.

In addition to the role of WNT signaling in development, it has later been shown that the pathway also plays an important role in regulating the maintenance, self-renewal and differentiation of mammalian adult stem cells. This role was first described for the intestinal epithelium.

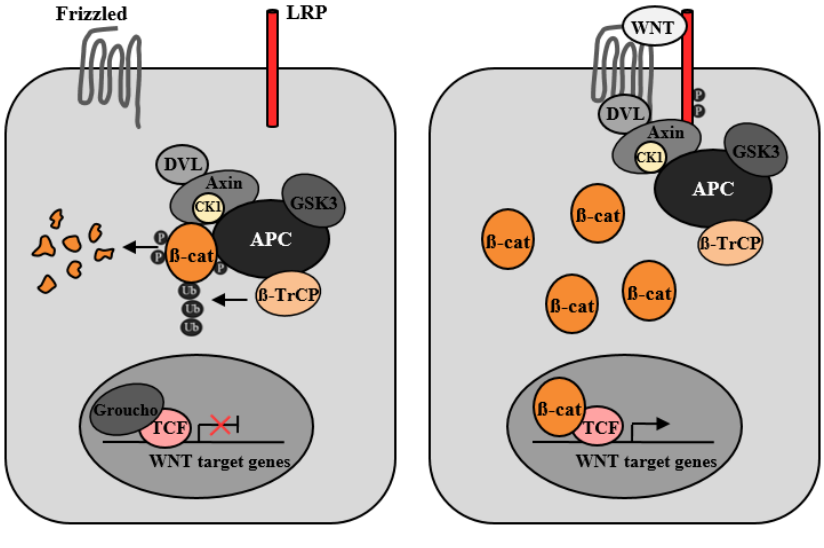

Figure 1. Canonical WNT signaling. In absence of a WNT signal, free cytoplasmic ß-catenin is targeted for degradation by the so-called destruction complex. This complex consists of two scaffolding proteins (APC and AXIN2), which bind to ß-catenin, two kinases (CK1 and GSK3ß) that sequentially phosphorylate ß-catenin at a set of highly conserved Thr and Ser residues, and the E3 ubiquitin ligase ß-TrCP that ub- iquitinates phosphorylated ß-catenin. This ubiquitination targets ß-catenin for degradation by the proteasome, resulting in the absence of cytoplasmic ß-catenin. In absence of ß-catenin, members of the TCF/LEF family together with transcriptional co-repressors (e.g. Groucho) repress WNT target gene expression. In presence of WNT signaling, WNT binds to its receptor Frizzled and the co-receptor LRP. This leads to DVL-mediated disruption of the destruction complex. Consequently, ß-catenin is able to accumulate in the cytoplasm and it can enter the nucleus. There it binds to TCF/LEF family members, converting them into transcriptional activators leading to WNT target gene expression.

WNT signaling in the intestinal epithelial layer

The intestinal epithelium is a single-cell layer that forms the barrier between the intestinal lumen and the rest of the body. It undergoes continuous stress due to the hazardous biological and chemical contents of the intestinal lumen. This may be the reason why it completely self-renews every 4-5 days, making it the fastest proliferating tissue in adult animals[9]. The epithelium of the small intestine is folded into repetitive units of villi and crypts of Lieberkuhn. Villi protrude into the intestinal lumen and contain differentiated cells that together perform food processing. Crypts of Lieberkühn are invaginations into the connective tissue that connect the villi at their base (**Figure 2**). This progenitor compartment produces all cell types that are required in the villi. The crypt harbors stem cells that reside at the bottom, and these stem cells are surrounded by Paneth cells. These Paneth cells are immune cells that have a niche function towards the stem cells[10]. The stem cells give rise to transit amplifying (TA) cells that rapidly divide 4-5 times. The TA cells start differentiating when crossing the crypt-villus boundary[11]. They predominantly differentiate into enterocytes, while a minority becomes goblet, enteroendocrine or Paneth cells. The Paneth cells migrate downwards to finally settle next to stem cells, while the other differentiated cells perform their specific functions while moving along the villus towards the tip. Upon arrival, they undergo apoptosis and are shed into the lumen.

This process of proliferation and differentiation is orchestrated by a small number of highly conserved signaling pathways, including Notch signaling pathways and the canonical WNT cascade. As mentioned earlier, this role for WNT signaling in the intestine was the first evidence that WNT also plays a role in adult tissues. In 1998, Clevers and colleagues found that the deletion of TCF4, the nuclear effector of WNT signaling, in mice completely blocks intestinal crypt development[12]. The same phenotype was observed when WNT signaling was blocked using the secreted WNT antagonist Dickkopf-1 (DKK1)[13,14]. Similarly, the induced deletion of β-catenin encoding genes[15] or TCF4[16] completely stopped crypt proliferation.

While inhibiting WNT signaling blocks self-renewal and proliferation, an uncontrolled increase of WNT signaling leads to hyperproliferation and ultimately cancer. The latter was first observed when the tumor suppressor Adenomatous Polyposis Coli (APC), a negative regulator of β-catenin stability, was found to be frequently (~80%) mutated in colon cancer. In absence of WNT signaling, the destruction complex including APC binds to β-catenin[17] and targets it for degradation. However, when WNT signaling is activated, the destruction complex becomes inactivated and no β-catenin degradation occurs. Very specific mutations in the core proteins of this degradation complex allow β-catenin to accumulate even in absence of WNT. β-catenin can then enter the nucleus and inappropriately activate WNT target genes[18]. Inactivating mutations in another destruction complex protein, AXIN2[19], and activating point mutations in β-catenin can also lead to aberrant WNT signaling and cancer[20,21]. WNT activating mutations are thought to account for nearly all cases of colon cancer[22].

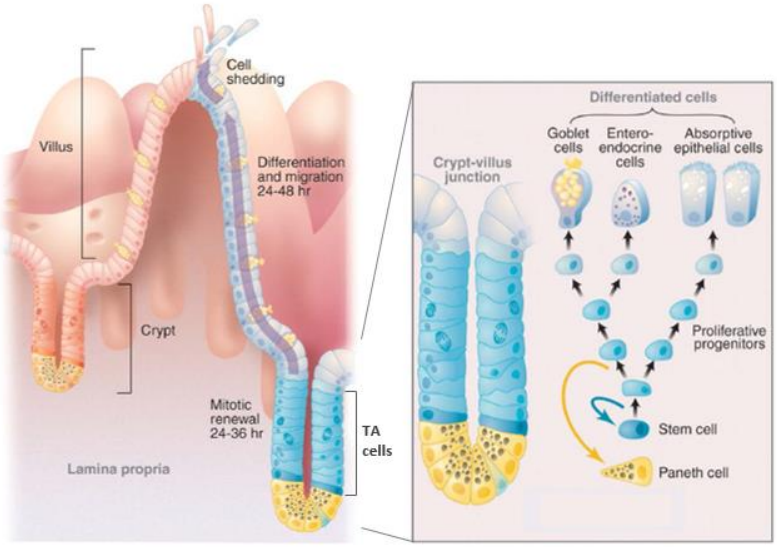

Figure 2. The structure of intestinal epithelium. The intestinal epithelium is dramatically enlarged by the formation of villi, which contain the different differentiated cell types necessary for nutrient absorption. At the bottom of these villi, the crypts of Lieberkühn are located, which contain the stem cells and their niche cells, the Paneth cells. These stem cells differentiate into fast proliferating transit amplifying (TA) cells. These TA cells differentiate into the different cell types, while moving upwards along the villus. Upon arrival at the tip, they undergo apoptosis and are shed into the lumen. (Courtesy of Wim de Lau)

LGR5 labels CBC cells

Since WNT signaling appeared to be such an important pathway in intestinal homeostasis and cancer, a micro-array was done in 2002 to determine the WNT-driven genetic program that is inappropriately activated in APC-mutant colon cancer cells[23]. The results showed that the program contains a core of approximately 80 WNT/TCF4 target genes[24]. The majority of these genes is expressed in the proliferating TA cells, while only a handful are specifically expressed in the postmitotic Paneth cells.

One TCF4 target gene, Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), especially drew the researchers' attention, because it was only expressed in the small, cycling cells in between the Paneth cells. Already in 1887, these so-called 'crypt base columnar' (CBC) cells were identified by Paneth[25] and in 1974 they were described in more detail using electron microscopy[26]. Already then, it was hypothesized that these cells represent the intestinal stem cells. However, these cells were forgotten until their rediscovery through the specific LGR5 expression. This CBC cell-specific LGR5 expression was confirmed using GFP- and LacZ-knock-in mouse models. In addition, an elegant lineage tracing experiment, using an LGR5 locus-specific CreERT2 and a Rosa26-LacZ Cre reporter mouse, provided definite proof for the stemness of CBC cells. Within five days after Cre induction, blue LacZ 'ribbons' spanned the whole crypt-villus axis and this persisted throughout life[27]. These ribbons included all differentiated intestinal cell types. Taken together, these studies showed that $LGR5^+$ CBC cells are long-lived, multipotent stem cells. Thus, revealing the stem cell identity of the CBC cells in the intestinal epithelium as was first suggested almost 40-years ago.

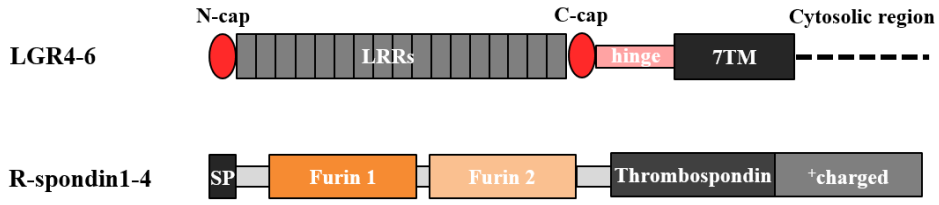

Figure 3. Linear structure of LGR4-6 and R-spondin1-4. (a) The extracellular domain (ECD) of LGR4-6 consists of leucine-rich repeats (LRRs) flanked by cysteine-rich caps. A hinge region links the LRRs to the 7TM domain. (b) R-spondins are characterized by two Furin domains in the ECD. A thrombospondin and a positively charged domain are located C-terminally.

Using similar lineage tracing strategies, LGR5 was subsequently also found to be specifically expressed in stem cells of many other organs and tissues, including the pancreas[28], stomach[29], liver[30], mammary gland[31], kidney[32] and hair follicles[33]. Thus, the WNT target gene LGR5 has been found to mark constitutively active stem cells and these findings led to the next big question: are LGRs important for stem cell function? Also, what are these receptors and how do they function?

Structure and function of LGR5

LGR4/5/6 belong to a subgroup of eight LGR receptors within the superfamily of Rhodopsin G-protein coupled receptors (GPCRs). Like all GPCRs, they contain a seven transmembrane (7TM) domain. In addition, all LGR receptors are characterized by a large extracellular domain that consist of a string of Leucin-Rich Repeats (LRRs), flanked by cysteine-rich sequences (**Figure 3**). Other members of this family include the hormone receptors LGR1 or Follicle Stimulating Hormone Receptor (FSHR), LGR2 or Luteinizing Hormone Receptor (LHR), and LGR3 or Thyroid Stimulating Hormone Receptor (TSHR). Upon hormone binding to the leucine-rich N-terminal ectodomain of these receptors, a conformational change of the 7TM region takes place which subsequently leads to G-protein-driven elevation of intracellular cAMP[34]. This mechanism of action, however, could never be confirmed for LGR4/5/6[35].

