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# RNF43 is frequently mutated in colorectal and endometrial cancers

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Contributions
M.G., E.H., W.C.H, G.G., S.B.G., E.S.L., S.O., C.S.F., and L.A.G. designed research. M.G., E.H., X.J.M., M.Y., J. R., K.C., G.S., M.S.L., Z.R.Q., R.N. and E.M.V.A. performed research. G.G., S.B.G., E.S.L., S.O., and C.S.F. contributed new reagents and analytic tools. M.G., E.H., X.J.M., E.M.V.A. and L.A.G. analyzed data. M.G., E.H. and L.A.G. wrote the manuscript.

Supplementary Table 1. *RNF43* Mutations in Colorectal Cancer, NHS/HPFS set ($n$ = 185)
Supplementary Table 2. *RNF43* Mutations in Colorectal Cancer, TCGA set ($n$ = 222)
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Supplementary Table 7. PCR/Sanger Primer Sequences.
Supplementary Table 8. Somatic Coding Mutations in Colorectal Cancer, NHS/HPFS set ($n$ = 185)
Supplementary Table 9. Somatic Coding Mutations in Colorectal Cancer, TCGA set ($n$ = 222)
Supplementary Table 10. Somatic Coding Mutations in Endometrial Cancer, TCGA set ($n$ = 248)

**URLs:**
CGHub, https://cghub.ucsc.edu/; Indelocator, http://www.broadinstitute.org/cancer/cga/indelocator.

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## Abstract

We report somatic mutations of *RNF43* in over 18% of colorectal adenocarcinomas and endometrial carcinomas. *RNF43* encodes an E3 ubiquitin ligase that negatively regulates Wnt signaling. Truncating mutations of *RNF43* are more prevalent in microsatellite-unstable tumors and show mutual exclusivity with inactivating *APC* mutations in colorectal adenocarcinomas. These results indicate that *RNF43* is one of the most commonly mutated genes in colorectal and endometrial cancers.

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The E3 ubiquitin-protein ligase RNF43 negatively regulates the Wnt signaling pathway (1). Somatic mutations in *RNF43* have been associated with heightened sensitivity to compounds that target the Wnt-specific acyltransferase porcupine (PORCN) in preclinical models (2). In parallel, clinical trials of small-molecule porcupine inhibitors (for example, LGK974) are ongoing in Wnt ligand-dependent malignancies (melanoma, pancreatic, breast, head and neck, and colorectal cancers). *RNF43* is frequently mutated in pancreatic cystic neoplasms (3) and in <5% of pancreatic carcinomas with acinar differentiation (4); however, *RNF43* mutations have not been reported in melanoma (5), breast cancer (6), or head and neck malignancies (7). In colorectal cancer, Wnt signaling is more commonly dysregulated through loss-of-function *APC* mutations (8), whereas *RNF43* has not been reported to be significantly mutated in prior sequencing studies (9, 10).

Unexpectedly, our whole-exome sequencing of colorectal cancers identified a large number of non-silent somatic mutations in *RNF43*. DNA isolated from a discovery set of 185 formalin-fixed, paraffin-embedded colorectal tumor and matched normal samples collected from participants in 2 prospective cohort studies, the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS) (11), showed *RNF43* mutations in 35 (18.9%) cases (median allelic fraction of 0.23, range of 0.01–0.68) (Supplementary Table 1). Frameshift mutations encoding p.Gly659fs and p.Arg117fs constituting insertions or deletions of 1 bp in homopolymeric tracts (microsatellite instability (MSI) loci) of seven and six C:G pairs, respectively, accounted for 41.7% (p.Gly659fs) and 8.3% (p.Arg117fs) of the *RNF43* mutations identified (Figure 1a). To exclude the possibility that these mutations represented technical artifacts, we validated 31 of the *RNF43* mutations (97% of 32 reactions that had leftover DNA available and achieved coverage of >50× in resequencing or successfully underwent Sanger sequencing) in the mutant tumors and their matched normal tissue (Supplementary Figure 1, Supplementary Table 1).

