A phospho-switch controls RNF43-mediated degradation of Wnt receptors to suppress tumorigenesis

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Frequent mutation of the tumour suppressor RNF43 is observed in many cancers, particularly colon malignancies. RNF43, an E3 ubiquitin ligase, negatively regulates Wnt signalling by inducing degradation of the Wnt receptor Frizzled. In this study, we discover that RNF43 activity requires phosphorylation at a triplet of conserved serines. This phospho-regulation of RNF43 is required for zebrafish development and growth of mouse intestinal organoids. Cancer-associated mutations that abrogate RNF43 phosphorylation cooperate with active Ras to promote tumorigenesis by abolishing the inhibitory function of RNF43 in Wnt signalling while maintaining its inhibitory function in p53 signalling. Our data suggest that RNF43 mutations cooperate with KRAS mutations to promote multi-step tumorigenesis via the Wnt-Ras-p53 axis in human colon cancers. Lastly, phosphomimetic substitutions of the serine trio restored the tumour suppressive activity of extracellular oncogenic mutants. Therefore, harnessing phospho-regulation of RNF43 might be a potential therapeutic strategy for tumours with RNF43 mutations.
ight regulation of the many signalling pathways that control cell fate is essential for proper development and homeostasis. The Wnt signalling pathway plays a prominent role in stem cell maintenance, embryonic development and tumorigenesis. The activities of key players in this pathway are regulated by post-translational modifications, such as phosphorylation and ubiquitination. Loss of these regulatory events often induces oncogenic transformation.

The intestinal stem cell (ISC)-specific ubiquitin ligase ring finger protein 43 (RNF43) negatively regulates Wnt signalling by triggering ubiquitin-mediated, endo-lysosomal degradation of the Frizzled (Fzd) family of Wnt receptors. RNF43-mediated negative regulation of Fzd requires an interaction between the extracellular domains of RNF43 and Fzd. The growth factor R-spondin (Rspo) is a vertebrate-specific Wnt agonist that reversibly counteracts negative regulation by RNF43 and also by its homologue zinc and ring finger protein 43 (ZNRF3). Rspo binds the stem cell-specific leucine-rich repeat-containing G-protein-coupled receptor Lgr5/6 and promotes formation of a trimeric complex with RNF43/ZNRF3. As a consequence, a RNF43/ZNRF3 target Lgr4/5/6 for degradation instead of Fzd, leading to an accumulation of Fzd Wnt receptors and subsequent elevation of Wnt signalling activity. Thus, Wnt signalling undergoes complex regulation not only in the cytoplasm but also at the plasma membrane by Rspo and RNF43/ZNRF3.

RNF43 itself is a downstream target gene of Wnt/β-catenin signalling and thus acts as an important negative feedback regulator to inhibit an excess of Wnt signalling activity. Indeed, missense or truncation mutations of RNF43 that compromise the negative feedback regulation of Wnt signalling have been identified in cancer. Tumours with these mutations are still dependent on Wnt for growth, but independent of Rspo. Missense mutations of RNF43 and ZNRF3 appear to function in a dominant-negative manner, consistent with the observation that many cancers exhibit a single mutation in either RNF43 or in ZNRF3 but not both. Many RNF43 cancer-associated mutations occur outside the N-terminal extracellular and cytoplasmic RING-finger domains, which regulate Fzd binding and ubiquitination, respectively, suggesting additional levels of RNF43 regulation. We previously showed that the C-terminal cytoplasmic region of RNF43 interacts with the Wnt signal transducer Dishevelled (Dvl) to suppress non-canonical Wnt signalling. This work also revealed that amino acid residues 442–478 within the cytoplasmic region of RNF43, which are not involved in the interaction with Dvl, are important for RNF43 regulation.

In addition, RNF43 suppresses p53-dependent transcription and cell death that are induced by DNA damage or viral infection. However, the molecular significance of oncogenic RNF43 mutations in the p53 pathway remained an unsolved issue. Here, we discover that intracellular phosphorylation of RNF43 is required for negative regulation of Wnt signalling. RNF43 mutations that abrogate this phosphorylation lead to loss of Fzd ubiquitination-degradation and de-repression of Wnt signalling, but do not compromise inhibition of p53. These RNF43 phospho-mutants cooperate with active Ras to promote tumorigenesis, and co-occurrence of RNF43 and KRAS mutations are associated with poor survival in human colon cancer. Strikingly, introducing phosphomimetic mutations into oncogenic RNF43 mutants restores RNF43-mediated ubiquitination and degradation of Fzd and inhibition of Wnt signalling, and abolishes oncogenic RNF43-Ras-mediated tumorigenesis in vitro and in vivo. Our results reveal RNF43 as a potential therapeutic target, and provide important insights into the mechanisms that promote multi-step colon cancer tumorigenesis along the Wnt-Ras-p53 axis.

Results

Multi-step phosphorylation of serines activates RNF43. Our previous report suggested that RNF43 binding to Dvl2 is not required to downregulate Fzd418. To verify that Dvl is not an essential cofactor for the recognition and degradation of Fzd, we determined whether the interaction with Dvl is required to regulate Fzd5. We found that a Dvl-interaction-defective RNF43 mutant (RNF43-ΔDvl-C) can still immunoprecipitate Fzd5 (Supplementary Fig. 1a, b) and induce its downregulation to the same degree as Dvl-interacting RNF43 in STF-Luc Wnt reporter assays using STF293 cells (i.e., HEK293 cells genetically carrying the SuperTopFlash-luciferase (STF-Luc) Wnt reporter, which is a specific and sensitive detector for endogenous Wnt activity) with Wnt3a conditioned media (CM) and flow cytometry (Supplementary Fig. 1c, d). These data further suggest that the Dvl-RNF43-Dvl interaction is dispensable for RNF43-mediated regulation of surface Fzd expression and Wnt/β-catenin signalling in this context.

We have reported that RNF43 amino acids 442–478, close to known Dvl binding regions, are important for RNF43 regulation. Our previous work suggested that the S478 is also indispensable for regulating Rnf43 in other species, were indispensable for RNF43-mediated suppression of Wnt signalling (Fig. 1a–c, Supplementary Fig. 1e, f, h). Based on these facts, we hypothesised that these serines are regulated by phosphorylation. To test this hypothesis, we replaced all three serines with aspartic (3SD) or glutamic acid (3SE) residues to mimic the phosphorylated form of serine. We found that the phosphomimetic RNF43 mutant maintained negative regulation of Fzd and Wnt/β-catenin signalling (Fig. 1c, d, Supplementary Fig. 1g). Conversely, replacing these serines in RNF43 with alanine (3SA) to prevent phosphorylation, or with threonine (3ST), impaired Fzd regulation in other species. However, RNF43(3SA) phospho-mutant did not autonomously activate Wnt signalling but facilitated the signal activity in a Wnt ligand-dependent manner (Supplementary Fig. 1h). Together, these results suggest that phosphorylation of the serine triplet is necessary for RNF43 function.