Until 2007, very little was known about the functional role of mammalian LGR5 in adult tissues. It had been recognized as a WNT target, due to its overexpression in colon cancer[36]. This overexpression was also observed in tumors of the ovary and the liver, possibly also due to aberrant WNT pathway activating[37-39]. The identification of LGR5 as an intestinal stem cell marker, however, prompted intense research to its ligand and putative signaling role.

LGR 4,-5,-6 R-spondin signaling

In 2011, R-spondins were identified as ligands for the LGR4-6. Initial studies claimed that R-spondins served as ligands for FZD[40], LRP6[41], or the Kremen receptor[41]. Clevers and colleagues performed mass spectrometry on surface receptors that were captured by tagged RSPO1 in HEK293T cells[42]. This identified LGR4 as an R-spondin receptor and a knock-down of LGR4 indeed resulted in reduced R-spondin sensitivity. LGR subfamily members

LGR4, -5 and -6 were able to rescue this defect and these three LGRs were found to bind to R-spondin with high affinity. In another study, Carmon et al. (2011) observed that tagged RSPO1 bound to LGR4 or -5 expressing cells. In addition, they observed that LGR and RSPO were internalized together upon binding[43]. Additional experiments also confirmed functionality of the LGR/R-spondin unit.

All vertebrate genomes encode four R-spondin (RSPO) proteins, small secreted proteins that contain two N-terminal cysteine-rich furin domains, a thrombospondin domain in the center of the protein and a positively charged C-terminal region. In 2004, Kazanskaya and collegues (2004) were the first to describe the role of R-spondins. By performing an expression screen for modulators of the WNT/β-catenin signaling in early frog embryos, they identified RSPO2 as a secreted agonist of this pathway[44]. When RSPO2 was depleted in a single blastomere at the 8-cell stage of an early frog embryo, the transcription of the WNT regulated muscle markers MyoD and MYF5 could not be activated, leading to impaired muscle development. These findings suggested a functional link between R-spondin activity and WNT/β-catenin signaling. In the same study, this link was confirmed in a WNT reporter assay in HeLa cells. In addition, it was shown in both Xenopus embryos and HeLa cells that R-spondins act immediately upstream of WNT proteins. Hence, the presence of the extracellular WNT antagonist Dickkopf-1 (DKK1) reduced R-spondin-driven WNT activation. In a subsequent study by Kim et al. (2005), R-spondin appeared to act as a potent growth factor for intestinal epithelial cells. Transgenic mice were generated in which RSPO1 was constitutively secreted by circulating lymphocytes. An enormous expansion was observed in the intestinal crypts of these mice. Involvement of the WNT pathway was evident by an increase phosphorylation of the Frizzled co-receptor LRP6 and stabilization of β-catenin[45]. Taken these two studies together, there could be concluded that R-spondins act immediately upstream of WNT proteins.

Chapter 2.

E3 ligases in WNT signaling

Since LGRs are GPCRs, G-protein-mediated signaling upon ligand binding was tested by monitoring β-arrestin, Ca^{2+} mobilization and cAMP production. However, no combination of R-spondins and LGRs led to G-protein activation[42,43]. Recently, the mechanism of R-spondin signaling was unraveled. Two E3 ubiquitin ligases, RNF43 and ZNRF3 present in the plasma membranes of intestinal stem cells were identified as essential signaling partners[46,47].

RNF43 and ZNRF3

In 2012, Koo et al. reported on the function of two related WNT target genes: the RING type E3 ubiquitin ligases RNF43 and ZNRF3[47]. Both proteins were shown to localize at the plasma membrane and, importantly, deletion of the genes in the intestine resulted in a marked expansion of the proliferative compartment. This proliferation coincided with high levels of β-catenin and upregulation of WNT target genes, indicating strong activation of WNT/β-catenin signaling in the absence of RNF43 and ZNRF3. A series of biochemical experiments revealed that RNF43 removes the WNT receptor Frizzled/LRP from the membrane by targeting Frizzled for degradation through ubiquitination of lysines in the 7TM domain. These findings were also reported in a study performed by Cong and colleagues[46]. They, importantly, added an unexpected new aspect on the role of the E3 ligases. A series of experiments

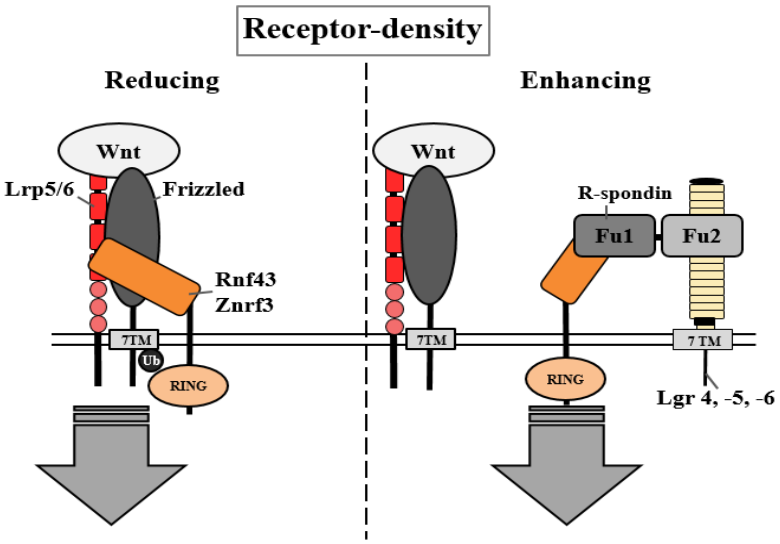

Figure 4. WNT receptor density regulation in stem cell. The Frizzled/LRP receptor complex is present on intestinal stem cells. Upon WNT binding to this complex, ~80 WNT target genes are activated, including LGR5. Two other WNT target genes are the membrane receptors RNF43 and ZNRF3, which contain an intracellular E3 ligase RING domain and an extracellular PA domain. These two receptors are part of a negative feedback mechanism (left panel). RNF43/ZNRF3 recognizes Frizzled, leading to poly-ubiquitination of the 7TM domain of Frizzled and subsequent degradation via endocytosis. This results in reduced WNT signaling. When R-spondin is present (right panel), its Furin1 domain binds to the PA domain of RNF43/ZNRF3, while its Furin2 domain binds LGR-4, -5 and -6 with high affinity. This results membrane clearance of both RNF43 and ZNRF3. In absence of RNF43 and ZNRF3, Frizzled/LRP receptor complexes can accumulate on the plasma membrane, leading to a maximized WNT signal. (Courtesy of Wim de Lau)

strongly indicated that R-spondin neutralizes the RNF43/ZNRF3-induced ubiquitination and subsequent endocytosis of Frizzled receptors[46]. R-spondin binding to LGR would enable it to also interact with RNF43/ZNRF3, resulting in membrane disappearance of the complete LGR/RNF43-ZNRF3 complex. In the absence of the E3 ligases, Frizzled receptors would accumulate at the membrane. This scenario would boost WNT signaling (**Figure 4**)

Both RNF43 and ZNRF3 belong to a class of E3 ligases with a domain composition also seen in the *Drosophila* Goliath and Godzilla proteins. All members of this class have an extracellular PA (Protease-Associated) domain, a single pass TM (transmembrane) and a cytoplasmic RING-type E3 ligase domain **(Figure 5)**. The human genome encodes 12 of these PM-TM-RING proteins and the function of most of these proteins is largely unknown. Both Goliath and Godzilla in *Drosophila*, and human RNF167 have been implicated to function in endosomal trafficking processes[48], while h-Goliath, or RNF130, has been described to be expressed in leukocytes[49]. Also, RNF13 appears to be important for cell proliferation and its expression has been associated with neural development, myogenesis and tumorigenesis[50]. The function of one family member GRAIL (or RNF128), however, has been described in more detail. GRAIL plays a major role in immune tolerance being a regulator of the expression of membrane receptors on T-lymphocytes[51]. Very recently, an ortholog of *RNF43* and *ZNRF3* was discovered in a genetic screen for mutations that affect the anteroposterior polarity of the unpaired interneuron AVG in *C. elegans*[52]. This gene was called *cell polarity defective 1* (*PLR-1*). Just like RNF43 and ZNRF3, PLR-1 is a negative regulator of WNT signaling.

Even though the function of the different family members appears to be very diverse, they all contain an extracellular PA domain. This extracellular PA domain, shared by all members, is a highly conserved ligand recognition motif found in proteases as well as in various receptors[53,54]. Different crystallographic studies confirmed that the RNF43 and ZNRF3-associated PA domains bind to LGR-bound R-spondin. Whether the PA also serves to direct the E3 ligase activity towards Frizzled is unknown but different clues will be discussed in a later paragraph.

Structure of R-spondin/LGR/RNF43-ZNRF3 binding

The exact structures of - and binding interfaces between - LGRs, R-spondins and the E3 ligases RNF43 and ZNRF3 were recently analyzed in a four independent crystallography studies.

LGR4-6

The large extracellular domain (ECD) of LGR4, -5 and -6 was found to form a horse shoe-like structure with its 17 LRRs, flanked by LGR1-type cysteine rich caps at both the C- and N-terminus. In contrast to LGR1-3 and the closely related GPCR follicle-stimulating hormone receptor (FSHR), LGR4-6 lack the hinge loop that in the other receptors is found to undergo a conformational change in the TM region upon ligand binding. These conformational changes

enables subsequent G protein coupling and the lack of the loop region in LGR4-6 may thus explain why G proteins are not essential in LGR4-6 signaling[55].

RNF43/ZNRF3

The ECDs of RNF43 and ZNRF3 contain a PA domain that adopts a distinctive PA fold. Crystal structures of the ECD of ZNRF3 show the typical β-sandwich structure with two peripheral α-helices. Two β-sheets (consisting of β2, β1, β7, β3 and β4, β5, β6 strands, respectively) accommodate an α-helix (αC) in between them[55-57]. Two additional α-helices (αA and αB) and two short 3_{10}-helices are located on the β4, β5 and β6 side of this β-sandwich, and a disulfide bond links the loop regions comprising the first and second 3_{10}-helices[56,57]. The overall fold of the ECD of RNF43 is very similar to this, with a root-mean-square deviation of 0.75Å. In addition, despite the low sequence identity of 15%, RNF128 also shows structural similarities with the ECD of RNF43 and ZNRF3[57] (**Figure 6a.**). Interestingly, ZNRF3 was found to dimerize, while RNF43 was found as monomer[56,57]. This observation was supported by the fact that the residues involved in ZNRF3 dimerization are not conserved between RNF43 and ZNRF3.