The unexpectedly high frequency of truncating *RNF43* mutations in our colorectal tumor cohort contrasted with the paucity of *RNF43* mutations reported by previous studies of a

comparable scale, including a TCGA (The Cancer Genome Atlas) study of 224 colorectal tumor-normal tissue pairs (9). We hypothesized that previous studies might have inadvertently filtered out many bona fide *RNF43* frameshift events owing to their similarity to the polymerase slip errors that may arise during the massively parallel sequencing process. Therefore, we reanalyzed 222 TCGA colorectal tumor-normal exomes (representing all TCGA colorectal exomes available on our local servers in September 2013). Of these, 49 cases (22%) were described in the published TCGA study (9). We discovered *RNF43* mutations with high allelic fraction at a frequency of 17.6% (median allelic fraction of 0.38, range of 0.04–0.77; 48.0% encoding p.Gly659fs and 12% encoding p.Arg117fs mutations). We then orthogonally validated these mutations by examining matched RNA sequencing (RNA-seq) data, additionally confirming mRNA expression of the mutant *RNF43* alleles (100% validation rate, 44 of 44 mutations in cases with at least 10-fold coverage at the relevant base pair; Supplementary Table 2). These results confirmed that *RNF43* is mutated at a high frequency in colorectal tumors (Figure 1b, Supplementary Table 2).

In light of this discovery, we reasoned that inactivating mutations in *RNF43* might also have been overlooked in previous whole-exome sequencing studies of endometrial cancer, another Wnt-dependent tumor type in which MSI is common. A reanalysis of all 248 endometrial tumor-normal exome pairs from the published TCGA study (12) identified the presence of non-silent *RNF43* mutations in 18.1% of cases (median allelic fraction of 0.31, range of 0.04–0.87) with the p.Gly659fs variant accounting for 47.3% and the p.Arg117fs variant for 3.6% of the alterations (Figure 1c, Supplementary Table 3). Matched RNA-seq data orthogonally validated these events (91% validation rate, 20 of 22 mutations in cases with at least 10-fold coverage of the relevant base pair, Supplementary Table 3).

The high frequency of truncating mutations at this locus (together with a low frequency of synonymous mutations) strongly suggested that *RNF43* mutations had undergone positive selection during colorectal and endometrial tumor evolution. To investigate this possibility, we analyzed *RNF43* in each tumor exome cohort using InVEx, an algorithm we previously developed to infer the presence of positive selection in tumors with high background mutation rates (5). InVEx uses the exon/intron mutational distribution at a given locus to determine whether a gene has more mutations of predicted functional consequence than expected under a model of no selection. *RNF43* emerged as significantly mutated in all three sample sets (NHS/HPFS colorectal cancer, $P = 1.4 \times 10^{-7}$; TCGA colorectal cancer, $P = 4.1 \times 10^{-10}$; TCGA endometrial cancer, $P = 2.0 \times 10^{-7}$). Furthermore, given the empirical somatic indel rate at exonic homopolymeric tracts of seven C:G pairs in tumors with MSI, *RNF43* indels causing p.Gly659fs occured significantly more often than expected by chance ($P < 2.2 \times 10^{-16}$, binomial test; 0.08–0.14% mutation frequency across all exonic 7-bp C:G tracts and 9.2–10.8% mutation frequency for the 7-bp C:G tract in *RNF43* causing p.Gly659fs, calculated in tumors with MSI). *RNF43* indels causing the p.Gly659fs alteration represented the most recurrent indels at exonic C:G homopolymeric tracts of any length in all three cohorts. Thus, *RNF43* mutations apparently conferred a fitness advantage to the colorectal and endometrial cancer cells in which they occurred.

As noted earlier, most *RNF43* mutations (73–75% of all non-silent variants) were truncating events (defined as frameshift indels, nonsense mutations and splice-site mutations), consistent with the previous characterization of *RNF43* as a tumor-suppressor gene (1). Additionally, 16 of 74 (21.6%) *RNF43*-mutant colorectal tumors (Supplementary Tables 1, 2) and 8 of 45 (17.8%) *RNF43*-mutant endometrial tumors (Supplementary Table 3) harbored at least 2 non-silent *RNF43* mutations, in line with the Knudson 2-hit hypothesis for tumor suppressor genes. We cannot exclude the possibility that the mutations affected the same allele in some two-hit cases, but this seems less likely given the overall loss-of-function pattern.

