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Ub and Dub of RNF43/ZNRF3 in the WNT signalling pathway

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The E3 ubiquitin ligases RING finger protein 43 (RNF43) and zinc and RING finger 3 (ZNRF3) have received great attention for their critical role in regulating WNT signalling during adult stem cell homeostasis. By promoting the turnover of WNT receptors, Frizzled and LRP5/6, RNF43 and ZNRF3 ensure that proper levels of WNT activity are maintained in stem cells. The molecular mechanism of RNF43/ZNRF3 activity is beginning to emerge from several recent studies, yet little is known about the regulation of RNF43/ZNRF3 at the post-translational level. A study in this issue of *EMBO Reports* identifies the deubiquitinating enzyme USP42 as a key regulator of WNT signalling, which acts by antagonizing the ubiquitin-dependent clearance of RNF43/ZNRF3 induced by R-spondins (Giebel *et al*, 2021).

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See also: **N Giebel *et al*** (May 2021)

WNT/β-catenin signalling (also known as canonical WNT signalling) is an evolutionarily conserved pathway involved in embryonic development of multicellular animals (Nusse & Clevers, 2017). In adult tissues, canonical WNT signalling controls self-renewal and maintenance of a variety of tissue-specific stem cells, most notably the intestinal stem cells (ISCs) residing at the bottom of the intestinal crypt (Nusse & Clevers, 2017). In order to maintain a correct balance between stem cell proliferation and differentiation, as in typical homeostatic conditions, WNT/β-catenin signalling needs to be tightly regulated. Several mechanisms have evolved to prevent WNT overactivation. Genetic mutations that override these safe-guard mechanisms, leading to sustained WNT activation, are often at the basis of different types of cancers. An example is colorectal adenocarcinoma, which is most frequently initiated by loss-of-function mutations in the WNT negative regulator, adenomatous polyposis coli (APC).

The two related paralogues RNF43/ZNRF3 (hereafter R/Z) represent a class of single-pass transmembrane RING-type E3 ubiquitin ligases that attenuate WNT signalling at the plasma membrane, by promoting ubiquitination and endo-lysosomal degradation of FZD and LRP5/6 (Hao *et al*, 2012; Koo *et al*, 2012). Both proteins contain a signal peptide, an extracellular protease-associated (PA) domain, a transmembrane domain and an intracellular RING domain with catalytic activity. R/Z are expressed in different types of stem cells, including ISCs, and are induced by WNT, forming a negative feedback loop that prevents uncontrolled stem cell proliferation. R/Z function is of fundamental importance for keeping stem cells in check, as evidenced by double knockout mice which show strong over-proliferation of the intestinal stem cells and formation of tumours, similar to APC mutations (Koo *et al*, 2012). R/Z mutations are indeed found in various human cancers, including colorectal and pancreatic carcinomas. Differently from APC, however, R/Z tumours still depend on a source of WNT ligands and are sensitive to PORCN inhibitors, small molecules that prevent WNT secretion and inhibit paracrine signalling (Koo *et al*, 2015). R/Z activity is also finely regulated, particularly by leucine-rich repeat-containing G protein-coupled receptor (LGR) 4/5/6, which binds secreted WNT agonists R-spondins (Rspo) and forms a ternary complex with R/Z, promoting their membrane clearance through an auto-ubiquitination mechanism (de Lau *et al*, 2011; Hao *et al*, 2012). So, by removing R/Z, Rspo increases FZD and LRP5/6 at the cell surface, boosting WNT signalling (Fig 1). Other players have been shown to modulate R/Z activity. Surprisingly, the cytosolic scaffold protein dishevelled (DVL), an essential component of the WNT pathway, is also required for R/Z activity, by recruiting the E3 ligases to WNT receptors and promoting their ubiquitination-dependent degradation (Jiang *et al*, 2015). More recently, several key studies showed that R/Z activity is regulated by post-translational modifications. For example, casein kinase 1 (CK1)-dependent phosphorylations were shown to be crucial for regulating RNF43 function (Spit *et al*, 2020; Tsukiyama *et al*, 2020), while the phosphatase PTPRK was shown to promote ZNRF3 activity by keeping it unphosphorylated (Chang *et al*, 2020).

The present work from Giebel *et al* (2021) adds another fundamental piece to the complex puzzle of R/Z regulation. The authors first performed a siRNA screening against deubiquitinating enzymes (DUBs) in HEK293T cells, using the well-established TOPflash luciferase reporter as a readout for WNT/β-catenin signalling. The authors identified ubiquitin-specific protease 42 (USP42) as a negative regulator of WNT signalling, since its knock-down upregulated WNT-induced luciferase activity. Interestingly, USP42 knock-down cooperated with Rspo treatment in the WNT reporter assay but, critically, did not further increase WNT signalling in R/Z double KO cells, suggesting that R/Z are epistatic to USP42. Further biochemical analysis revealed that USP42 knock-down increased, while its overexpression diminished, R/Z polyubiquitination. Importantly, a catalytically inactive mutant $USP42^{C120A}$ was unable to inhibit WNT signalling, validating the idea that USP42 functions in the WNT pathway through de-ubiquitination of