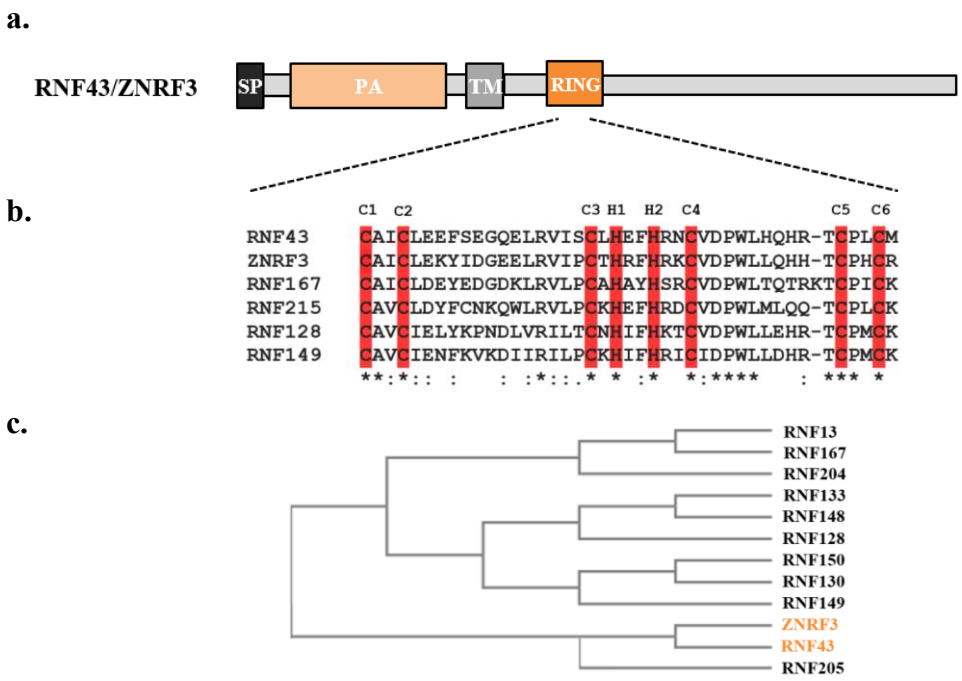

Figure 5. RNF43 and ZNRF3 encode PM-TM-RING E3 ligases. (**a.**) Schematic representation of the domains in RNF43 and ZNRF3. SP: signal peptide; PA: protease-associated domain; TM: transmembrane, and RING domain. (**b.**) Sequence alignment of RING domains of different family members. RNF128 is also known as GRAIL. The RING domain contains two conserved Histidine (H1 and H2) and six conserved Cysteine (C1-C6) residues (in red), which are important for E3 ligase activity. (**c.**) Phylogenic tree of PA-TM-RING E3 ligase family members. (Adapted from Ref. 48)

R-spondin

R-spondin contains two Furin domains, each comprised of three cysteine-knotted β-hairpins stabilized by four disulfide bridges[55,56]. The pattern of these disulphide bonds appears to be conserved between Furin domains in different proteins[58,59]. The hinge region between both Furin domains may generate differences in orientation between bound and unbound structures.

LGR/R-spondin/RNF43-ZNRF3 complex

The first furin domain (Fu_1) of R-spondin binds to RNF43/ZNRF3, while the second furin domain (Fu_2) binds to LGR4/5. This way, R-spondin forms a bridge between the two receptors without direct interaction between RNF43/ZNRF3 and LGR (**Figure 6b.**).

The Fu_1 domain binds to the ECD of RNF43 or ZNRF3. Binding of RSPO1 to the ECD of RNF43 is carried out by a β-hairpin protrusion (residue F61-L76) of Fu_1 that fits into the narrow binding groove in between the two β-sheets of RNF43[55]. In contrast to the mainly hydrophobic interaction between RSPO1 and LGR5, hydrophilic interactions form the basis of this recognition. The key residues of RNF43 that are involved in this interaction are Gln84, His86, Leu88, Asp97, Lys108 and Glu110 (**Figure 6c.**), of which all are conserved between RNF43 and ZNRF3 except for Asp97 (Glu in ZNRF3)[55,56]. The conserved RSPO1 residues in this interface are Ser48, Asn51, Cys53, Leu64, Arg66, Arg70 and Gln71 (**Figure 6c.**). R66A and Q71A mutations in Fu_1 indeed completely annul RSPO-ZNRF3 interaction[60].

Fu_2 of RSPO1 uses its first β-hairpin to bind to the LRR domains 3-9 of LGR4/5[61]. This β-hairpin contains a ^{106}FSHNF^{110} loop of which the aromatic amino acids Phe106 and Phe110 form a clamp that target Ala190 on LGR5[56]. This clamping mediates subsequent hydrophobic interactions with surrounding residues. Single mutations in this clamp, like F106E, F106A, F110E and F110A, disrupt ligand-receptor binding[60,61], showing that these residues are essential for RSPO1-LGR interaction. In addition, the essential residues on RSPO1 and LGR are highly conserved between RSPO1-4 and LGR4-6, in various species[55,56,61], explaining the observed promiscuity in receptor-ligand interaction[42].

The crystallography studies have provided us with a detailed insight in both the RSPO-LGR and RSPO-RNF43/ZNRF3 interactions. A remaining, puzzling aspect of this signaling mechanism is whether - and how - the ECD of RNF43/ZNRF3 interacts with the WNT receptor complex.

RNF43/ZNRF3 substrate recognition and modification

Understanding how other RING E3 ubiquitin ligases recognize and modify their substrate could help in the understanding how RNF43 and ZNRF3 would interact with the WNT receptor complex.

Role of RING domain in substrate modification

Ubiquitination is a three step enzymatic cascade involving three different enzymes. First, the ubiquitin (ub) unit is activated by an E1 activating enzyme. Subsequently, the active ub is conjugated to an E2 ubiquitin-carrier enzyme. Finally, the RING E3 ubiquitin ligase catalyzes the transfer of the activated ubiquitin, carried by E2, to the substrate[62]. The E3 ligase binds to the substrate and is thus responsible for the specificity of this process. This substrate recognition is performed by a domain outside the RING domain, however, the RING domain still simultaneously has to recruit both the substrate protein exposing the acceptor Lysine and an E2 enzyme, harboring the activated Ubiquitin. These E2-ub and Lys-substrate binding sites occupy independent positions within the RING domain[63].

RING-type E3 ligases can be split into three classes: monomers, dimers and multisubunit complexes. Dimerization is generally mediated by the RING domain and/or its surrounding region, and both homodimeric and heterodimeric RING E3 ligases occur. In heterodimeric operating RING E3 ligases, one partner can have an inactive RING domain that serves to stabilize the active RING domain of the other partner or helps to transfer the activated Ub from E2 to E3[64]. Within multisubunit E3 ligases, the Cullin RING ubiquitin ligases, or CRLs, comprise the largest class[65]. CRL-1 is one of these class members and it is an E3 ligase complex consisting of the scaffold Cullin1, the adaptor SKP1, RING protein Rbx1, and one of more than 70 specific substrate binding F-box proteins. One such F-box protein is β-TrCP and it is involved in the degradation of β-catenin, a process that forms an essential part of WNT signaling (see **Figure 1** for an overview of the WNT signaling pathway).

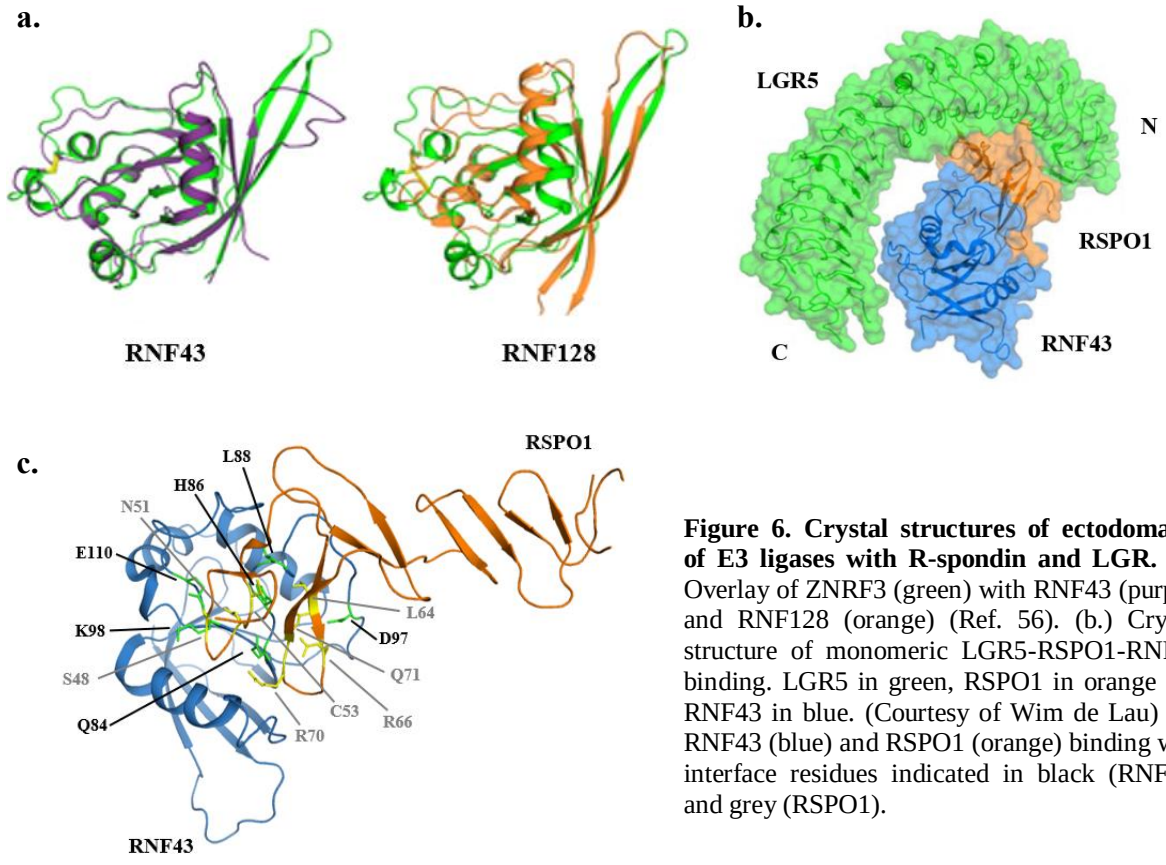

Figure 6. Crystal structures of ectodomains of E3 ligases with R-spondin and LGR. (a.) Overlay of ZNRF3 (green) with RNF43 (purple) and RNF128 (orange) (Ref. 56). (b.) Crystal structure of monomeric LGR5-RSPO1-RNF43 binding. LGR5 in green, RSPO1 in orange and RNF43 in blue. (Courtesy of Wim de Lau) (c.) RNF43 (blue) and RSPO1 (orange) binding with interface residues indicated in black (RNF43) and grey (RSPO1).

Substrate specificity in RING-type E3 ligases

Multisubunit E3 ligases, as indicated in the previous paragraph, exhibit a sophisticated mechanism for substrate binding. Firstly, the substrate recognition resides in a separate protein component. Secondly, recognition and subsequent binding are often dependent on phosphorylation of a phosphodegron sequence in the substrate protein. Usually, this phosphorylation needs the consecutive action of two different kinases, indicating a high level of regulation.

An important question is how substrate recognition is achieved by single-chain E3 ligases. In addition, the question is how these E3 ligases can recognize multiple substrates. RNF43 and ZNRF3 use their PA domains as part of R-spondin/LGR-mediated enhancement of WNT signaling. However, whether this recognition directs ubiquitination of LGRs, analogous to the function of F-box proteins, is unknown. Also, recognition of the Frizzled-LRP5/6 complex as a starting point in the ubiquitination of Frizzled awaits confirmation.

Similar to Frizzled regulation by RNF43 and ZNRF3, the family member GRAIL regulates the level of plasma membrane receptors involved in T-cell unresponsiveness[66]. Its plasma membrane-localized substrates include CD83[67], CD40L[68] and the tetraspanins CD151 and CD81[69]. For all substrates listed, the PA domain of GRAIL appears to be responsible for substrate recognition/binding. Substrate binding, through involvement of the PA domain, results in all cases in RING domain-mediated ubiquitination of residues in the cytosolic part of the substrate and subsequent proteasomal degradation of the substrate. An important issue is how this multi-substrate recognition of a single PA domain is achieved. F-box proteins, like β-TrCP, in multisubunit E3 ligases also mediate interaction with a multitude of substrate proteins, but they recognize a consensus phosphodegron that is shared by all substrates. Primary protein sequence analysis of the GRAIL substrates does not identify such similarities between the extracellular TNF-R domain of CD40L, the Ig-like domain of CD83 and the loop domains of CD81 and CD151[67]. However, topology predictions have shown that these molecules may share a secondary structural domain. Crystallography studies would be necessary to reveal these possible shared domains. Thus, the question is whether all RNF128 substrates carry a motif that is seen by the RNF128-PA domain. If so, do the RNF43/ZNRF3 substrates Frizzled and R-spondin also share a motif for RNF43/ZNRF3-PA domain recognition? Or do RNF43 and ZNRF3 have different recognition sites for Frizzled and R-spondin?