To demonstrate that these serines are phosphorylated in the normal cellular environment, we performed Phos-tag SDS-PAGE, which can distinguish phosphorylated proteins by a band-shift. This analysis indicated that a signal corresponding to phospho-RNF43 was lost in the phospho-deficient mutant RNF43(3SA) (Supplementary Fig. 2a). In addition, 2D-PAGE revealed the loss of a phospho-RNF43 signal in cells expressing RNF43(3SA), or when cells expressing RNF43(WT) were cultured under phosphate-depleted conditions (Supplementary Fig. 2b, c). Furthermore, 32Pi metabolic labelling of cells revealed significantly lower phosphorylation of RNF43(3SA) than RNF43(WT) (Fig. 1e, Supplementary Fig. 2d). These data support our hypothesis that under normal cellular conditions RNF43 phosphorylation regulates its function.

Our previous report suggested that S478 is also indispensable for regulating RNF43 function. Indeed, we found that substitution of S478 with A or D/E led to the inhibition or activation, respectively, of RNF43 function in STF-Luc assays, similar to substitutions at the conserved serine triplet (Fig. 2a).
The S478A substitution in RNF43 increased the cell surface levels of Fzd compared to WT RNF43 (Fig. 2b), suggesting that the S478 phospho-status also regulates Fzd levels and Wnt signalling.

The serine-rich region of β-catenin, which is similar to SRR2 of RNF43, is sequentially phosphorylated by CK1 and GSK-3β. Therefore, we examined whether stepwise phosphorylation also regulates RNF43. Our STF-Luc reporter assay showed that RNF43 containing 3SA-S478D substitutions impaired its function, whereas RNF43(3SD-S478A) retained function (Fig. 2c). These data suggest that S478 phosphorylation might be upstream of serine triplet phosphorylation and required only to prime the subsequent phosphorylation of the serine triplet.

Next, we examined whether CK1 or GSK-3β phosphorylate RNF43. Flow cytometric analysis showed that GSK-3β inhibition did not alter RNF43-mediated regulation of Fzd surface levels (Fig. 2d). In contrast, CK1 inhibition led to restored surface levels of Fzd in RNF43(WT)- and RNF43(S478D)-expressing cells, but not in RNF43(3SD)-expressing cells (Fig. 2d). These results suggest that the serine triplet is phosphorylated by CK1. Indeed, an in vitro kinase assay detected the CK1-dependent phosphorylation of RNF43(WT) but not RNF43(3SA) (Fig. 2e, Supplementary Fig. 2e) and also revealed the phosphorylation of endogenous RNF43 by CK1 (Supplementary Fig. 2f). Interestingly, the total amount of phosphorylation signal was significantly
increased with both the RNF43(S478D) and the RNF43(3SD) mutant, suggesting that the phosphorylation of the serine triplet is required for additional RNF43 phosphorylation events outside the SSSDS sequence, which we confirmed by MS/MS analysis (Supplementary Fig. 2g, h). Furthermore, a lower level of phosphorylation was observed on oncogenic RNF43(R127P), which accumulates in the ER (Supplementary Fig. 2j) compared to WT RNF43 (Supplementary Fig. 2i). This result suggests that RNF43 might be phosphorylated in a localisation-dependent manner and activated after it leaves the ER.

Collectively, these data demonstrate that RNF43 activity is regulated by multi-step phosphorylation events (Fig. 2h).

**Exogenous RNF43 functions similarly to endogenous RNF43.** First, we confirmed that the STF293 cells we used in this study
The role of a priming phosphorylation was investigated using STF-Luc assay (a, c) and flow cytometric analysis (b) using RNF43 mutants. Luciferase activity or surface Fzd level in empty vector-transfected (NC) or mock cells was set to 1. Grey or black lines, or grey fills indicate not stated, RNF43 stably expressed or mock cells, respectively. Characters shown in red indicate amino acids after substitution (a, c). Surface expression of Fzd was examined via flow cytometric analysis following addition of kinase inhibitors (GSK-3p, CHIR-99021; CK1, IC261). Phosphorylation of RNF43 and mutant forms was examined by an in vitro kinase assay with CK1/2. Phospho-RNF43 levels were normalised to total RNF43 protein levels and normalised phospho-RNF43(WT) levels were set to 1. f The effects of a loss of endogenous RNF43 (KO) or removal of the RNF43 phospho-switch (ΔPS; similar to serine-rich region (SRR2) and SRR2-2 in Supplementary Fig. 1f) were examined in STF293 cells using a STF-luciferase assay. The luciferase activity in parental STF293 cells was set to 1. g Surface Fzd expression on RNF43 KO or ΔPS STF293 cells was evaluated using flow cytometry with pan-Fzd antibodies (Abs). h Schematic of localisation-dependent RNF43 activation via multi-step phosphorylation. RNF43 activity is acquired via phosphorylation at a post-ER stage during or after protein trafficking toward the cell surface. Bar graphs and error bars in this figure represent mean ± standard deviation (sd) of at least three biologically independent experiments. Red circles indicate individual values of each sample. The P values for the indicated comparisons were determined by one-way ANOVA (P < 0.05). n = 3 (a–c, f), n = 4 (e) biologically independent samples. Asterisks or ND indicates significant or no significant difference in indicated comparisons, respectively. All FACS data in this figure was acquired and displayed with same strategy shown in Supplementary Fig. 1g. Each coloured line indicates the property of RNF43 expressing in cells (d, g).

RNF43 phosphorylation promotes ubiquitination of Fzd. To further investigate how RNF43 function is regulated via serine phosphorylation, we examined protein-protein interactions and subcellular localisation of the phospho-mutants. Immunoprecipitation and immunofluorescent experiments suggested that RNF43(3SA) and RNF43(3 SD) behave similarly to RNF43(WT) with regard to protein-protein interactions (Supplementary Fig. 4a), homodimer formation (Supplementary Fig. 4b), heterodimer formation with ZNRF3 (Supplementary Fig. 4c) and endosomal/ER/nuclear localisation (Supplementary Fig. 4d). In addition, expression of ZNRF3 was not perturbed by any of the RNF43 mutants (Supplementary Fig. 4e). Co-immunoprecipitation analysis also revealed that the RNF43 phosphomimetic (3SD) and phosphoresistant (3SA) mutants maintained the interactions with Dvl2 or Fzd5, similar to WT RNF43 (Supplementary Fig. 4f, g). We then considered that serine phosphorylation may change the conformation of the intracellular portion of RNF43 to expose the RING finger domain, which is essential for interactions with E2 enzymes. However, phospho-mutations did not alter binding to the E2 enzyme UbcH5C, which is essential for RNF43-mediated ubiquitination of Fzd26 (Supplementary Fig. 4f). Previously, we and other groups reported the localisation of RNF43 in the nuclear membrane18,28–30 and demonstrated that this protein negatively regulated Wnt/β-catenin signalling by depleting the Tcf4 transcription factor from Wnt target genes29,30. Accordingly, we examined the role of the phospho-switch in this mechanism by directly activating nuclear Wnt/β-catenin signalling with ΔN-β-catenin. As reported, WT RNF43 suppressed Wnt/β-catenin signalling downstream of receptor control, although a marginal level of negative regulation was also observed at the nuclear level (Supplementary Fig. 4i). The functions of all other RNF43 phospho-mutants were similar to the WT.

