Germline Mutations in FAF1 Are Associated With Hereditary Colorectal Cancer

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BACKGROUND & AIDS: A significant proportion of colorectal cancer (CRC) cases have familial aggregation but little is known about the genetic factors that contribute to these cases. We performed an exhaustive functional characterization of genetic variants associated with familial CRC. METHODS: We performed whole-exome sequencing analyses of 75 patients from 40 families with a history of CRC (including early-onset cases) of an unknown germline basis (discovery cohort). We also sequenced specific genes in DNA from an external replication cohort of 473 families, including 488 patients with colorectal tumors that had normal expression of mismatch repair proteins (validation cohort). We disrupted the Fas-associated factor 1 gene (FAFI) in DLD-1 CRC cells using CRISPR/Cas9 gene editing; some cells were transfected with plasmids that express FAFI missense variants. Cells were analyzed by immunoblots, quantitative real-time polymerase chain reaction, and functional assays monitoring apoptosis, proliferation, and assays for Wnt signaling or nuclear factor (NF)-κB activity.

RESULTS: We identified predicted pathogenic variant in the FAF1 gene (c.1111G>A; p.Asp371Asn) in the discovery cohort; it was present in 4 patients of the same family. We identified a second variant in FAF1 in the validation cohort (c.254G>C; p.Arg85Pro). Both variants encoded unstable FAF1 proteins. Expression of these variants in CRC cells caused them to become resistant to apoptosis, accumulate b-catenin in the cytoplasm, and translocate NF-κB to the nucleus. CONCLUSIONS: In whole-exome sequencing analyses of patients from families with a history of CRC, we identified variants in FAF1 that associate with development of CRC. These variants encode unstable forms of FAF1 that increase resistance of CRC cells to apoptosis and increase activity of b-catenin and NF-κB.

Keywords: Wnt Signaling; Programmed Cell Death; Gene Editing; Functional Genomics.
Colon cancer (CRC) is one of the most frequent neoplasms worldwide, accounting for approximately 8% of all cancer-related deaths. A familial component, defined by the presence of 2 or more affected relatives, is estimated to be involved in 12% to 35% of all CRC cases. However, only 5.2% of all CRC cases are caused by known high-penetration CRC genes (APC, MUTYH, the DNA polymerases POLE and POLD1, and the mismatch repair genes MLH1, MSH2, MSH6, and PMS2). Despite these hereditary syndromic forms, a large proportion of patients with CRC have a family history of the disease but the underlying germline cause remains unexplained. Part of this familial risk could be related to uncommon and highly penetrant mutations in genes yet to be discovered. Numerous genes involved in CRC and/or polyposis predisposition have been proposed, although, so far, strong evidence of association has only been demonstrated for NTHLI, MSH3, GREML1, RNPF4, RSP20, and MLH3.

In the past years, genome-wide next-generation sequencing and copy-number technologies have been widely used for the identification of new germline variants involved in cancer predisposition. Nevertheless, recognizing the correct causal pathogenic variant among the large number of variants identified by using these genome-wide techniques is not straightforward. The functional characterization of the identified variants is a helpful approach to establish the link to disease predisposition. To standardize and facilitate this approach, key guidelines and experimental scanning pipelines have been developed to assess the functional relevance of the genetic variants. On the other hand, CRISPR/Cas gene editing has transformed functional genomics, enabling researchers to potentially edit any desired region of the genome. Modeling by CRISPR/Cas has allowed the characterization of several known hereditary CRC genes (e.g., MLH1 and POLE), as well as somatic CRC mutational events (KRAS, TP53, SMAD4, and APC). The integration of next-generation sequencing results with these models can help monitor the genomic changes that trigger CRC development and allow more personalized therapy designs. Nevertheless, these cancer modeling tools have been scarcely used to decipher the pathogenicity of many variants of uncertain significance.

In this study, we aimed at identifying novel causal genes for CRC germline predisposition by performing whole-exome sequencing in CRC families with an unknown germline basis. The detected rare variants were functionally evaluated to demonstrate their pathogenicity and implications in predisposing to CRC, permitting a more accurate and adequate diagnosis of patients, as well as facilitating genetic counseling and prevention.