Competitive substrate recognition model for RNF43/ZNRF3

The majority of the surface-exposed residues conserved between RNF43 and ZNRF3 cluster on the R-spondin binding site, forming a single binding platform on the PA domain[56] **(Figure 7a. and b.)**. This suggests that this is the only evolutionary conserved binding platform of ZNRF3 and RNF43. Consequently, the PA domain would bind both R-spondin and the Frizzled (FZD) complex at the same or overlapping site. This notion is supported by the observation that there is only a weak interaction between R-spondin and RNF43/ZNRF3, with an affinity of 1-10μM, while R-spondin strongly binds to LGR, with an affinity of 2-5nM[42,46,55-57]. This could indicate that R-spondin and the FZD complex compete for binding with the

PA domain. This way, ZNRF3 and RNF43 could exist in equilibrium between FZD complex and R-spondin binding. This would balance ubiquitination and degradation of Frizzled, and degradation of RNF43/ZNRF3 itself leaving Frizzled on the surface.

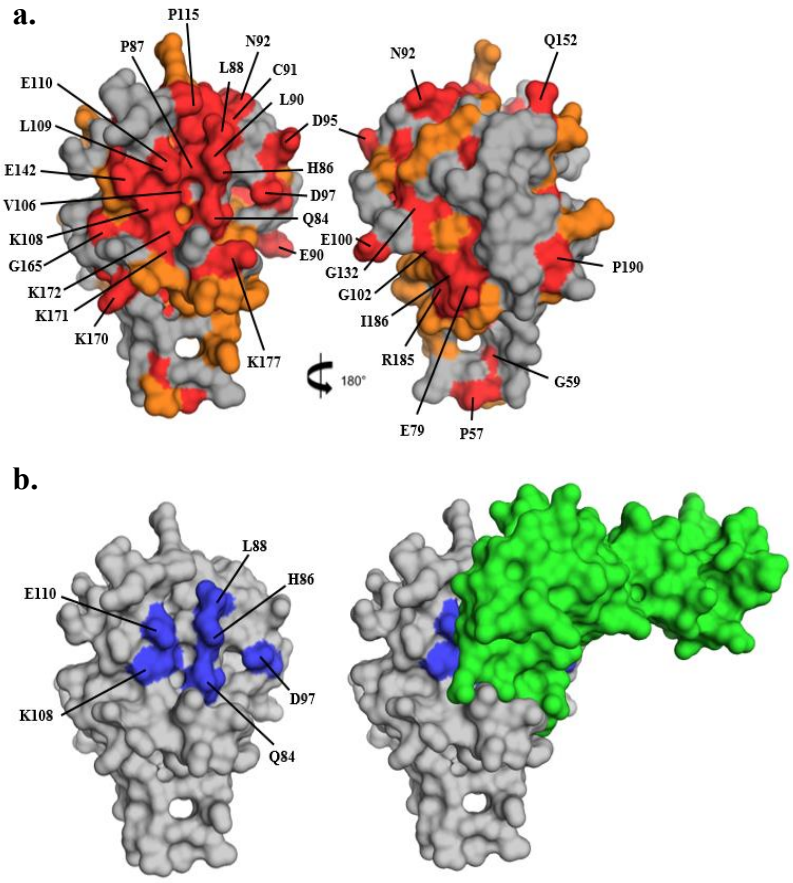

Figure 7. RSPO1 binding sites at conserved sites of RNF43. (a.) Conserved (orange) and identical (red) residues between RNF43 and ZNRF3 are shown on the surface of RNF43. Identical residues are labeled. Two views are shown (Adapted from Ref. Peng, 2013). (b.) Critical residues for RSPO1 binding are shown on the surface of RNF43 (left panel). RSPO1 (green) binding to RNF43 (right panel).

The very recent study performed on PLR-1 in *C. elegans* contributes to the plausibility of the PA domain of RNF43/ZNRF3 binding to the FZD complex. It was shown that PLR-1 uses its RING domain to ubiquitinate the invariant second intracellular loop lysine of the Frizzled orthologs CFZ-2, LIN-17, MIG-1 and MOM-5[52]. For this, both the WNT binding cysteine rich domain (CRD) of the Frizzled orthologs, and the PA domain of PLR-1 were necessary, confirming the role of the PA domain in substrate recognition.

Since R-spondin seems to be a vertebrate invention, PLR-1, in contrast to RNF43 and ZNRF3, does not have to recognize R-spondin in addition to Frizzled. However, it appears to bind to two other proteins: CAM-1 (Ror) and LIN18 (Ryk)[52]. These proteins are WNT receptors or Frizzled co-receptors[70]. Like Frizzled, Ror contains a CRD with ten conserved cysteines. Ryk contains a Frizzled-unrelated WIF domain. PLR-1's function seems to be regulating the membrane levels of Ror and Ryk, since both receptors are internalized upon PLR-1 overexpression[52]. This internalization is dependent on the presence of Frizzled receptors. It is unknown whether this Frizzled-dependent internalization is due to a physical linkage. Thus, the PA domain of PLR-1 either only binds to Frizzled, leading to passive Ryk/Ror internalization because of the direct interaction or PLR-1 binds to Frizzled and Ryk/Ror separately. For the latter option, the PA domain should be able to recognize both the CRD and the WIF domain. A detailed study is required to elucidate the mechanism underlying these observations.

Table 1. Assay for determining Frizzled complex and R-spondin binding sites.

WT RNF43/ZNRF3			**FZD-binding mutant RNF43/ZNRF3**			**RSPO-binding mutant RNF43/ZNRF3**		
	FZD	LGR		FZD	LGR		FZD	LGR
- RSPO	*Absent*	*Present*	- RSPO	*Present*	*Present*	- RSPO	*Absent*	*Present*
+ RSPO	*Present*	*Absent*	+ RSPO	*Present*	*Absent*	+ RSPO	*Absent*	*Present*

To experimentally test whether overlapping or independent binding epitopes exist in RNF43/ZNRF3 for recognition of Frizzled and/or R-spondin, an assay could be developed in which N-terminally-tagged versions of Frizzled, LGR and variants of RNF43/ZNRF3 are overexpressed. Monitoring the plasma membrane levels of Frizzled and LGR should indicate mutations affecting the binding site(s). Hereby is assumed that the R-spondin mediated tethering of LGRs and E3 ligases not only induces membrane clearance of the E3 ligases[46], but also of the LGR partner since it is co-endocytosed[71]. A mutation abolishing R-spondin binding to RNF43/ZNRF3 would still lead to Frizzled degradation in the presence of R-spondin. Yet, when a mutation would inhibit FZD complex recognition, no Frizzled degradation would occur. High Frizzled signals should be seen, even in the absence of R-spondin. See **table 1** for clarification.

Conserved domain in C-terminal region of RNF43/ZNRF3

Protein alignment of RNF43 and ZNRF3 of different vertebrate species shows that RNF43 and ZNRF3 contain a highly conserved C-terminal region (RNF43: a.a. 443-512, ZNRF3: a.a. 525-601) **(Figure 8)**. This region shows no homology with other proteins and does not contain a conserved domain sequence, according to the NCBI Conserved Domain Search. It is a serine-rich region and the high degree of conservation indicates functional importance. Remarkably, this region appears to only be present in vertebrates. One could speculate on a role in Frizzled or R-spondin recognition.

Monomeric or dimeric function of RNF43/ZNRF3

As mentioned earlier, some RING E3 ligases function as a dimer. In these dimers, one RING domain is thought to exert the E3 function, while the other RING domain locks the ub tail into an optimal conformation for transfer to the substrate[72,73]. Dou et al. (2013) asked the question how monomeric RING E3 ligases optimize the ub transfer without a second RING domain. They found that the monomeric E3 ligase CBL-B requires a domain adjacent to the conserved RING domain for optimal ub transfer[74]. A phosphorylated tyrosine in a linker helix in CBL-B appears to replace the tail of a second subunit and enhances the efficiency of catalysis by ~200-fold[74]. A similar ub-locking mechanism was found in SUMO RING E3 ligases. The SUMO ligase SIZ1 was suggested to use an acidic patch on an α-helix for ub stabilization[75], while the SUMO ligase RanBP2 used an N-terminal β-sheet to correctly position ub[76].

Two crystallography studies showed that the ECD of ZNRF3 forms a dimer[56,57], while the ECD of RNF43 was only found as a monomer. However, whether the RING domain of ZNRF3 and RNF43 functions as a dimer is unknown. It is unlikely that RNF43 and ZNRF3 would function as a heterodimer. The mouse knock-out experiments performed by Koo and colleagues (2012) strongly indicated that single deletion of RNF43 or ZNRF3 did not have any effect on intestinal stem cells[46]. If RNF43 and ZNRF3 would have complementary roles, e.g. as a heterodimer, in down regulating Frizzled, removal of either of the two E3 ligases would have affected the stem cells. In addition, the *C. elegans* PLR-1 seems to act alone, arguing also against a heterodimeric role for RNF43/ZNRF3. If RNF43 and/or ZNRF3 function as a monomer, an additional region in the protein could be present mediating Ub transfer.

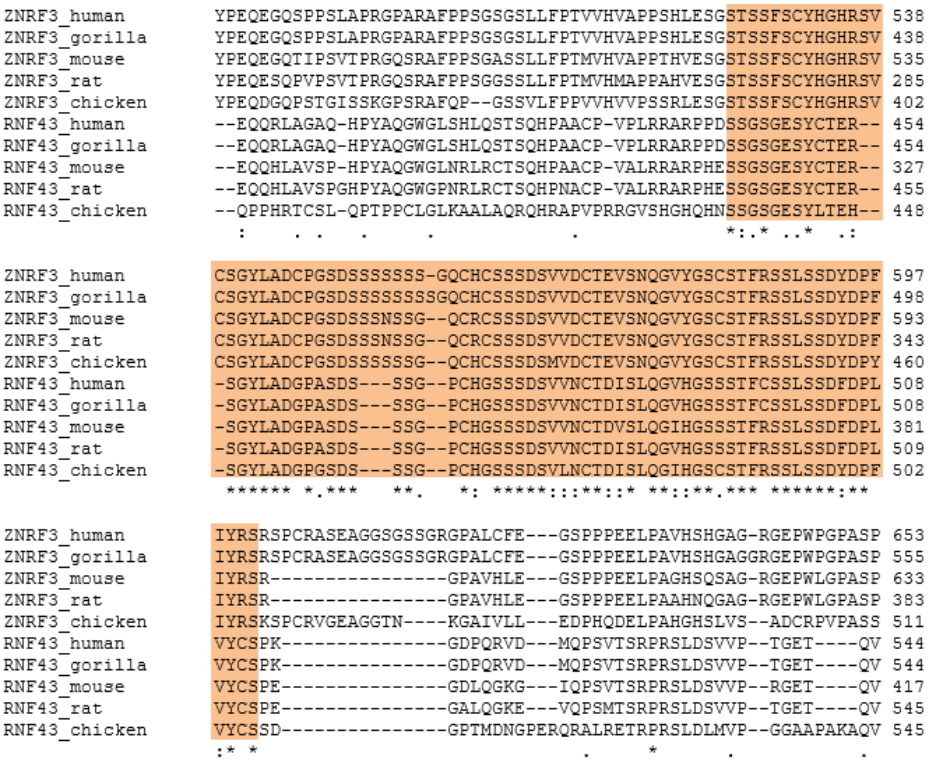

Figure 8. C-terminal conserved region of RNF43 and ZNRF3. ClustalW[119] alignment of the conserved cytoplasmic region between RNF43 and ZNRF3 of human, gorilla, mouse, rat and chicken. Conserved region is highlighted; residues 443-512 of RNF43 and residues 525-601 of ZNRF3.