Based on these negative results, we next hypothesised that RNF43 phosphorylation affects its ability to ubiquitinate Fzd. To test this hypothesis, we treated cells co-expressing Fzd5 and RNF43 with bafilomycin to inhibit lysosome-dependent degradation and monitored the levels of polyubiquitinated Fzd5. Indeed, cells co-expressing RNF43(3SA) displayed less polyubiquitinated Fzd5 than cells co-expressing RNF43(3SD) or WT RNF43 (Supplementary Fig. 4j, see also Fig. 6d). These data suggest that phosphorylation promotes RNF43-mediated ubiquitination, and in turn endocytosis and lysosomal degradation, of Fzd5.

It is currently thought that RNF43-mediated degradation of the substrate Fzd is regulated by RNF43 levels and by R-spondin. Our results reveal an additional layer of regulation in Wnt signalling. In general, phosphorylation of the substrate is known to regulate its degradation, however, we have uncovered phospho-regulation of the ubiquitin ligase RNF43 itself as another way to control degradation of Fzd. We clearly show that phosphorylation of
RNF43 directly facilitates its ubiquitin ligase activity. This phosopho-dependent regulation may function downstream from the well-known RNF43-Rspo-Lgr module.

Phospho-RNF43 regulates morphogenesis and ISC maintenance. We next examined the biological significance of RNF43 phospho-regulation in developing zebrafish embryos. Injection of mRNA encoding RNF43(3SA) into Wnt reporter zebrafish embryos resulted in increased expression of both endogenous Wnt/β-catenin target genes, such as β-catenin, axin2 and nkd1, as well as the reporter gene, egfp. In contrast, injection of mRNA encoding RNF43(WT), but this defect was absent from embryos injected with mRNA encoding RNF43(3SA) (Supplementary Fig. 5b). The activation and expression of target genes in other signalling pathways, including FGFR5 and BMP5, were not perturbed by RNF43 expression in developing zebrafish embryos (Fig. 3b). In addition, we observed defective anterior–posterior (A–P) axis elongation at later stages of development in embryos injected with mRNA encoding RNF43(WT), but this defect was absent from embryos injected with mRNA encoding RNF43(3SA) (Supplementary Fig. 5b). Impaired A–P axis elongation can arise from defective mesoderm induction due to a lack of proficient Wnt/β-catenin signalling activity, or from the loss of convergent extension movement due to deficient non-canonical Wnt signalling. Therefore, we investigated whether non-canonical Wnt signalling activity is altered by RNF43(3SA) expression. In situ hybridisation for the expression of axis-related marker genes showed that the short and wide non-canonical Wnt phenotype induced by expression of RNF43(WT) was mostly absent in embryos expressing RNF43(3SA) (Supplementary Fig. 5c). Together, these data suggest that the defects in A–P axis elongation originate from dysfunction of both canonical Wnt/β-catenin signalling and likely non-canonical Wnt signalling, with minimal influences from other signalling pathways. They also establish a critical role for the S474–476 phospho-switch of RNF43 during embryonic development.

We have reported that RNF43 is expressed in ISCs, where it regulates normal crypt development and stem cell maintenance. RNF43 and ZNRF3 strongly inhibit Wnt/β-catenin signalling to suppress excess proliferation of ISCs, while maintaining Wnt/β-catenin signalling is also important for the self-renewal of ISCs. These paradoxical roles suggest that RNF43 expressed in ISCs might be regulated to fine-tune its activity and maintain ISC self-renewal. Based on this idea, we employed a mouse intestinal organoid model to examine whether the phospho-regulation of RNF43 function contributes to ISC maintenance. Expression of RNF43(3SD) during short-term culture of organoids in the presence of EGF, Noggin and Rspo (the ENR condition) significantly suppressed organoid growth only when the Rspo concentration in the culture medium was low (1% Rspo-conditioned medium). By contrast, expression of RNF43(3SA) did not suppress organoid growth under either low or high (10%) Rspo concentrations but instead improved their viability in the 1% Rspo ENR condition (Fig. 3c, Supplementary Fig. 5d, e). These results suggest that phospho-regulation of RNF43 is required for the survival and the growth of crypts, and that this regulation occurs independently of the RNF43-Rspo-Lgr regulatory module. We then added exogenous Wnt3a to ENR medium (the WENR condition) to rule out a function of the Paneth cell-niche. Again, ISCs failed to maintain the organoid culture after the second passage when expressing RNF43(3SD) (Fig. 3d, Supplementary Fig. 5f), suggesting the importance of phospho-regulation of RNF43 in ISC maintenance during long-term culture. These results reveal that phospho-regulation of RNF43 functions as an additional regulatory layer of Wnt signalling and suggest the functional importance of maintaining RNF43 in a low-phosphorylation state in ISCs. This RNF43 phospho-regulation ensures tighter control of ISC activity together with the Rspo-Lgr4/5 regulator of RNF43.

RNF43(3SA) cooperates with active Ras to induce tumours. Inactivation of RNF43 and ZNRF3 induces Wnt-dependent adenoma formation, and these genes are frequently mutated in various human cancers. These studies suggested essential roles of RNF43/ZNRF3 in suppressing tumorigenesis in both mice and humans. Indeed, several cancer-associated mutations in the N-terminal extracellular domain of RNF43 greatly increase Wnt/β-catenin signalling activity in a Wnt-dependent, but Rspo-independent, manner. To further investigate the role of RNF43 in tumorigenesis, we identified patient mutations downstream of the RING-finger domain using the COSMIC database for mutations in cancer. Twenty-three patient tumour mutations within the cytoplasmic region after the RING-finger domain, but outside the SSSDS sequence, did not alter RNF43 function in the STF-Luc reporter assay (Supplementary Fig. 6a), suggesting that these are passenger mutations. In contrast, we found that four naturally occurring cancer-associated mutations within the SSSDS sequence inhibit or are predicted to inhibit RNF43-mediated repression of Wnt/β-catenin signalling (Figs. 1b, 2a, Supplementary Fig. 6b, c), suggesting that loss of RNF43 phospho-regulation contributes to tumorigenesis.