Given that the *RNF43* mutations were predominated by small insertions or deletions in homopolymeric tracts in tumor types with a high prevalence of MSI, we hypothesized that these mutations might be enriched in MSI tumors. Indeed, truncating mutations of *RNF43* were statistically linked to the microsatellite instability high (MSI-H) phenotype in both tumor types ($P < 2.2 \times 10^{-16}$ for colorectal cancer and $P < 9.0 \times 10^{-16}$ for endometrial cancer, Fisher's exact test; Figure 2a, b). Overall, *RNF43* was mutated in 79.7% of MSI-H colorectal tumors and 50.7% of MSI-H endometrial tumors (Supplementary Table 4). This association suggests that mismatch repair deficiency creates a permissive environment for the predominant *RNF43* frameshift alterations affecting codons 659 and 117.

Inactivating *RNF43* mutations should result in augmented Wnt signaling, owing to increased cell surface abundance of the Wnt receptor Frizzled (1, 2). We thus asked whether any of the significantly mutated Wnt pathway genes displayed a mutually exclusive pattern of mutation with *RNF43* in colorectal or endometrial cancer. Indeed, truncating mutations in *APC*, the most frequently mutated gene in colorectal cancer, exhibited profound mutual exclusivity with truncating mutations in *RNF43* (6 of 74 *RNF43*-mutant samples had co-occurring truncating mutations in *RNF43* and *APC*, $P < 2.2 \times 10^{-16}$, Fisher's exact test; 4 of 55 mutant samples had equivalent co-occurring truncating mutations when analysis was restricted to MSI-H tumors, $P = 3.6 \times 10^{-5}$; Figure 2a). Moreover, we detected *RSPO3-PTPRK* fusions (which activate Wnt signaling (10)) in two tumors, neither of which harbored *RNF43* or *APC* mutations. The mutually exclusive relationship between mutations in *RNF43* and *APC* provides additional genetic evidence in support of an activating role for truncating mutations of *RNF43* in the Wnt pathway in colorectal cancer.

Our study demonstrates that *RNF43* is a frequently mutated gene in colorectal and endometrial cancers, enriched in the setting of MSI. These findings further predict that stomach cancer, a third tumor type characterized by Wnt pathway dysregulation and frequent MSI (13), should also harbor frequent mutations in *RNF43*. Indeed, we found that 3 of 37 (8.1%) published stomach cancer exomes (13, 14) had non-silent *RNF43* mutations, including a mutation encoding p.Gly659fs. Moreover, while this manuscript was under review, two studies reported RNF43 mutations in gastric cancer (15, 16). These studies also demonstrated a higher percentage of RNF43 mutations in microsatellite-unstable samples in comparison to microsatellite-stable ones (54.6% versus 4.8% and 33% versus 3% in refs. 15 and 16, respectively). The discovery of multiple tumor types with frequent mutations in *RNF43* may have near-term clinical relevance in light of preclinical studies showing that *RNF43* mutations render Wnt-driven cancer cells susceptible to pharmacologic porcupine

inhibition (2). More generally, these findings suggest that large-scale genomic analyses remain a fruitful means to discover biologically and clinically relevant changes in human cancer.

## Methods

### Tumor specimens

We collected 185 colorectal tumor-normal pairs from the participants of NHS and HPFS who were diagnosed with colorectal carcinomas (Supplementary Table 5). All of the colorectal tumor and adjacent normal colorectal tissue samples were collected according to Partners Human Research Committee institutional review board (IRB)-approved protocols, and informed consent was obtained from all subjects. Hematoxylin and eosin slides prepared from formalin-fixed, paraffin-embedded blocks were examined for cancer extent, tumor grade and other tumor characteristics through pathology review (S.O.). MSI status for each colorectal cancer sample was determined using standard protocols (11). We extracted genomic DNA from formalin-fixed, paraffin-embedded samples using the Qiagen QIAamp DNA FFPE Tissue Kit. DNA quality was evaluated by quantification using the Quant-iT Pico Green dsDNA Assay Kit (Invitrogen) according to the manufacturer's protocol.