Possible DUB regulation of RNF43/ZNRF3

Often, ubiquitin ligases are inactivated by autoubiquitination. This modification can be reversed by a de-ubiquitinating enzyme (DUB). The PM-TM-RING ligase family member GRAIL, for instance, is regulated by two isoforms of the DUB Otub 1 (Otub 1 and Otub 1-ARF 1) that associate with GRAIL at its coiled-coil domain[77]. Otub 1 binds to ub-GRAIL without deubiquitinating it **(Figure 9a.)**. However, it functions through blocking USP8, a DUB for ub-GRAIL. USP8 needs to be ubiquitinated to function as a DUB for GRAIL, and thus deubiquitination of USP8 by Otub 1, blocks USP8 function allowing degradation of ub-GRAIL. This process is affected by an alternative reading frame of Otub 1, Otub 1-ARF1. Otub 1-ARF1 is also able to interact with GRAIL, however it lacks DUB activity. It thus blocks Otub 1 binding and allows USP8 to deubiquitinate GRAIL[66]. RNF43 and ZNRF3 both

do not contain a coiled-coil domain that is necessary in GRAIL for Otub 1 binding **(Figure 9b.)**. However, this does not exclude regulation by DUBs. The earlier described conserved domain in the cytoplasmic tail of RNF43/ZNRF3 may function in DUB binding.

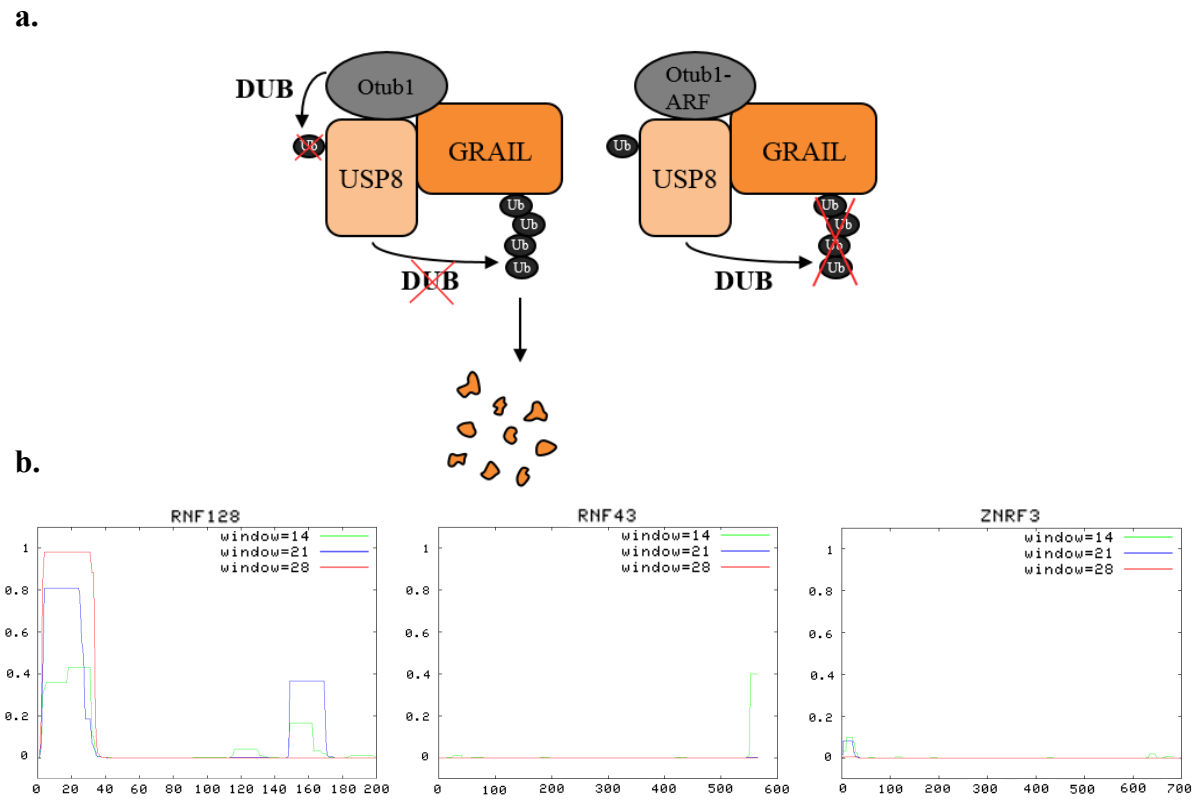

Figure 9. Regulation by DUBs at the coiled coil domain. (a.) Two isoforms of the ubiquitin-specific protease otubain 1 (Otub 1 and Otub 1-ARF) associate with GRAIL. In addition, USP8 binds to both GRAIL and Otub 1/Otub 1-ARF in a trimolecular complex. USP8 is a deubiquitinating enzyme (DUB) for the auto-ubiquitinated GRAIL. USP8 has to be ubiquitinated to be able to function. However, another DUB, Otub 1, deubiquitinates ub-USP8, which inactivates it and allows auto-ubiquitinated GRAIL to be degraded (left panel). The alternatively spliced isoform Otub 1-ARF can also bind to GRAIL, however, it cannot deubiquitinate USP8. Consequently, USP8's function is not blocked, GRAIL is deubiquitinated and it is not degraded by the proteasome (right panel). (Adapted from Ref. 77) The panels shows the output from COILS[117], a program that compares a sequence to a database of known coiled coil sequences and this results in a similarity score. This score is compared to the distribution of scores in coiled coil proteins, resulting in a probability that the sequence will adopt a coiled coil conformation. Here these scores are shown for the cytoplasmic tail of GRAIL (RNF128), RNF43 and ZNRF3.

Chapter 3.

Tumor-related somatic mutations in RNF43 and ZNRF3 genes.

Mutations leading to the complete absence of protein production or subtle mutations inactivating the RING domain activity might indicate a functional role of the protein itself in tumor initiation or maintenance. Mapping of somatic missense mutations in tumors, outside the RING domain, could provide information on the role of other known, or functionally uncharacterized, domains. Missense mutations impairing FZD complex binding, for instance, could lead to uncontrolled WNT signaling, facilitating tumor formation. Conversely, occurrence of such mutations in various tumor types may reveal involvement of WNT signaling in those tissues.

WNT pathway mutations in colon cancer

Canonical WNT signaling promotes proliferation in the intestinal crypt. Tight control of this pathway by positive- and negative-feedback loops is necessary to directly balance WNT activity and indirectly tissue homeostasis. Disruption of this WNT homeostasis by mutations in components of the pathway, have been shown responsible for the initiation of colon cancer. Such aberrations have been found in more than 90% of all colon cancers. ~80% of colon cancers carry an inactivating *APC* mutation, while ~5% carry activating *CTNNB1* (the gene encoding β-catenin) mutations in the third exon[20,78]. Remarkably, *RSPO2* and *RSPO3* gain-of-expression gene fusions are found in ~10% of colon cancers[79]. Other mutations that have been implicated to be involved in colon cancer are mutations in *AXIN, SOX9, TCF4* and *DKK* family members[80].

RNF43 and ZNRF3 are WNT target genes, acting as negative feed-back loop components of WNT signaling in stem cells by targeting Frizzled receptors for degradation. This immediately suggests the possibility that these gene products would act as tumor suppressors. Indeed, Koo et al. (2012) showed that simultaneous deletion of *RNF43* and *ZNRF3* leads to unrestricted expansion of the intestinal stem cell zone and subsequent adenoma formation[47]. In addition, *RNF43* mutations have been found in two colon cancer cell lines[81] and in a transposon-based screen for intestinal adenoma formation[82]. *RNF43* mutations also have been identified in other cancer types, e.g. in human intraductal papillary mucinous neoplasm[83] and in cholangiocarcinoma[84]. Taken together, these observations are suggestive for a tumor suppressor role of these E3 ligases.

Table 2. RNF43 mutations listed in the Sanger COSMIC database

#	Sample ID	AA mutation	CDS mutation	Type	Primary tissue	Histology subtype	Zygosity	APC	β-cat	ZNRF3	Exome seq	Ref
1	1749376	p.C119R	c.355T>C	MS	Biliary tract	Adenocarcinoma	unkn				X	84
2	1749385	p.N167I	c.500A>T	MS	Biliary tract	Adenocarcinoma	unkn				X	84
3	1749356	p.T204R	c.611C>G	MS	Biliary tract	Adenocarcinoma	unkn				X	84
4	1749364	p.R113*	c.337C>T	NS	Biliary tract	Adenocarcinoma	unkn				X	84
5	1749386	p.Q723*	c.2167C>T	NS	Biliary tract	Adenocarcinoma	unkn				X	84
6	1301775	p.A35S	c.103G>T	MS	Breast	Carcinoma, unkn	unkn				X	118
7	1899956	p.S607L	c.1820C>T	MS	Breast	Carcinoma, unkn	Hetero.				X	118
8	1927071	p.E541K	c.1621G>A	MS	CNS	Astrocytoma Grade II	unkn				X	119
9	1927072	p.E541K	c.1621G>A	MS	CNS	Astrocytoma Grade II	unkn				X	119
10	1735432	p.R650*	c.1948C>T	NS	CNS	unkn	unkn				X	120
11	1783425	p.A78T	c.232G>A	MS	Endometrium	Endometrioid carcinoma	unkn				?	?
12	1783421	p.S85F	c.254C>T	MS	Endometrium	Endometrioid carcinoma	unkn				?	?
13	1783488	p.H183R	c.548A>G	MS	Endometrium	Endometrioid carcinoma	unkn				?	?
14	1783423	p.R286W	c.856C>T	MS	Endometrium	Endometrioid carcinoma	unkn				?	?
15	1783534[a]	p.Y357C	c.1070A>G	MS	Endometrium	Endometrioid carcinoma	unkn		X		?	?
16	1783470	p.S607L	c.1820C>T	MS	Endometrium	Endometrioid carcinoma	unkn	X			?	?
17	1783534[a]	p.L647I	c.1939C>A	MS	Endometrium	Endometrioid carcinoma	unkn		X		?	?
18	1783357	p.Q22*	c.64C>T	NS	Endometrium	Endometrioid carcinoma	unkn				?	?
19	1783366	p.P587S	c.1759C>T	MS	Endometrium	Endometrioid carcinoma	Hetero.	X			?	?
20	1998457	p.G102E	c.305G>A	MS	Haematopoietic	ALL	Hetero.				X	121
21	1998488	p.V107L	c.319G>C	MS	Haematopoietic	Hairy cell leukaemia	Hetero.				X	121
22	1780023	p.M173T	c.518T>C	MS	Kidney	CCRCC	unkn				?	?
23	1779986	p.S532F	c.1595C>T	MS	Kidney	CCRCC	unkn				?	?
24	1998442	p.R117fs*41	c.344delC	Del	Colon	Carcinoma, unkn	Homo.		X		X	121
25	1651033	p.G659fs*41	c.1976delG	Del	Colon	Adenocarcinoma	unkn	X			X	108
26	1766779	p.G659fs*41	c.1976delG	Del	Colon	Adenocarcinoma	unkn				X	79
27	1651071	p.P441fs*2	c.1321_1322insA	Ins	Colon	Adenocarcinoma	unkn	X			X	108
28	1651030	p.I48T	c.143T>C	MS	Colon	Adenocarcinoma	unkn	X			X	108
29	1651648	p.L82S	c.245T>C	MS	Colon	Adenocarcinoma	unkn	X			X	108
30	1651015	p.M83T	c.248T>C	MS	Colon	Adenocarcinoma	unkn				X	108
31	1766803	p.G133E	c.398G>A	MS	Colon	Adenocarcinoma	unkn				X	79
32	1766784	p.T142A	c.424A>G	MS	Colon	Adenocarcinoma	unkn			X	X	79
33	1998443	p.L214M	c.640C>A	MS	Colon	Carcinoma, unkn	Hetero.	X			X	121
34	1651002	p.S216L	c.647C>T	MS	Colon	Adenocarcinoma	unkn	X			X	108
35	1651109	p.S216L	c.647C>T	MS	Colon	Adenocarcinoma	unkn	X			X	108