To directly investigate the role of the RNF43 phospho-regulation in tumorigenesis, we established cell lines that stably express RNF43 constructs (Supplementary Fig. 7a). We first used the non-tumour cell line NIH3T3 to avoid unexpected genomic variations that frequently occur in cancer-derived cell lines due to their genomic instability. NIH3T3 cells expressing dominant-negative RNF43(3SA) did not acquire a transformed phenotype, as assessed by anchorage-independent cell growth (Supplementary Fig. 7b, left panel) and allograft transplantation into nude mice (Fig. 4a, left panel). These data suggest that the gain of RNF43-mediated facilitation of Wnt/β-catenin signalling alone is not sufficient for tumorigenesis. Given the high co-occurrence of RNF43 mutations with activating KRAS mutations in human pancreatic tumours, we next investigated the oncogenic properties of RNF43(3SA) in NIH3T3 cells that contain mutant, active Ras (Cle-H3 cells). Strikingly, Cle-H3 cells expressing RNF43(3SA) exhibited greatly accelerated anchorage-independent colony formation, spheroid formation and tumour growth in nude mice (Fig. 4a–d, Supplementary Fig. 7a–c). In fact, RNF43(3SA) displayed similar oncogenic properties as an established cancer-associated R127P mutation in RNF43, which is within the extracellular protease-associated (PA) domain and abolishes inhibition of Wnt signalling. These data suggest that the phospho-switch mutation (3SA) has a similar dominant-negative effect observed in previous known oncogenic mutations found in the PA domain and that the loss of RNF43 phosphorlation cooperates with active Ras to promote tumorigenesis in vivo. These established tumours arising from Cle-H3 cells expressing RNF43(3SA) displayed a strong cytoplasmic accumulation of β-catenin as previously described in RNF43/ZNRF3 DKO intestine (Supplementary Fig. 7d).
response in the absence of endogenous RNF43 overexpression induced by aberrant Wnt signalling. We found that the RNF43 mutants (3SA, 3SD, R127P) retained DNA damage-induced p53 expression and its nuclear localisation, and suppressed p53-dependent induction of p21 and Bax, similar to WT RNF43, suggesting that the nuclear function of RNF43 does not require phosphorylation and ubiquitinating activity (Fig. 5a, Supplementary Figs. 4i, 8). Furthermore, downregulation of p53 target genes by RNF43 and its derivatives were completely abolished in MB352 cells with the lack of TP53 gene, whereas it was maintained in the culture of STF293 cells with iCRT3, which inhibits β-catenin and Tcf/Lef binding (Supplementary Fig. 8a, b). These data provide further support that RNF43 does not degrade p53 and does not suppress p53 target genes via the Wnt-myc-p21 pathway\textsuperscript{43} but suppresses p53-dependent transcription\textsuperscript{20}, and that it controls multiple signalling pathways via distinct...
activating mutations in Ras have the potential to facilitate tumorigenesis (Fig. 5d). The positive feedback circuit of Wnt signalling, which we have reported previously, not only enhances oncogenic RNF43 induces its own expression by a positive feedback circuit of Wnt signalling, which we have reported previously, and then further suppresses the p53 pathway to facilitate tumorigenesis (Fig. 5d).

Our data suggest that RNF43 mutations combined with activating mutations in Ras have the potential to fulfil not only two but all three steps of the traditional multi-step model of colon carcinogenesis (concurrent Wnt activation and p53 inactivation by RNF43 mutation and Ras activation by KRAS mutation).

To investigate the relevance of our findings to human cancer, we performed a comprehensive analysis of The Cancer Genome Atlas (TCGA) database to determine whether mutations in these genes affect patient outcome. This analysis confirmed that...
mutations in RNF43 or ZNRF3 occur independently, supporting our theory that oncogenic RNF43 and ZNRF3 mutations act as dominant negatives.18 (Supplementary Fig. 9a). Furthermore, we found that co-occurrence of KRAS and TP53 mutations is significantly associated with poor outcome (Supplementary Fig. 9b), as reported recently.47 RNF43 mutations were generally associated with poor outcome in patients with colorectal cancer, regardless of the microsatellite instability phenotype (MSI) status (Supplementary Fig. 9c). Importantly, Kaplan–Meier analysis revealed that co-occurrence of RNF43 and KRAS mutations was associated with a poorer outcome for colorectal cancer, compared to single mutations of each gene (Fig. 5b), although the samples size was not enough to obtain significant differences. In contrast, TP53 mutations did not alter the outcome of colon cancer patients with RNF43 mutations (Fig. 5c), supporting that RNF43 functions in the p53 pathway as shown in Fig. 5a and Supplementary Fig. 8. Moreover, colorectal tumours with mutations in RNF43 lacked mutations in APC and TP53. Overall, our findings provide insight into why the co-occurrence of mutations in two genes, RNF43 and Ras, greatly

**Fig. 5** Cooperation of mutant RNF43 with active Ras establishes Wnt-Ras-p53 axis. a Induction of p53 and p21 protein was examined by immunoblotting (IB) with Eto treatment. Expression of p53 and p21 in empty vector-transfected NC cells with DMSO treatment was set to 1. Bar graphs and error bars represent mean ± sd. Red circles indicate individual values of each sample. Asterisks indicate significant differences from NC cells stimulated with Eto (P < 0.05, one-way ANOVA, n = 3 biological replicates). ND indicates no significant difference. b, c Prognosis of patients with colorectal tumour that carry genetic mutations in RNF43 with or without KRAS (b) or in RNF43 with or without TP53 (c) is shown. Sample number and P value determined by log-rank test (b, c) with Holm adjustment for multiple comparisons (b) are indicated in each graph. d Schematic of biological role of RNF43 oncogenic mutations in multi-step tumorigenesis. Oncogenic RNF43 mutants that promote Wnt signalling and inhibit the p53 pathway cooperate with activating Ras mutations to complete all the steps of multi-step colorectal tumorigenesis.
accelerate tumorigenesis: RNF43-KRAS mutations cooperate to establish the Wnt-Ras-p53 axis, supporting the multi-step model of colorectal carcinogenesis (Fig. 5d).

Phosphorylation converts onco-RNF43 to a tumour suppressor. Our data suggest that the 3SA and 3SD mutations abrogate and facilitate, respectively, RNF43-mediated ubiquitination of Fzd and inhibition of Wnt/β-catenin signalling (Figs. 1c, d, 6d, Supplementary Fig. 4j). To examine whether the phosphorylation of RNF43 can modulate the activity of distant oncogenic mutations in the PA domain or in the RING-finger domain of RNF43, we introduced the 3SA or 3SD mutations into RNF43(I48T), RNF43(R127P) and RNF43(H292R) backgrounds. We previously showed that the RNF43(I48T) and RNF43(R127P) mutations affects the subcellular localisation of RNF43 and impairs the function of RNF43. Interestingly, the introduction of the 3SD, but not 3SA, mutations into the oncogenic RNF43 (I48T) and RNF43(R127P) backgrounds could partially rescue RNF43-mediated inhibition of Wnt signalling, as assessed by the STF-Luc assay (Fig. 6a, Supplementary Fig. 10a). In contrast, 3SD phosphomimetic substitution did not recover the RING domain-dependent ubiquitination activity of RNF43, which further supports the presence of a phospho-switch (Supplementary Fig. 10a). In addition, the 3SD substitution almost completely abolished the oncogenic activity of the RNF43(R127P) both in vitro and in vivo (Fig. 6b, c, Supplementary Fig. 10b). However, the introduction of 3SD did not alter the levels of RNF43(R127P) protein (Supplementary Fig. 7a) or its aberrant ER localisation that we reported previously (Supplementary Fig. 10c). Thus, phosphomimetic mutations within the conserved serine triplet can revert diverse Rspo-independent dominant-negative RNF43 mutants back to functional negative regulators of Wnt signalling, irrespective of whether the oncogenic mutation is extracellular (I48T, R127P; Fig. 6a, Supplementary Fig. 10a) or intracellular (S478A; Fig. 2a). Further, our results suggest that forced phosphorylation or substitution of the conserved serine triplet might represent a promising therapeutic approach (Fig. 5d).