### DNA sequencing

TCGA exome sequence data were downloaded from CGHub (see URLs). The generation of sequencing data from the NHS and HPFS cohorts was performed using a protocol for formalin-fixed, paraffin-embedded samples that has been detailed previously (17). Briefly, whole-exome capture libraries were constructed from tumor and normal DNA after sample shearing, end repair, phosphorylation and ligation to barcoded sequencing adaptors. DNA was then subjected to solution-phase hybrid capture with SureSelect v2 Exome bait (Agilent Technologies), and we performed multiplexing of the samples and sequencing on Illumina HiSeq 2000 instruments. Average mean target coverage was 96× (Supplementary Table 6). For validation, we used the Fluidigm Access Array microfluidic device. PCR products were barcoded, pooled and subjected to Illumina sequencing on a MiSeq instrument to a mean coverage of 11000×. For six samples, validation of the c.1976delC (p.Gly659fs) mutation was performed by amplifying and Sanger sequencing (Genewiz) a 211-bp product containing the change of interest (Supplementary Figure 1 and Supplementary Table 7).

### Exome sequencing data analysis

Sequence analysis was performed as previously described (17, 18) to identify somatic single-nucleotide variants and short insertion or deletion events (Supplementary Tables 8–10). Somatic mutations were called with the MuTect algorithm (18), and somatic indels were called with the Indelocator algorithm (see URLs). Manual review of all *RNF43* indels in the NHS and HPFS cohorts was conducted using the Integrated Genomics Viewer (19), leading to deletions at base pair 56,435,160 being considered sequencing artifacts and excluded from further analysis. During manual review, it was discovered that several indels in *RNF43* resulting in p.Gly659fs were discarded as 'germline' indels by Indelocator owing to the presence of a small number of reads supporting the variant in the normal exome. Indels likely to be somatic but originally filtered out as germline by Indelocator were

rescued across the exome if they passed the following conservative criteria: coverage of at least 50 reads in both tumor and normal samples, >20% of reads supporting the variant in tumor samples and <5% of reads supporting the variant in normal samples.

### RSPO fusion determination

TCGA RNA-seq data corresponding to the 222 samples in the analyzed colorectal cancer exome TCGA set were downloaded from CGHub (219 of 222 samples had available RNA-seq data). Gene fusions were called with TopHat-Fusion (20).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing Interests

L.A.G. is a consultant for an equity holder in Foundation Medicine. L.A.G. is also a consultant to Novartis, Millenium/Takeda, and Boehringer Ingelheim and is a recipient of a grant from Novartis.