#	Sample ID	AA mutation	CDS mutation	Type	Primary tissue	Histology subtype	Zygosity	APC	β-cat	ZNRF3	Exome seq	Ref
36	1766787	p.R454C	c.1360C>T	MS	Colon	Adenocarcinoma	unkn			X	X	79
37	1651115	p.S495Y	c.1484C>A	MS	Colon	Adenocarcinoma	unkn	X			X	108
38	1651100	p.R529W	c.1585C>T	MS	Colon	Adenocarcinoma	unkn	X			X	108
39	1766767	p.R561Q	c.1682G>A	MS	Colon	Adenocarcinoma	unkn	X			X	79
40	1766793	p.R609Q	c.1826G>A	MS	Colon	Adenocarcinoma	unkn	X		X	X	79
41	1650979	p.Q653R	c.1958A>G	MS	Colon	Adenocarcinoma	unkn				X	108
42	1651033	p.A689T	c.2065G>A	MS	Colon	Adenocarcinoma	unkn	X			X	108
43	1651117	p.A752T	c.2254G>A	MS	Colon	Adenocarcinoma	unkn				X	108
44	1766754	p.R132*	c.394C>T	NS	Colon	Adenocarcinoma	unkn			X	X	79
45	1766751	p.R132*	c.394C>T	NS	Colon	Adenocarcinoma	unkn	X		X	X	79
46	1651224	p.R145*	c.433C>T	NS	Colon	Adenocarcinoma	Hetero.	X		X	?	?
47	1781584	p.S607L	c.1820C>T	MS	Lung	Squamous cell carcinoma	unkn				?	?
48	1780938	p.A696V	c.2087C>T	MS	Lung	Squamous cell carcinoma	unkn				?	?
49	1782751	p.W747*	c.2241G>A	NS	Lung	Squamous cell carcinoma	unkn				?	?
50	1890864	p.G659fs*41	c.1976delG	Del	Oesophagus	Adenocarcinoma	unkn				X	122
51	1890958	p.G659fs*41	c.1976delG	Del	Oesophagus	Adenocarcinoma	unkn			X	X	122
52	1860013	p.I48V	c.142A>G	MS	Oesophagus	Squamous cell carcinoma	unkn				X	122
53	1890868	p.R145*	c.433C>T	NS	Oesophagus	Adenocarcinoma	unkn				X	122
54	1474931	p.P118T	c.352C>A	MS	Ovary	Serous carcinoma	Hetero.				X	107
55	1475106	p.A146G	c.437C>G	MS	Ovary	Serous carcinoma	Hetero.				X	107
56	1998467	p.W302C	c.906G>T	MS	Ovary	Carcinom, unkn	Homo.				X	121
57	1691651	p.R127P	c.380G>C	MS	Pancreas	Cystic tumour	unkn				X	83
58	1691642[b]	p.A169T	c.505G>A	MS	Pancreas	Adenoma	unkn	X			X	83
59	1691653	p.S41*	c.122C>G	NS	Pancreas	Cystic tumour	unkn				X	83
60	1691645	p.R113*	c.337C>T	NS	Pancreas	Adenoma	unkn				X	83
61	1691639	p.R145*	c.433C>T	NS	Pancreas	Adenoma	unkn				X	83
62	1691641	p.Q152*	c.454C>T	NS	Pancreas	Adenoma	unkn				X	83
63	1691640	p.Y177*	c.531C>A	NS	Pancreas	Adenoma	unkn				X	83
64	1691644	p.S216*	c.647C>A	NS	Pancreas	Adenoma	unkn				X	83
65	1691642[b]	p.R371*	c.1111C>T	NS	Pancreas	Adenoma	unkn	X			X	83
66	1691650	p.R371*	c.1111C>T	NS	Pancreas	Cystic tumour	unkn				X	83
67	1604873[c]	p.D230V	c.689A>T	MS	Skin	Squamous cell carcinoma	unkn			X	X	123
68	1604873[c]	p.V725L	c.2173G>T	MS	Skin	Squamous cell carcinoma	unkn			X	X	123
69	1943744	p.G659fs*41	c.1976delG	Del	Stomach	Adenocarcinoma, unkn	unkn				X	124
70	1943738	p.L82S	c.245T>C	MS	Stomach	Adenocarcinoma, unkn	unkn				X	124
71	1944674	p.W194*	c.581G>A	NS	Stomach	Intestinal adenocarcinoma	unkn				X	125

AA, amino acid; ALL, acute lymphoblastic leukemia; CCRCC, Clear cell renal cell carcinoma; CDS, coding DNA sequence; Del, deletion; Exome seq, exome sequencing; Ins, insertion; MS, missense; NS, nonsense; Ref., reference.
a, b, c: same samples.

RNF43 mutations in COSMIC database

To further analyze the assumed tumor suppressor role of RNF43 and ZNRF3, I analyzed the mutations listed in the Sanger COSMIC Database. This database stores information about mutations in human cancers described in primary literature (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). This database contains information about 947.213 samples. Out of these samples, 107 contain an *RNF43* mutation (excl. silent mutations), of which 71 mutations in 67 samples appeared to be somatically acquired. For the analysis presented here, only those mutations are included. They consist of 7 frameshifts, 19 nonsense and 45 missense mutations (**Table 2**). Unfortunately, the majority of *ZNRF3* mutations (59 of the 75 mutations) in the COSMIC Database are not somatically confirmed. Therefore, I focused on the *RNF43* mutations.

To see whether any hot spots would occur in specific regions, *RNF43* missense mutations were mapped along a linear representation of the protein and its domains (**Figure 10a.**). As expected, mutations are located in the RING domain [R286W (14) and W302C (56), number between brackets is number found in Table 2]. Both mutations occur at residues conserved between RNF43 and ZNRF3, indicating that these residues might be required for RING E3 ligase function. However, these residues are not part of the RING consensus sequence. Also, two mutations are located at conserved residues in the TM domain [T204R (3) and L241M (33)], possibly blocking TM localization. Two mutations at a conserved residue are found in the C-terminal conserved region (previous paragraph) of *RNF43* [R454C (36) and S495Y (37)].

A remarkably high number of mutations cluster at the PA domain. The range of observed mutations [A78T (11), L82S (29), M83T (30), S85F (12), G102E (20) and V107L (21)] (**Figure 10b.**) overlaps with the R-spondin interface with the critical residues being Q84, H86, L88, D97, K108 and E110. Mutations disrupting R-spondin binding would oppose tumor formation, since RNF43 and ZNRF3 would be allowed to completely silence WNT signaling. Therefore, mutations at this site could indicate that this area also facilitates FZD complex binding. Disruption of FZD complex binding would lead to elevated Frizzled membrane levels and enhanced WNT signaling. Thus, these mutations support the notion that the PA domain may bind to both R-spondin and the FZD complex. Notably, the mutations V107L (21) and G133E (31) are located at residues conserved between *RNF43*, *ZNRF3* and *PLR-1* (Supplementary Figure 1). This indicates that these residues may be important for FZD complex binding. In addition, a P79L mutation in *ZNRF3* (*RNF43*, P160) is also located at a residue conserved between the three genes. Lastly, the A169T (58) mutation in *RNF43* should be noted since the same residue is mutated in *ZNRF3* (A188V).

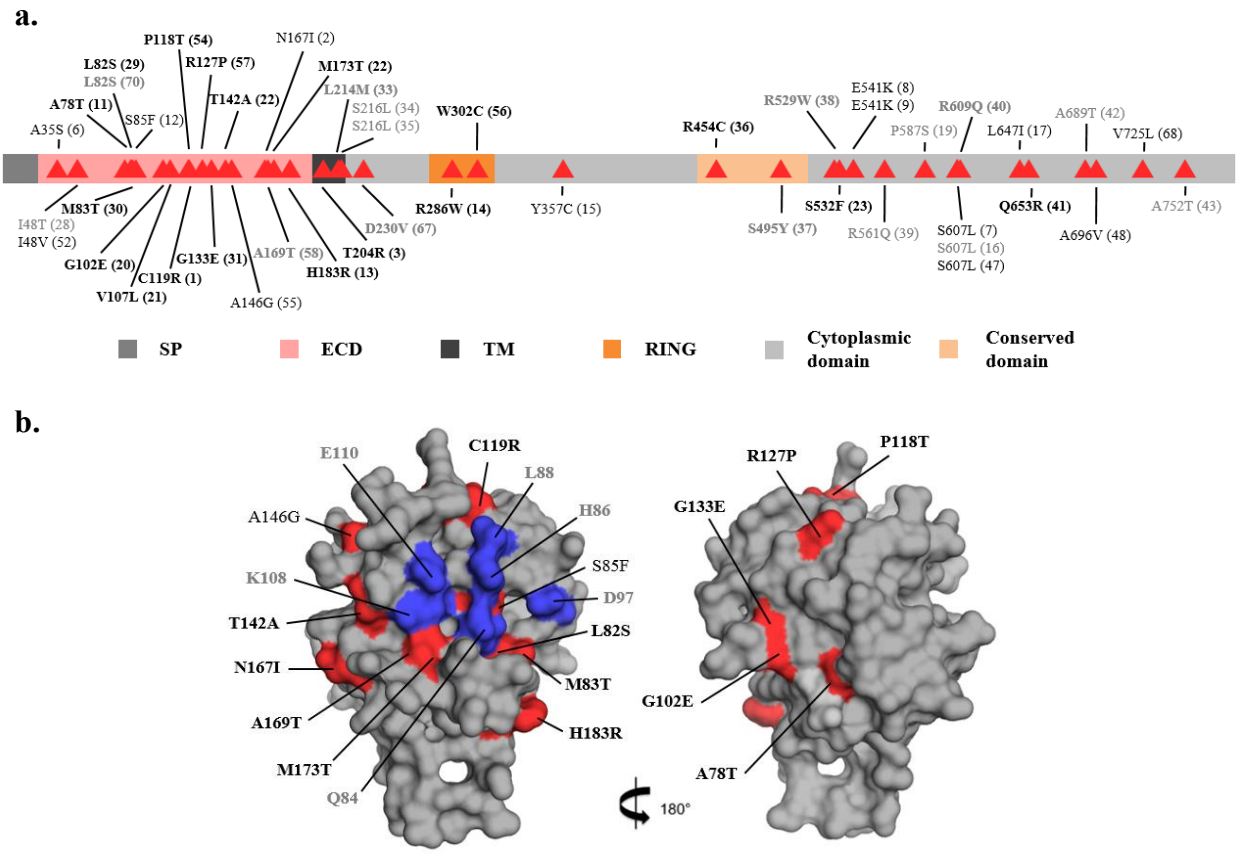

Figure 10. RNF43 mutations in cancer. (a.) RNF43 missense mutations from the COMSIC database mapped on a linear representation of the RNF43 gene. Mutations in samples also containing an *APC* mutation are indicated in grey, mutations in samples without *APC* mutation are indicated in black. Mutations at residues conserved between RNF43 and ZNRF3 are indicated in bold. (#) is the number found in table 2. Different domains are indicated. SP, signal peptide; ECD, extracellular domain; TM, transmembrane. (b.) The mutations (black) shown on the RNF43 surface in red and critical residues for R-spondin binding (grey) in blue. Two views are shown.