Based on these data, and our discovery that phosphorylation of RNF43 affects ubiquitination of Fzd5 (Supplementary Fig. 4j), we hypothesised that the R127P oncogenic mutation reduces RNF43-mediated ubiquitination of Fzd5, and that the 3SD mutations restore ubiquitination activity to the R127P mutant. To test this hypothesis, we again treated cells co-expressing Fzd5 and RNF43 mutants with bafilomycin to inhibit lysosome-dependent degradation and monitored the levels of ubiquitinated Fzd5. As expected, we found that cells co-expressing Fzd5 and RNF43 mutants with bafilomycin to inhibit lysosome-dependent degradation and monitored the levels of ubiquitinated Fzd5. As expected, we found that cells co-expressing Fzd5 and RNF43 displayed less ubiquitinated Fzd5 than cells co-expressing wild-type RNF43 (Fig. 6d). Strikingly, the RNF43(R127P-3SD) showed restored ubiquitination of Fzd5 relative to RNF43(R127P) (Fig. 6d). Thus, mislocalisation of RNF43(R127P) reduces RNF43-mediated ubiquitination of Fzd5, and the 3SD phosphomimetic substitution can restore the ubiquitination and tumour suppressor activity to RNF43(R127P). These findings reveal phosphorylation of RNF43 as a potential therapeutic target to restore the inhibitory role of RNF43.

Discussion

Our work shows that phosphorylation of three conserved serines regulates the function and oncogenic potential of RNF43 by influencing the ubiquitination and subsequent lysosomal degradation of Fzd. Phosphorylation of RNF43 is required to negatively regulate canonical and non-canonical Wnt signalling during embryonic development and in adult stem cells. Dysregulation of RNF43 phosphorylation leads to a breakdown in homeostasis and an increase in oncogenic activity. Thus, both multi-step phosphorylation and Rspo-Lgr4/5/6 regulate RNF43 to control the surface level of Fzd and Wnt signalling activity (Fig. 6e). Furthermore, phosphorylation status seems to be the most critical regulator of RNF43, as it acts downstream of Rspo/Lgr5. Therefore, the phosphatase and the upstream signal that regulates RNF43 phospho-status should be identified to fully understand the mechanism of Wnt signalling regulation.

Importantly, combining phosphomimetic substitution with distant oncogenic RNF43 mutations (i.e. I48T and R127P) restores RNF43-mediated tumour suppression. The R127P mutation leads to mislocalisation of RNF43, which preclude phosphorylation at the conserved serine triplet. The 3SD mutation restored RNF43(R127P)-mediated ubiquitination of Fzd, apparently without restoring the localisation of this mutant. In addition, RNF43(R127P) exhibited a lower level of phosphorylation relative to RNF43(WT), suggesting that the function of RNF43 is not regulated directly by localisation, but by localisation-dependent phosphorylation. Our data thus indicate that RNF43 is a therapeutic target for patients harbouring oncogenic mutations outside the serine triplet at the RING-finger domain. Suppressing RNF43 mutation-dependent tumorigenesis using Wnt inhibitors such as porcupine inhibitors (e.g., IWP-2) may be effective, because oncogenic RNF43 facilitates signalling activity in the presence of Wnt, as shown in our reporter assays, and we have already reported that tumorigenic hyperplasia of intestinal organs in the absence of both RNF43 and ZNRF3 is inhibited by IWP-2 treatment. However, such a general inhibition of Wnt production affects a broad array of cell types and organs that maintain homeostasis under the control of Wnt, regardless of RNF43 expression, and so may have serious side effects. Additionally, inhibiting the Rspo-Lgr module may not be effective for anti-cancer therapy, as we have previously found that oncogenic RNF43 does not require Rspo for the acceleration of Wnt signalling. In contrast, the recovery of RNF43 activity by targeting serine phosphorylation could be a potential approach for tumour suppression with milder side effects, since it is predicted to only affect RNF43 mutant cells.

Recently, another research group observed the apparent hyperproliferation of the gastric mucosa in RING-dead RNF43 mutant mice, although these mice developed healthy intestines and did not exhibit neoplastic expansion of the ISC region, as reported previously in RNF43/ZNRF3 DcKO mice. These results may indicate the importance of another function of RNF43, namely suppression of the p53 pathway. We demonstrate in Figs. 5a and 6d and Supplementary Fig. 8 that this suppression does not require the RING-finger domain-dependent ubiquitinating activity of RNF43, which is essential for the degradation of Fzd. RNF43 mutants that lack ubiquitination activity because of extracellular mutation (R127P) or a broken phospho-switch (3SA) retain the ability to suppress p53, whereas a lack of RNF43 expression causes a loss of suppression of both the Wnt and p53 pathways. Therefore, the ability of ubiquitination-dead mutants to suppress the p53 pathway may depend on the binding of RNF43 to p53 but not on phosphorylation, similar to the suppression of Wnt signalling by the nuclear RNF43-Tcf4 interaction. In Supplementary Fig. 4i, we indeed demonstrate suppression of Wnt signalling via nuclear RNF43-Tcf4 binding, but at a marginal level relative to the mechanism associated with Fzd degradation at the surface. We previously reported that RNF43 is a direct target of Wnt signalling; namely, a feedback loop is established (Fig. 5d), and mutations can further induce Fzd accumulation and p53 inhibition. In this study, we demonstrated that a RNF43 mutation can cooperate with KRAS to induce Wnt-RAS-p53 axis activity and thus drive tumorigenesis.

Furthermore, we demonstrated that mutations in the phosphoregulated serines that affect Wnt signalling did not alter RNF43-
mediated p53 inhibition. Unfortunately, the number of patient tumour samples containing specific RNF43 missense mutations that reliably induce excessive Wnt signalling and/or maintain p53 inactivation is currently insufficient for database analysis (Supplementary Fig. 6a, b)\(^1\). Thus, we could not complete the analyses for the prognosis of colorectal tumour patients to clarify the roles of RNF43 phospho-regulation in tumorigenesis due to an insufficient numbers of samples.