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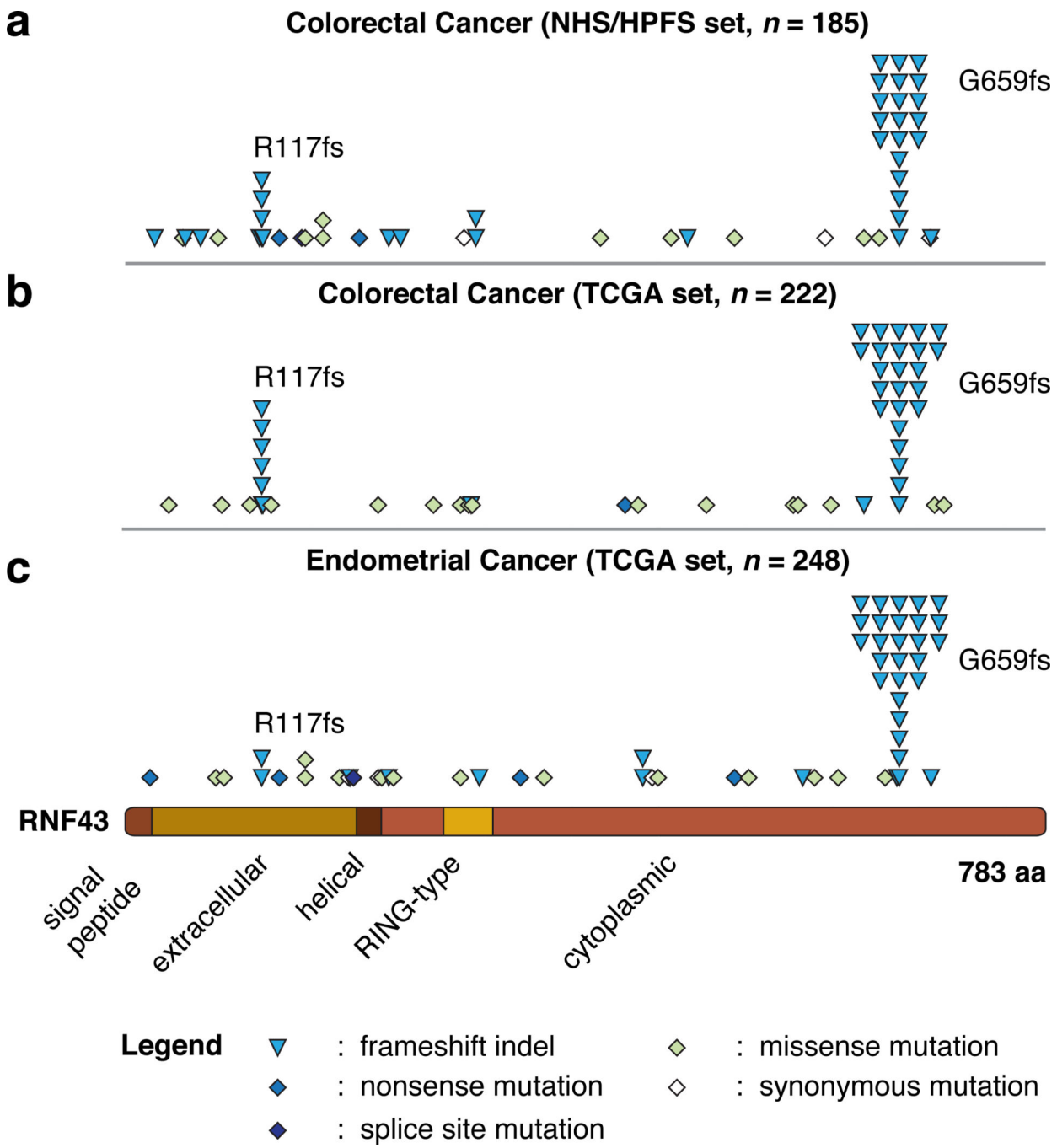


**Figure 1.**

*RNF43* mutations in colorectal and endometrial cancers. (a–c) Distribution and type of *RNF43* mutations in colorectal cancer, NHS and HPFS set (a); colorectal cancer, TCGA set (b); and endometrial cancer, TCGA set (c). The domains of *RNF43* are depicted schematically at the bottom.