Interestingly, the majority of the samples containing *RNF43* mutations clustering in the PA domain do not harbor an *APC* mutation (black in Figure 10). Conversely, the *RNF43* mutations in samples that also contain an *APC* mutation (grey) do not cluster at a specific site but are spread over the gene. In addition, more than half of these mutations are located at non-conserved residues, while *RNF43*-PA mutations in samples without an *APC* mutation are mainly located at conserved residues. This might suggest that disruption of the substrate recognition function of the RNF43-associated PA domain may drive adenoma formation analogous to the classical *APC* and ß-catenin mutations.

No clusters of mutations are found outside the known domains in *RNF43*, revealing no other functional domains. As previously mentioned, some mutations are located in the cytoplasmic region that is poorly conserved. The majority of these mutations, in addition, occur at non-conserved sites. There are four exceptions: D230V (67), R529W (38), R609Q (40) and Q653R (41). At present, no conceivable role is available for these mutations.

RNF43 mutations in different tissues

As mentioned before, aberrant WNT signaling is responsible for the development of colon cancer. In most cases, *APC* or *CTNNB1* mutations are the drivers of this tumorigenesis. Since the deletion of both *RNF43* and *ZNRF3* led to colon adenoma formation in mice[47], the question is now whether *RNF43* mutations can also be drivers for colon tumorigenesis in human. In addition, the question arises whether tumor formation in other tissue types might be dependent on aberrant WNT signaling.

Colon

The majority of the *RNF43* mutations are found in the colon, with 23 out of 67 samples (**Table 2**). As previously mentioned, worldwide studies agree that about 80% of the colon cancer patients carry an *APC* mutation and approximately 5% carry an activating mutation in *CTNNB1*. In 40% of all samples with an *RNF43* mutation, however, no overlap is seen with the classical *APC* and *CTNNB1* mutagenic events. This may be explained as an indication that *RNF43* mutations are not just neutral events, but rather have an active role in tumorigenesis in the colon. Since mouse knock out experiments seem to indicate that both *RNF43* and *ZNRF3* have to be mutated in mice for colon adenomas to form, *RNF43* mutations would be expected to coincide with mutations in *ZNRF3* in all those cases where no accompanying *APC* or *CTNNB1* mutations can be detected. Remarkably, in the 23 colon samples in which *RNF43* appeared mutated only in six cases *ZNRF3* appeared mutated as well. Three of these samples do not harbor an *APC* or *CTNNB1* mutation. This would suggest, in contrast to the mouse gene knock-outs, that *ZNRF3* mutations, in addition to an *RNF43* mutations, are no prerequisite in the initiation of colon cancer in humans. In the COSMIC database, eight colon samples are seen that contain *ZNRF3* mutations in the absence of *RNF43* mutations (**Table 3**) but almost invariably in the presence of mutations in the *APC* gene. This again supports a minor role for the tumor suppressor activity of *ZNRF3* in human colon cancer. It should be noted that we in fact do not know whether human intestinal stem cells express ZNRF3 like seen in mice. The eight *ZNRF3* mutations mentioned here were, moreover, not somatically confirmed.

One might also speculate about another substrate for RNF43 that is more important in tumorigenesis than Frizzled, since the low frequency of samples containing mutations in both E3 ligases. However, the cluster of mutations in the PA domain supports the Frizzled vs. R-spondin competition model.

Pancreas

In addition to colon cancer samples, nine pancreas samples are listed in the COSMIC database that contain *RNF43* mutations. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic malignancy and it develops through the progression of ductal precursor lesions, including pancreatic intraepithelial neoplasms (PanINs), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs)[85]. The majority of these neoplasms acquire an activating mutation in *KRAS*, a GTP-binding protein

Table 3. ZNRF3 mutations in colon cancer samples listed in the Sanger COSMIC Database.

Sample ID	AA mutation	CDS mutation	Type	Somatic confirmed?	Zygosity	APC	β-cat	RNF43	Ref.
1651142	p.P79L	c.236C>T	MS		Hetero	X			
1651337	p.A88V	c.263C>T	MS		Hetero	X			
1766754	p.R102*	c.304C>T	NS	X	unkn			X	79
1766751	p.R102*	c.304C>T	NS	X	unkn	X		X	79
1651162[a]	p.R108H	c.323G>A	MS		Hetero	X			
1766779[b]	p.R111*	c.331C>T	NS		unkn				79
1651233[c]	p.R145*	c.433C>T	NS		Hetero	X	X		
1766779[b]	p.?	c.444+2T>C	?		unkn				79
1651162[a]	p.R174H	c.521G>A	MS		Hetero	X			
1650960	p.A181fs*58	c.538delG	Del		Hetero	X	X		
1650947	p.V249fs*117	c.739_740delGT	Del		Hetero	X	X		
1651233[c]	p.R393fs*77	c.1174delC	Del		Hetero	X	X		
1766793	p.N476K	c.1428C>G	MS		unkn	X		X	79
1766784	p.R502Q	c.1505G>A	MS		unkn			X	79
1863429	p.S513fs*68	c.1529_1530insG	Ins	X	unkn	X			79
1766787	p.P743S	c.2227C>T	MS		unkn			X	79
1651224	p.R759*	c.2275C>T	NS		Hetero	X		X	

AA, amino acid; CDS, coding DNA sequence; Del, deletion; Exome seq, exome sequencing; Ins, insertion; MS, missense; NS, nonsense; Ref., reference.
a, b, c: same samples.

of the RAS family that mediates a variety of functions including proliferation, differentiation and survival. This mutation is detected in about 30% of the early neoplasms and it can rise to almost 100% in advanced PDACs[86]. Indeed, seven out of nine pancreatic samples in the COSMIC database containing an *RNF43* mutation, also harbor a *KRAS* mutation.

Because of the high prevalence in pancreatic malignancies, *KRAS* is thought to be one of the main driver mutations of pancreatic tumorigenesis. However, activation of KRAS alone is not sufficient to induce PDAC in acinar cells, one of the cell types of PDAC origin[87]. This indicates that additional driver mutations might be necessary in different types of neoplasms. In IPMN, a point mutation in codon 201 of the *GNAS* gene might be such an additional driver mutation since it is found in as many as 66% of the cases[83,88]. This mutation was also found in four out of six IPMN samples containing an *RNF43* mutation.

Since *RNF43* mutations are frequently observed in IPNMs and MCNs[83,88], aberrant WNT signaling through an inactivating *RNF43* mutation might also have a driver effect on pancreatic tumorigenesis. WNT signaling is essential for pancreas development during embryogenesis[89,90] but it becomes inactive in the adult pancreas[91]. Upon injury, however, WNT signaling is reactivated and the stem cell marker LGR5 is then expressed in the neo-formed ducts[28]. The presence of LGR5 and active WNT signaling in these ducts indicates that RNF43 should also be present to regulate WNT signaling levels. Absence of RNF43 might then lead to hyper proliferation in the ducts and subsequent tumor formation. Indeed, there is a growing body of evidence showing that WNT pathway activation may contribute PDAC

progression[92] and a recent study by Jiang et al. (2013) described a role for RNF43[93]. They observed that PDAC cell lines that are WNT dependent all harbor *RNF43* loss-of-function mutations. These *RNF43* mutations were found to enhance WNT signaling and drive proliferation, while suppression of WNT signaling in the *RNF43* mutant cell lines induced cell cycle arrest and differentiation. Remarkably, these WNT dependent *RNF43* mutant cell lines also all had a *KRAS* mutation. This suggests that RNF43 may act as a tumor suppressor and that loss of RNF43 may be the additional stimulus necessary to promote proliferation and pancreatic tumorigenesis. In colon cancer, active WNT signaling due to an *APC* or activating β-catenin mutation drives of tumorigenesis, while a *KRAS* mutation is a secondary event promoting progression to carcinomas[94]. The same mechanism might also drive PDAC progression.

Remarkably, none of the pancreatic tumor samples also contains a *ZNRF3* mutation. It would be interesting to investigate whether the pancreatic duct cells, the cells of PDAC origin, do express ZNRF3. If not, this would explain the lack of ZNRF3 mutations and the apparent bigger role of *RNF43* mutations in pancreas than in the colon cancer.

Biliary tract

Inactivating *RNF43* mutations have also been found in 9.3% of cholangiocarcinomas[84] (five samples in COSMIC database, see **Table 2**), which are relatively rare, malignant tumors in the bile duct[95, 96]. These *RNF43* mutations are associated with poor survival and can serve as an independent predictive value for cholangiocarcinoma patient survival[84]. Like pancreatic carcinomas, at least a proportion of cholangiocarcinomas is thought to develop from precancerous lesions in the biliary tract, including mucin-producing bile duct tumors (MPBT), intraductal papillary neoplasms of the bile duct (IPNB) and bile duct cystadenomas (BDCA). These lesions have been recognized as the biliary equivalents of the pancreatic neoplastic lesions [96-99]. The bile duct and pancreas are also thought to be linked through a stem cell compartment in the biliary tree. This compartment has been implicated to contain stem cells for the liver, pancreas and bile duct both during development and in adulthood[100]. Thus, the pancreas and bile duct are thought to have a common embryonic origin and a shared adult stem cell compartment, which might explain the shared mutational characteristics between PDAC and cholangiocarcinoma (CCA). Genes that are commonly found to be mutated are for instance *KRAS, TP53, SMAD4* and now also *RNF43*[84]. Notably, none of the *RNF43* mutated samples in the pancreas display *SMAD4* mutations, while 4/5 CCA samples do. Since SMAD4 inactivation often occurs late in neoplastic progression[101], this can be explained by the fact that the listed pancreas samples are neoplasms that not yet have developed into PDAC, while the CCA samples are from advanced malignancies.