The current databases do not classify correctly or in detail the type of mutations within a gene. It would be necessary to classify the type of RNF43 mutations as complete or partial deletions, or missense mutations, and to link them to functional changes, in order to fully understand the molecular and cellular roles for these mutations in tumorigenesis. This is especially so because RNF43 suppresses the p53 and Wnt signalling pathways by different mechanisms. It was recently reported that an RNF43
Serine phosphorylation reverses oncogenic RNF43 to a tumour suppressor. a The role of serine phosphorylation was examined using STF-Luc assay in an RNF43(R127P) mutant background. Luciferase activity in mock-transfected cells was set to 1 (mean ± sd). Schematic of RNF43 mutants used in Figs. 5, 6. Supplementary Fig. 7, 9 is shown. Independent measurements of each sample were performed in triplicate. Asterisks indicate significant differences (P < 0.05, one-way ANOVA, n = 3 biologically independent samples) from RNF43(R127P) cells. b Colony-forming activity was evaluated following expression of RNF43 phospho-mutant forms in Cle-H3 cells via soft agar assay and volume of colonies was estimated. Scale bars, 100 µm. Asterisks indicate significant differences from RNF43(R127P) tumour. c Tumour growth was examined in nude mice with Cle-H3 cells following the expression of RNF43 phospho-mutant forms at 5 wks after Cle-H3 injection and tumour weight was measured. Images for all of the tumours are shown. Scale bar, 1 cm. NT indicates no tumour observed. Bar graphs and error bars in (b, c) represents mean ± sem of biologically independent samples. Red circles indicate individual values of each sample. P values for the indicated comparisons were determined by one-way ANOVA (P < 0.05). n = 98–134 (b), n = 6–12 (c) biologically independent samples. Asterisks or ND indicates significant or no significant difference in indicated comparisons, respectively. d Ubiquitination of Fzd5 by RNF43 phospho-mutants was examined with baflomycin A1, by immunoprecipitation (IP)-IB experiments. e Schematic of molecular mechanism and biological role of RNF43 phosphorylation in Wnt signalling and multi-step tumorigenesis. Wild-type RNF43 is activated by serine mutation. wild type RNF43 is activated by serine mutation. The Wnt antagonist DKK1 and putative post-translational modification, the phospho-switch, demonstrated that many cytoplasmic mutations are passengers, except those in the RING domain and the phospho-switch, whereas mutations in the PA domain confer an oncogenic effect. Our careful analysis of missense mutations in RNF43 has provided significant insights into the operation of this tumour suppressor with three functional domains: the extracellular PA domain for interaction with Fzd or Rsps, the RING domain for ubiquitination and a phospho-switch for functional control. We also identified a critical role of the phospho-switch by demonstrating its epistatic control of mutations in the PA domain. Our findings suggest that therapeutic phosphomimetics could revert an oncogenic RNF43 mutant to a functional tumour suppressor.

Methods

Accession numbers of RNF43 and ZNRF3. Human RNF43, NP_001294273. Human ZNRF3, NP_001193927. Mouse Dvl2, NP_031914. Mouse Fzd5, NP_001036124. Mouse RNF43, NP_766036. Naked-mole rat RNF43, NP_001036124. Bengalese finch RNF43, XP_021332049. Pigeon raven RNF43, XP_021124322. Bengalese finch RNF43, XP_021396062. Three-toed box turtle RNF43, XP_026514312. Tropical clawed frog RNF43, XP_002935238. Zebrafish RNF43, XP_02132049.

Plasmids. The mammalian expression vectors pDNA3-hRNF43(WT, Δ366–441, Δ366–478, 148T, R127P)-HA, pcS2 + FLAG-hRNF43 and pcS2-myc-mdR2 were described in our previous reports18. A series of deletion mutants, pcDNA3-Δ476A:S478D (Δ5SA-SD), 3SA, 3SD, 3SE, S474Δ, S476A (3SA), S474Δ, S476D (3SD), S474Δ, S476E (3SE), S474Δ, S476A (3SA), S474Δ, S476E (3SE), S474Δ, S476A (3SA), S474Δ, S476D (3SD), S478E (SE), S478P (SP), S474Δ, S476A:S478D (3SA:SD), S474Δ, S476A:S478E (3SA:SE), H292R, S539A, E318D, Q344H, Y357C, R389I, L148M, G427E, V479L, T883M, S532F, S532D, R519Q, E541K, H549N, R554G, P569H, P587S, R600S, S607L, D628G, E662K, H726L, Δ12, ΔSRR2-2, ΔSRR2-3 were inserted into pMX-puro vectors for the derivation of stable cell lines (3SA, 3SD, 3SE, S478A, R127P and R127-3SD) bearing HA or x2 FLAG-HA tags were lysed in 100 μl of cell culture lysis reagent. The luciferase activity was measured with the Luciferase Assay System (E1501, Promega) using 10 μl of cell lysate. p53-dependent p21 expression. Luciferase assays. STF293 cells stably expressing HA-tagged RNF43 WT or mutant forms or STF293 control cells were seeded into 24-well plates (5 x 104 cells) and transfected with RNF43 expression plasmids using FuGENE HD Transfection Reagent (E231A, Promega). Cells were incubated according to the manufacturer’s protocol. For retrovirus-mediated gene transduction, NIH3T3, Cle-H3, NIH3T3 and STF293 cells were infected with retroviruses produced in Plat-E or Plat-A packing cells53. These cells were then cultured in the presence of 5 μg/ml puromycin (P8833, Sigma) for 1 week. Stable expression of RNF43 constructs was confirmed by immunoblot analysis. CHIR99021 (3 μM; SML-1046, Sigma) and IC261 (2 μM; ab-initio) were used to inhibit the kinases GSK-3β and CK1 respectively. The IC50 (50 μM; SML-0211, Sigma) was used to inhibit the binding between β-catenin and Tcf/Lef. Etoposide (20 μM, VP-16, E1383, Sigma) was used for 12 h to induce p53-dependent p21 expression.

Immunoprecipitation and immunoblotting. Cells expressing RNF43 constructs were lysed with IP lysis buffer containing 30 mM Tris-HCl (pH 7.6), 130 mM NaCl, 1 mM Na3VO4, 0.4% NP-40, 1 mM PMSF, 10 mM sodium pyrophosphate and Complete Mini EDTA-free (155410, Roche). Lysates were incubated on ice for 20 min and then centrifuged at 16,000g for 20 min at 4°C. After determination of protein concentration via the Bradford assay (500-0006, Protein Assay, Bio-Rad), cell lysates (10 μg/lane) were subjected to SDS-PAGE on 8–10% acrylamide gels in the presence or absence of 50 μM Phos-tag Acrylamide (AAL-107, FujiFilm-Wako) and separated proteins were transferred to an Immobilon-P membrane (IPVH00100, Millipore). The membranes were probed with antibodies against HA (HA-11-16812, MMS-101R, Covance) at 1:5000 dilution, Myc (9E10, PRB-150P, Covance) at 1:5000 dilution, Flag (F13E6, M2, Covance) at 1:5000 dilution, c-myc (sc-764, Santa Cruz Biotechnology) and 1:800 dilution, ubiquitin (sc-8017, Santa Cruz Biotechnology) at 1:5000 dilution, HH2B (sc-165128, Cell Signaling Technology) at 1:1000 dilution (both), p53 (sc-126, DO-1, Santa Cruz Biotechnology) at 1:1000 dilution, p21 (sc-6246, F-5, Santa Cruz Biotechnology and 614014, Cell Signaling Technology) at 1:1000 dilution (both), β-actin (sc-8432, Cell Signaling Technology) at 1:10000 dilution, tubulin (sc-8017, Santa Cruz Biotechnology) at 1:10000 dilution, IRE1α (sc-3929, Cell Signaling Technology) at 1:10000 dilution, m-RTK (sc-6442, Cell Signaling Technology) at 1:1000 dilution, Cdc25A (sc-13685, Santa Cruz Biotechnology) at 1:500 dilution, β-actin (sc-8432, Cell Signaling Technology) at 1:10000 dilution, IRE1α (sc-3929, Cell Signaling Technology) at 1:10000 dilution, m-RTK (sc-6442, Cell Signaling Technology) at 1:1000 dilution.
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Phosphate depletion culture and 32P, metabolic labelling. Depletion of phosphate and 32P, metabolic labelling of RNF43 were performed as described below. Cells expressing exogenous RNF43 or carrying HA-tagged endogenous RNF43 were incubated under normal conditions for 42–48 h, washed twice before culture with sodium phosphate-free DMEM (11971, Gibco) containing 10% dextran FCS (04-311-113, Biological Industries) and then cultured for 2 h to deplete phosphate from cellular proteins. These cells were either directly subjected to 2D-PAGE and immunoblot experiments or underwent metabolic labelling of RNF43. For metabolic labelling, phosphate-depleted cells were subsequently cultured in phosphate-depleted culture media containing 1 mM of 32P orthophosphoric acid (32P) (NE05835, Parkin Elmer) for 4 h in order to label cellular proteins. After extensive washing, labelled cells were lysed with IP lysis buffer and underwent immunoprecipitation as detailed above using anti-HA agarose beads or anti-FLAG M2 beads (A2220, Sigma) or anti-HA agarose beads (A2095, Sigma) for 8–12 h. Beads were washed 5 times with IP lysis buffer and the retained proteins were eluted by incubation with 200 μg/ml 3XFLAG peptide for 20 min at room temperature (F4799, Sigma) or by boiling with SDS sample buffer for immunoblot analysis. Protein levels were quantified with densitometry using ImageJ software (NIH) and normalised to an internal loading control (GAPDH).