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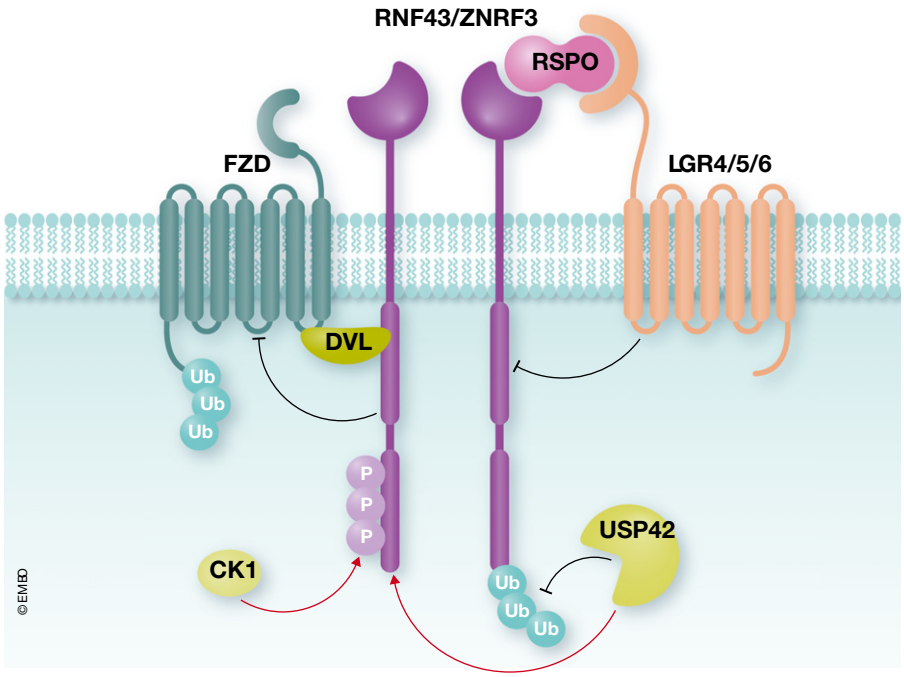

Figure 1. The deubiquitinating enzyme USP42 stabilizes RNF43/ZNRF3 at the plasma membrane.

When bound to RSPO and LGR, RNF43/ZNRF3 are removed from the cell surface as a result of auto-ubiquitination and endocytosis, inhibiting the E3 ligase activity. USP42 counteracts R/Z auto-ubiquitination, stabilizing their levels on the plasma membrane. Consequently, R/Z are available to inhibit WNT signalling by promoting FZD ubiquitination and endo-lysosomal degradation. CK1 phosphorylates R/Z cytoplasmic tail, enhancing its anti-WNT activity. DVL bridges R/Z to FZD, allowing downregulation of the WNT receptors.

R/Z (Fig 1). Interaction between USP42 and R/Z was confirmed by co-immunoprecipitation, and using different deletion constructs, the authors could map the binding in the Dvl interacting region (DIR), found in the cytoplasmic domain of R/Z and already known to play an important regulatory role (Jiang *et al*, 2015). On the other hand, USP42 required an intact N-terminal domain and catalytic activity for its interaction with ZNRF3, while other regions were dispensable. Elegant cell-surface biotinylation experiments showed that USP42 overexpression reduced the ubiquitin "earmarks" on R/Z, while increasing R/Z plasma membrane levels. Notably, co-expression of R/Z and USP42 decreased cooperatively FZD levels, through an active endocytic process, while knock-down of USP42 increased endogenous LRP6 levels in cultured cells. Altogether, the evidence brought by Giebel and colleagues shows that USP42 synergizes with R/Z in promoting WNT receptor turnover. Perhaps the most significant insight at a mechanistic level was that USP42 stabilized the formation of a ternary complex comprising ZNRF3, Rspo and LGR4, preventing at the same time ubiquitin-dependent clearance of ZNRF3 induced by Rspo/LGR. This led the authors to suggest that USP42 directly oppose Rspo/LGR-mediated clearance of R/Z, by keeping the E3 ligases in a de-ubiquitinated form and stalling the ternary complex between Rspo, LGR and R/Z.

USP42 mRNA is often upregulated in various cancers. To understand its role in cancer cells, Giebel *et al* (2021) performed bulk-RNA sequencing on HCT116 colorectal cancer cells upon USP42 knock-down, showing upregulation of genes associated with intestinal stem cell features as well as epithelial–mesenchymal transition (EMT). The latter was corroborated by loss of cell–cell adhesion molecule E-cadherin, whose expression could be rescued by treatments that inhibited WNT secretion. Thus, loss of USP42 confers cancer cell EMT characteristics and hypersensitivity to WNT signalling, a finding paralleled by experiments on mouse intestinal organoids. Wild-type organoids died when Rspo was withdrawn from culture medium; however, USP42 KO intestinal organoids could still grow, indicating independency from Rspo factors, as a consequence of hypersensitivity to WNT. However, when endogenous WNT secretion was blocked, USP42 KO organoids also died, confirming their growth advantage still depended on a source of paracrine WNT signalling, similar to R/Z double KO organoids (Koo *et al*, 2012; Koo *et al*, 2015). Altogether, the work from Giebel and colleagues defines a novel role for USP42 in WNT signalling and reveals important insights into its molecular function as a key factor that stabilizes R/Z at the cell surface. However, some key questions remain still open. For example, the precise role of USP42 in the intestinal stem cells or other tissue-specific stem cells was not investigated. Due to the importance of R/Z and WNT in stem cell regulation, it is tempting to speculate that USP42 (or perhaps other cell type-specific DUBs) may play a fundamental role in stem cell homeostasis. Another emerging point is the importance of the cytosolic tail of R/Z in modulating the E3 ligase activity, by integrating different types of post-translational modifications. Elucidating how different modifications are coordinated on R/Z will provide fundamental insights on the complexity of its regulation.

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