As mentioned before, aberrant WNT signaling due to loss-of-function mutations in *RNF43* may play a role in driving pancreatic tumorigenesis. Similarly, there are indications that WNT signaling may also be involved in CCA development. For instance, different levels of WNT pathway activation and target gene expression were found in four CCA cell lines[102]. When WNT signaling was blocked in these cell lines, proliferation was suppressed while apoptosis was enhanced. Furthermore, different studies have described reduced membranous β-catenin

Table 4. RNF43 mutations in ovary tumors described by Ryland et al., 2013

Sample ID	Tumor type	AA mutation	CDS mutation	Type	Genomic status
IC263	MBT	c.182_183delTG	p.Leu61GlnfsX13	Del	LOH
4515	MBT	.856C > T	p.Arg286Trp	MS	LOH
IC080	MOC	c.16C > T	p.Gln6X	NS	LOH
IC050	MOC	c.1389_1392dupCAGT	p.Asp465GlnfsX2	Dup	
IC138	MOC	c.850G > T	p.Glu284X	NS	LOH
IC321	MOC	c.583-1G > T	p.Pro195IlefsX7	NS	LOH
IC557	MOC	c.760delC	p.Gln254ArgfsX165	Del	
IC588	MOC	c.1009C > T	p.Arg337X	NS	Al

Al, allelic imbalance; Del, deletion; Dup, duplication; LOH, loss of heterogeneity; MBT, mucinous borderline tumor; MOC, mucinous ovarian carcinoma; MS, missense; NS, nonsense.

expression levels in a large part of CCAs[103,104]. Taken together, this implicates that the WNT signaling pathway may be activated during CCA progression and that *RNF43* mutations might lead to this WNT activation.

Ovary

In the COSMIC database, three somatic *RNF43* mutated samples of the ovary are listed; two serous carcinoma samples and one of unknown subtype. Recently, Ryland et al. (2013) identified additional *RNF43* mutations in 2/22 (9%) of the ovarian mucinous borderline tumors and in 6/29 (21%) of the ovarian mucinous carcinomas[105] (**Table 4**), which are thought to partly arise from ovarian mucinous borderline tumors. Moreover, Zou et al. (2013) also identified *RNF43* as a gene that is frequently mutated in ovarian mucinous carcinomas (2/15, 13.3%)[106]. Interestingly, the overall frequency of *RNF43* mutations in ovarian mucinous tumors and carcinomas (~15%) is considerably higher than the frequency in ovarian serous carcinomas (~0.6%); only two *RNF43* mutations were detected in 316 cases of ovarian serous carcinoma characterized by The Cancer Genome Atlas[107]. This, in combination with *RNF43* mutations found in mucin-producing cystic tumors of the pancreas[83,88] and in cholangiocarcinomas of which a subset secretes mucin[84], suggests that RNF43 inactivation may be a key event in other mucinous tumors of other organs as well. However, this does not exclude a role for *RNF43* mutations in non-mucinous malignancies. The frequency of *RNF43* mutations in colorectal mucinous tumors (6.3%) was not observed to be significantly higher than mutations in non-mucinous colorectal tumors (4.9%)[108].

Seven out of eight *RNF43* mutations in the ovarian mucinous borderline tumor and carcinomas described by Ryland et al. were of deleterious nature, i.e. frameshifts or nonsense [105]. Moreover, five out of eight mutations were also associated with loss of heterogeneity (LOH) of the wild-type allele. This suggests that *RNF43* indeed functions as a tumor suppressor. This is supported by the observations made by Wu et al. in mucinous pancreatic neoplasms[83]. Eight out of ten *RNF43* mutations were of deleterious nature and six mutations were also combined with LOH. In addition, silent mutations were found in neither ovarian mucinous borderline tumor and carcinomas nor in mucinous pancreatic neoplasms. Thus,

RNF43 mutations are likely to be pathogenic rather than random mutational events, at least in mucinous pancreatic and ovarian malignancies.

Endometrial cancer

Eight endometrioid endometrial carcinoma (EEC) samples containing a somatic *RNF43* mutation are listed in the COSMIC database. In these carcinomas, genetic aberrations in *phosphatase and tension homolog* (*PTEN*), *APC* and *CTNNB1* have frequently been observed[109,110]. However, it was unclear how these aberrations were involved in tumor

initiation and/or progression. Recently, it was shown that constitutive activation of WNT signaling in the endometrium by loss of *APC* results in squamous cell metaplasia (SCM)

without malignant transformation[111]. Conversely, *PTEN* loss-of-function mutations led to SCM and subsequent malignant transformation into EEC. When *PTEN* and *APC* were lost simultaneously, early onset of EEC took place and these EECs had a more aggressive malignant behavior than EECs with *PTEN* mutations alone. This indicates that the WNT and PTEN signaling pathways act synergistically in EEC onset and progression. In addition, it indicates that EECs with activated WNT signaling most likely also harbor a *PTEN* mutation.

Normally, 40% of EECs harbor a PTEN mutation[110]. Therefore, when the *RNF43* mutations would be random mutagenic events, 40% of the samples would be expected to contain a *PTEN* mutation. However, all *RNF43* mutated endometrial samples in the COSMIC database harbor a *PTEN* mutation, while only three out of eight contain an *APC* or *CTNNB1* mutation. This suggests that these *RNF43* mutations may lead to aberrant WNT signaling and tumorigenesis, since all EECs with aberrant WNT signaling are expected to have a *PTEN* mutation. Thus, RNF43 may function as a tumor suppressor in the endometrium.

Lastly, it is also interesting to note that LGR5 has recently been found to be expressed in endometrial stem cells. However, whether these stem cells, and LGR5/R-spondin/RNF43 signaling, are involved in EEC development still has to be explored[112].

Confliction role of RSPO-LGR signaling in colorectal cancer

Since R-spondins are WNT activators, R-spondin overexpression is thought to play a role in tumorigenesis. Indeed, recurrent RSPO2 gene fusions are found in colon tumors[79] and in a transposon-based genetic screen in mice, it was identified as a candidate colorectal cancer (CRC) driver gene[113]. However, a very recent study by Wu et al. (2014) described that RSPO2 acts negatively on CRC tumor growth[114]. Overexpression of RSPO2 together with LGR5 was shown to result in suppression of WNT signaling and CRC tumor growth. They showed that this was due to the stabilization of ZNRF3 on the cell membrane by RSPO2 and LGR5. These findings led to the proposal of a novel RSPO2-induced WNT signaling feedback model. This suggests that RSPO2 enables transient WNT pathway activation resulting in LGR5 upregulation. LGR5 then interacts with RSPO2 to stabilize ZNRF3 at the cell membrane, resulting in LRP6/Frizzled degradation. This would then create a negative

feedback loop limiting β-catenin accumulation and WNT target gene expression, eventually resulting in growth suppression of CRC cells. In this model, RSPO2 depletion would lead to WNT pathway activation and consequent CRC cell growth. Indeed, loss of RSPO2 due to promoter hypermethylation was frequently observed in CRCs[114]. This model, therefore, contradicts the described working mechanism for R-spondin as presented by Cong and colleagues (2013)[46]. Tumor growth suppression was, moreover, not seen in all colon carcinoma-derived cell lines. Clearly, this model is not particularly obvious, and needs confirmation from experiments performed on colon carcinoma tissue directly isolated from patients.

Conclusion

The WNT pathway is a highly conserved signaling cascade involved in embryonic development and adult stem cell homeostasis. In adult stem cells, this pathway is highly regulated by positive and negative feedback mechanisms and dysregulation of WNT signaling is often seen in cancer patients. A recently discovered negative feedback mechanism involves the transmembrane RING E3 ubiquitin ligases RNF43 and ZNRF3[46]. These two E3 ligases are stem cell specific WNT target genes. They target the WNT receptor complex for degradation by ubiquitination, resulting in decreased Frizzled membrane levels and consequently reduced WNT signaling. RNF43 and ZNRF3 are regulated by R-spondin-bound LGR4-6. These LGRs are also WNT target genes and LGR5 is specifically expressed in stem cells, while LGR4 is also expressed in the transit amplifying compartment[115]. Upon trimeric complex formation of RNF43/ZNRF3, LGR and R-spondin, RNF43 and ZNRF3 are removed from the surface resulting in increased WNT signaling. Thus, stem cells appear to have a sophisticated regulatory network that balances WNT signaling and homeostasis.

To be able to function in this regulatory network, RNF43 and ZNRF3 should be able to bind to both R-spondin and to the Frizzled complex. RNF43/ZNRF3 and R-spondin binding takes place at the highly conserved PA domain of RNF43/ZNRF3 and this has been described in much detail[55-57]. However, it has not yet been described how RNF43/ZNRF3 binds to the Frizzled complex. In this review, we tried to get clues about the binding FZD complex site by looking at RNF43/ZNRF3 homologues. Looking at the close family member GRAIL provides us with the first clue since it showed that that the PA domain of GRAIL can recognize multiple substrates[66]. However, the different substrates should possibly contain a similar recognition motif. A different clue, supporting Frizzled binding by the PA domain, came from a recent study on a RNF43/ZNRF3 homolog PLR-1[52]. In *C. elegans*, PLR-1 uses its PA domain to bind to the Frizzled homologs CFZ-2, LIN-17, MIG-1 and MOM-5. PLR-1 does not bind to R-spondin, since R-spondin is a vertebrate invention. However, it does potentially bind to the WNT (co-)receptors CAM-1 (Ror) and LIN18 (Ryk), also indicating that the PA domain may recognize different substrates. The possibility that RNF43 and ZNRF3 bind to both R-spondin and frizzled leads to a model of competitive substrate recognition. This model is supported by the observation that R-spondin and RNF43/ZNRF3 only weakly interact. Thus, R-spondin and Frizzled may compete for binding with the PA domain, resulting in equilibrium of WNT signaling and consequently homeostasis.

Since aberrant WNT signaling is a driver of colon cancer, disruption of RNF43/ZNRF3 function could also be involved in tumorigenesis. Indeed, Koo et al. (2012) showed that simultaneous deletion of *RNF43* and *ZNRF3* results in hyperproliferation and colon tumor formation in mice[47]. Mapping of the somatic missense mutations in *RNF43* listed in the COSMIC database showed mutations in both the RING domain and the transmembrane domain possibly resulting in impaired function. In addition, a cluster of *RNF43* mutations at residues conserved between *RNF43* and *ZNRF3* was found at the PA domain. Since the

disruption of R-spondin binding would lead to reduced WNT signaling opposing tumor formation, this suggests that the PA domain should also binds to the FZD complex. Lastly, no mutation clusters were found in the cytoplasmic region outside the RING domain, indicating that this region may not play a major role in RNF43 function.

When looking at the *RNF43* mutations in different organs, it became clear that *RNF43* mutations are likely to be pathogenic rather than neutral mutational events. This seems the case not only in the colon but also in other organs, including the pancreas, biliary tract, ovary and endometrium. Whether simultaneous *ZNRF3* mutations are needed for tumorigenesis in the colon or ovary is unclear, but the analysis of COSMIC database samples seems to argue against. If E3 ligase-mediated Frizzled downregulation functions as tumor suppressor in the pancreas, biliary tract or endometrium, mutations in *ZNRF3* are not required.

Taken together, it is known that a sophisticated regulatory network in adult stem cells maintains homeostasis and that this network includes a negative feedback mechanism in which RNF43 and ZNRF3 are key players. However, it is still unknown how RNF43 and ZNRF3 function exactly and a lot of questions remain. For instance, how do RNF43 and ZNRF3 recognize Frizzled and how do they target it for degradation? In addition, how are RNF43 and ZNRF3 involved in cancer? Should they both be mutated for tumor formation or is a mutation in a single gene enough? And, are mutations in other genes possibly linked to RNF43/ZNRF3 driven tumorigenesis? Thus, new studies should be performed to provide the answers these questions.

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Supplementary information

Supplementary figure 1. Alignment of *RNF43*, *ZNRF3* and *PRL-1*. ClustalW[116] alignment of *RNF43* and *ZNRF3* of human, rabbit, mouse and chicken, and *PLR-1* from *C. elegans*. Bold and underlined residues are identical in all three genes. Residues critical for R-spondin binding are indicated in red, while other conserved residues are green.