Flow cytometry. Cells expressing RNF43 constructs were cultured in the presence or absence of small molecule kinase inhibitors (GSK-3β, 3 μM CHIR-99021 for 24 h or Cki1, 2 μM IC261 for 4 h) or Rapo (10 ng/ml for 3 h) and then harvested using PBS containing 1 mM EDTA and resuspended in FACS staining buffer (PBS containing 0.1% BSA and 0.02% sodium azide). Single-cell suspensions of STF293 cells (1 × 106 cells) were transferred for 45 min on ice with a combination of anti–pan Fzd (OMPR-18R5, gift kind of A. Gurney, Oncomed) at 1:100 dilution and anti-human IgG-ICTIC (109-095-098, Jackson ImmunoResearch) antibodies at 1:250 dilution. All analyses were performed using a FACS Calibur flow cytometer and CellQuest Ver. 3.3 (Becton Dickinson, BD) software. All graphs are presented with normalised scales for every histogram. All SDS data in this study were acquired and displayed with the same strategy shown in Supplementary Fig. 1g.

Cellular localisation analysis. Localisation of RNF43 mutants was examined with the expression vectors for RNF43(WT, 3SA and 3SD)-EGFP or RNF43(WT, 3SA, 3SD, R127P and R127P-3SD)-HA with anti-HA-Ab or Anti-HA antibodies and Alexa488 Tyramide SuperBoost kit (B49012, Invitrogen) in Ha-1 cells fixed with 2% formalin, stained according to manufacturer's standard protocol, then images of cells were taken with BX51 fluorescent microscope, DP71 camera, DP controller Ver. 3.1.1.267 and DP Manager software Ver. 3.1.1.208 (Olympus).

Phosphorylation analysis. Mass spectrometry analysis of the RNF43 constructs was performed with an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with a nanoLC instrument (Advance, Michrom Bioresources, Auburn, CA) and HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). Peptide separation was performed with an in-house pulled fused silica capillary (internal diameter: 0.1 mm; length: 10 cm; tip internal diameter, 0.05 mm) packed with 3-μm C18 L-column (Chemicals Evaluation and Research Institute, Japan). The mobile phases consisted of 0.1% formic acid (A) and 100% acetonitrile (B). Peptides were eluted with a gradient of 5-35% B for 40 min at a flow rate of 300 nL/min. Collision-induced dissociation (CID) spectra were acquired automatically in the data-dependent scan mode with the dynamic exclusion option. Full MS spectra were obtained with Orbitrap in the mass/charge (m/z) range of 300-2000 with a resolution of 60,000 at m/z 400. The 12 most intense precursor ions for in the full MS spectra were selected for subsequent ion-trap MS/MS analysis with the automated gain control (AGC) mode. The AGC were set to 1.0 × 106 for full MS, 1.0 × 105 for CID MS/MS. The normalised collision energy values were set to 35%. Lock mass function was activated to minimise mass error during analysis. The peak lists were generated by Msn.exe (Thermo Fisher Scientific) with a minimum scan/group value of 1 and were compared with IPI_Human database using the Mascot for determining serine, threonine and tyrosine phosphorylation on RNF43.

Identification of phospho-proteins by LC-MS/MS analysis. STEF293 cells stably expressing RNF43 were lysed with IP lysis buffer 48 h after seeding. Lysates underwent immunoprecipitation as detailed above using anti-HA agarose beads before being washed 7 times and RNF43 eluted by incubation using 100 μg/ml HA peptide (E1149, SIGMA) for 20 min at room temperature. Eluted proteins were separated by SDS-PAGE on 8% acrylamide gels before undergoing silver staining. The bands corresponding to RNF43 (~100 kDa) were excised from the stained gel and the protein therein was subjected to in-gel digestion with trypsin or chymotrypsin. Identification of proteins was performed using our standard protocol as described below. Resulting peptides were dissolved in a solution containing 0.1% trifluoroacetic acid and 2% acetonitrile and analysed by an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with a nanoeLC instrument (Advance, Michrom Bioresources, Auburn, CA) and HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). Peptide separation was performed with an in-house pulled fused silica capillary (internal diameter: 0.1 mm; length: 10 cm; tip internal diameter, 0.05 mm) packed with 3-μm C18 L-column (Chemicals Evaluation and Research Institute, Japan). The mobile phases consisted of 0.1% formic acid (A) and 100% acetonitrile (B). Peptides were eluted with a gradient of 5-35% B for 40 min at a flow rate of 300 nL/min. Collision-induced dissociation (CID) spectra were acquired automatically in the data-dependent scan mode with the dynamic exclusion option. Full MS spectra were obtained with Orbitrap in the mass/charge (m/z) range of 300-2000 with a resolution of 60,000 at m/z 400. The 12 most intense precursor ions for in the full MS spectra were selected for subsequent ion-trap MS/MS analysis with the automated gain control (AGC) mode. The AGC were set to 1.0 × 106 for full MS, 1.0 × 105 for CID MS/MS. The normalised collision energy values were set to 35%. Lock mass function was activated to minimise mass error during analysis. The peak lists were generated by Msn.exe (Thermo Fisher Scientific) with a minimum scan/group value of 1 and were compared with IPI_Human database using the Mascot for determining serine, threonine and tyrosine phosphorylation on RNF43.
(12,000g, 20 min) and cytoplasmic fractions (supernatant). ER membrane was col-
lected by IP with Anti-IRE1 antibodies (#3294, LC10, Cell Signaling Technology) at 1:40 dilution. Endogenous RNF43 protein was concentrated from each cellular fraction via IP with anti-HA antibodies in a cell lysis buffer. Subcellular localisa-
tions of RNF43 were confirmed by immunoblotting using the antibodies indicated.