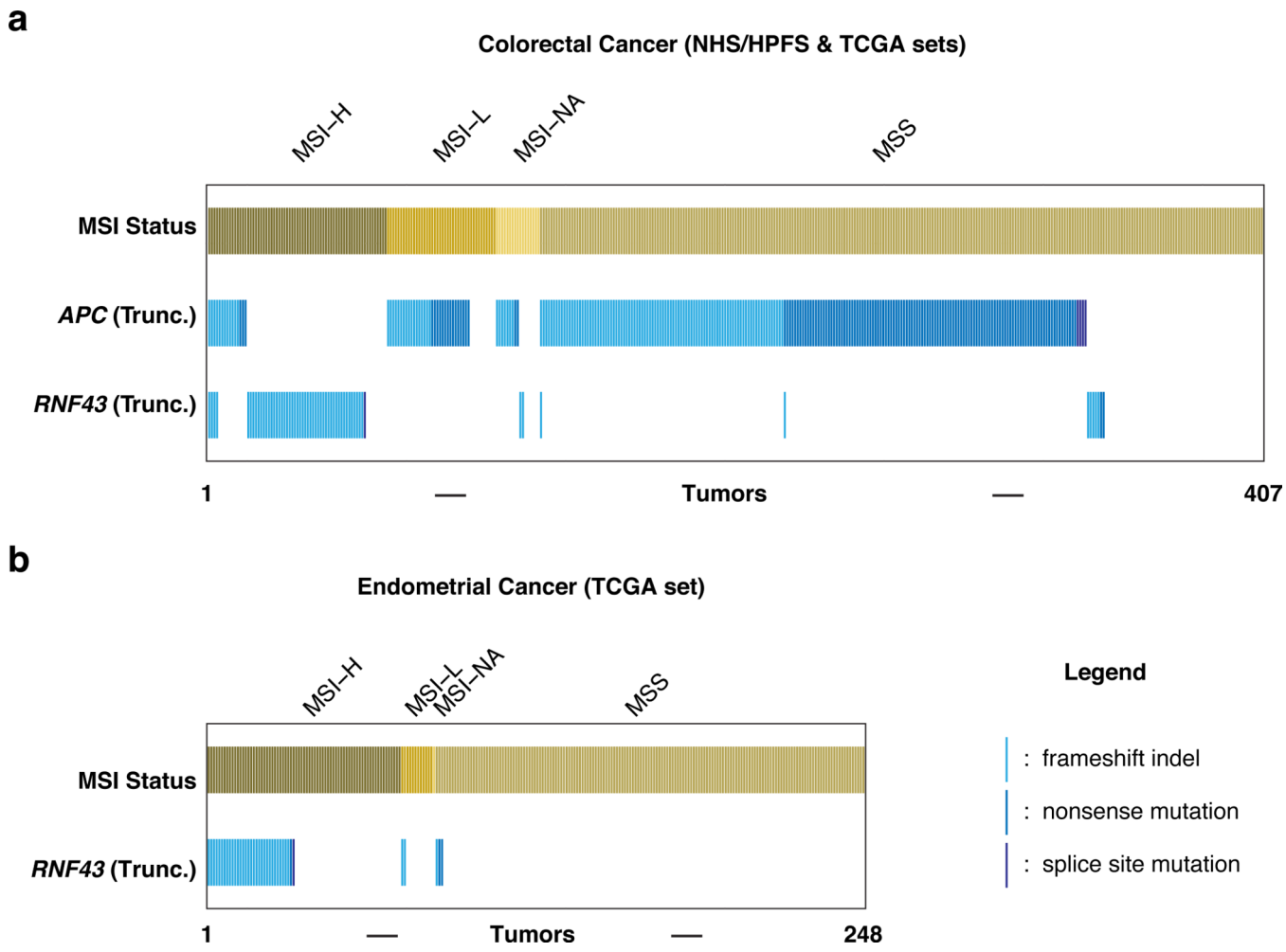


**Figure 2.**

Association of *RNF43* mutations with MSI status and *APC* mutations. (a) Matrix displaying MSI status, *APC* truncating events and *RNF43* truncating events for each of 407 colorectal tumor samples (NHS set and HPFS set and TCGA set). Each vertical line represents one colorectal cancer. Top, MSI status. MSI-H, microsatellite instability high; MSI-L, microsatellite instability low; MSI-NA, MSI status not available; MSS, microsatellite stable. Middle, a filled box shows that an *APC* truncating mutation is present in that tumor. Bottom, a filled box shows that an *RNF43* truncating mutation is present in that tumor. (b) Matrix displaying MSI status and *RNF43* truncation status for each of 248 endometrial tumor samples (TCGA set). Each vertical line represents one endometrial cancer. Top, MSI status. Bottom, a filled box shows that an *RNF43* truncating mutation is present in that tumor. Mutations in *APC* and *RNF43* are colored by type. In cases where multiple mutations were present in the same cancer sample, the presence of a frameshift mutation took priority in determining the displayed color, followed by the presence of a nonsense mutation.