Zebrafish maintenance and injection into zebrafish eggs. Wnt reporter zebr-
afish (AB, OTMz2EGFP-transgenic fish) were raised and maintained under
standard conditions12. Experimental zebrafish care was performed in accordance
with institutional (Gunma and Osaka University) and national guidelines and
regulations. For all injections, 50–150 pg mRNA encoding human RNF43 was
injected into zebrafish eggs at the one-cell stage.

Quantitative PCR (qPCR). Total RNA was isolated from HCT116, STF293 cells or
staged OTM:d2EGFP-transgenic zebrafish homogenised in TriZol reagent (15596018, Invitrogen). 1 μg RNA was used for cDNA synthesis using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (FSQ-301, Toyobo). Quantitative
real-time PCR (qPCR) for d2EGFP, Hbax, axin2, nk1l, mzrf3 and β-actin or hCDKNA1,
hK2, εZNF3 and GADPH (primers detailed in Supplementary Method) was performed using standard PCR conditions on a StepOnePlus Real-Time PCR System
(Applied Biosystems) or Mx3000P Real-Time qPCR System (Agilent) with Power
SYBR Green PCR Master Mix (436793, Applied Biosystems) or TUNDERBRD SYBR qPCR Mix (QPS-101, Toyobo). Expression levels were normalised to β-actin
expression. All experiments were performed in triplicate. The sequence of all primers
used for qPCR in this study are detailed in Supplementary Methods.

Whole mount in situ hybridisation. Digoxigenin-labelled RNA antisense probes for
in situ hybridisation were generated via in vitro transcription using plasmids
containing full-length cDNA for myoD18, RelB and dlx3b according to the
manufacturer’s protocol (DIG RNA labelling kit; 11175025910, Roche Life Sci-
ence). Whole mount in situ hybridisation was performed according to a standard
protocol. Zebrafish embryos were fixed in 4% paraformaldehyde in PBS, antisense
probes hybridised and stained with BM purple (1144207401, Roche Life Science).

Organoid experiments. Small intestine crypts were isolated from Vil-creERT2
mice and organoids were established using general procedure23, 25. Mouse intestine
washed with cold PBS, segmented, and removed villi by scraping. Then, crypts
isolated using Gentle Cell Dissociation Reagent (07174, STEMCELL technologies).
After counting isolated crypts, 100 crypts embedded in Matrigel (356255, Corning)
with supplement of growth factors. Medium refreshed every other day. Organoids
were maintained in organoid culture conditioned medium for retrovirus-mediated
genesis transcription. Retrovirus containing human RNF43, 3SD or SSA constructs were
produced in Plat-E cells53 (RV-101, Cell Biolabs). Retrovirus containing media was
concentrated using Retrovirus Concentrator (631456, Takara) and introduced to
organoid cultures in the standard condition54. Organoid cultured in the presence of WonD3a and Nicotinamide 3 days before infection. On the infection
day, organoids were inactivated by mechanical dissociation followed by chemical
dissociation using TriPLE (12605-010, Invitrogen) at 37°C. Fragmented organoid
fragments and collected embedded in Matrigel with culture medium containing Y-27632
(70503, Sigma). Infected organoids were selected from 3 days post-infection for
1 week with 2.5 μg/ml puromycin in order to remove uninfected organoids.
Expression of RNF43 proteins was induced by treating transduced organoids
with 1 μM 4-hydroxytamoxifen (4-OHT, T5648, Sigma) for 6 h at 37 °C. Organoids were
infected on day 6 or 10 of culture. The minor axis (S) and major axis (L) of each colony were measured at day
10, organoids fragmented by mechanical dissociation followed by chemical dis-
integration (1.5% NGS and 0.1% BSA in TBST after blocking with 5% NGS in TBST.
Tumour images were taken using a confocal laser scanning microscope (Carl Zeiss,
Axio Imager Z1 & LSM700) equipped with a water-immersion ×40 objective lens
(C-Apochromat 40 ×1.20 W Corr M27) and ZEN Black 2011 software (Zeiss). Z-
stack images were processed and arranged using Imagej software (1.52 v, NIH) and
Photoshop CSS (12.0 × 64, Adobe).

TCGA database analysis. All TCGA analysis was performed on The Cancer
Genome Atlas website with datasets of patients with any tumour type (all tumour)
or colorectal tumour containing mutations in RNF43, KRAS and/or TP53. All
analyses were performed on the public website, The Cancer Genome Atlas
(https://cancergenome.nih.gov) and GDC Data Portal (https://portal.gdc.cancer.gov).

Statistics and reproducibility. All P values between samples in all experiments were
determined via one-way analysis of variance (one-way ANOVA), or log-rank test
on GDC Data Portal (https://portal.gdc.cancer.gov). Error bars represent standard
deviation (sd) or standard error of the mean (sem) as indicated. All raw data and
exact P values in the analyses of this study are shown in Source Data file. The reproducibility of all experiments is shown as number of repeated/number of similar results. Supplementary Fig. 5a showed n = 3/3 reproducibility. Supplementary
Figures 2a–c, g, j, 5a, 4a–d, f, h, 7d, 10c showed n = 2/2 reproducibility. Experiments in Figs. 2c, 3a,d,e, 4j, 6d, 7a, 8a–c, 10b,c were performed once. The result in Fig. 6d and Supplementary Fig. 4i directly supports each other. Result in all experiments that were not repeated was highly consistent in fact/theory with the results from other experiments in this or in our past study.

Website. All mutations in the human RNF43 gene were retrieved from the Cat-
ologue of Somatic Mutations In Cancer database (https://cancer.sanger.ac.uk/
cosmic), The Cancer Genome Atlas (https://cancergenome.nih.gov) GDC Data
Portal (https://portal.gdc.cancer.gov) and cBioPortal database (http://www.
cbiportal.org).

Reporting summary. Further information on research design is available in the Nature
Research Reporting Summary linked to this article.

Data availability
The mass spectrometric datasets used in Supplementary Fig. 2h have been deposited in the
ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/) via
the via the iPOST partner repository under dataset identifiers PXD020598 and
PXDD02059. All full scan images of our blotting data used in this study are shown in Source Data file. Source data are provided with this paper.

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Author contributions
T.T. performed most of the biochemical, cellular and mouse experiments. J.Z., S.O. and T.M. performed zebrafish experiments. J.K. and A.M. performed intestinal organoid experiments. M.M. performed MS/MS experiments and data analysis. Y.S performed TCGA database analysis. Y.F. and T.H. performed immunofluorescent experiments. H.T. and S.T. provided technical assistance. T.I. supervised J.Z., S.O. and T.M. B-K.K supervised J.K. and A.M. H.T. supervised T.H. K.I.N. supervised M.M. Y.O. supervised Y.F. T.T. supervised Y.S., S.T. and the project as a whole. T.T. and B-K.K. wrote the manuscript together with T.I., S.H. and input from all other authors.

Competing interests
The authors declare no competing interests.

Additional information
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