Materials and Methods

Patients

We selected 75 patients from 40 families with strong CRC aggregation where other known hereditary cancer syndromes had been ruled out (unaffiliated) and compatible with an autosomal dominant pattern of inheritance. The selection criteria were as follows: 3 or more relatives with CRC, 2 or more consecutive affected generations, and at least 1 CRC diagnosed before the age of 60. The presence of germline alterations in well-known genes related to hereditary CRC syndromes (APC, MUTYH, and the DNA MMR genes) had been previously ruled out for all probands. The tumors developed by the probands were microsatellite stable, negative for MLH1 promoter methylation, and showed normal expression of the MMR proteins MLH1, MSH2, MSH6, and PMS2. The entire cohort was previously described in detail. For replication purposes, an independent hereditary nonpolyposis CRC cohort was available, and comprised 473 families including 488 MMR-proficient cancer affected patients, 96% of them affected with CRC. The mean age at cancer diagnosis was 49 (range: 16–82). Among the 473 studied families, 58 (12.2%) fulfilled the Amsterdam criteria, 385 (81.4%) the Bethesda guidelines, and the remaining 30 (6.3%) none of the established criteria for hereditary nonpolyposis CRC. Detailed description of the cohort may be found in Belhadj et al.

Abbreviations used in this paper: CETSA, cellular thermal shift assay; CRC, colorectal cancer; DEDID, death effector domain-interacting domain; FAF1, Fas-associated factor 1; FID, Fas-interacting domain; LOH, loss of heterozygosity; NF-kB, nuclear factor-xB; PCR, polymerase chain reaction; pNA, p-nitroanilide; SNP, single nucleotide polymorphism; sgRNA, single guide RNA; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; UAS, ubiquitin-related domain.

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This study was approved by the institutional ethics committee (IDIBAPS: 2011/6440; IDIBELL: PR247/15) and written informed consent was obtained in all cases.

For details on exome sequencing and variant prioritization in initial cohort, mutation identification in pooled samples in replication cohort, variant validation, segregation analysis, and tumor loss of heterozygosis (LOH), as well as additional pathogenicity prediction tools, see the Supplementary Material.

**Immunohistochemistry**

Immunostains were performed on histological 2-μm sections from colon tumor and normal mucosa from patient III-5 of family FAM13. More detailed information is provided in the Supplementary Material.

**Functional Characterization of Genetic Variants**

**Cell lines.** The DLD-1 human CRC cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, Waltham, MA), at 37 °C in 5% CO2.

**Plasmids.** LentiCRISPRv2-Puro (#98290; Addgene, Cambridge, MA) and pcDNA3-β-catenin vectors were available. FAF1 ORF (NM_007051) cloned into pcDNA3.1-+C-(K)-DYK expression vector (OHu13027D, FLAG-tagged) was purchased from GenScript (Nanjing, China).

**Antibodies.** Monoclonal antibody against FAF1 (ab183045) was from Abcam (Cambridge, UK). The DYKDDDDK antibody (A00187) was from GenScript. Anti-p65 (D14E12), anti-GAPDH (14C10), and anti-β-Catenin (D10A8) were purchased from Cell Signaling (Danvers, MA). Anti-PCNA (sc 9857-R) was from Santa Cruz Biotechnology (Dallas, TX). Goat anti-rabbit (SA5-10036) and anti-mouse (SA5-10176) DyLight 800 secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA).

**CRISPR/Cas9-mediated FAF1 knockout generation.** The Benchling (http://benchling.com) and the MIT (http://crispr.mit.edu) CRISPR tools were used to design the single guide RNA (sgRNA) against the coding region of the FAF1 gene. The sgRNA was cloned into the LentiCRISPRv2-Puro vector and transiently transfected into the DLD-1 CRC cell line. Two days later, transfected cells were puromycin-selected (4 μg/mL) and immediately snap-frozen in a dry ice/ethanol bath. Subsequently, cells were lysed by 3 freeze-thaw cycles. After heating, stable proteins remain soluble while unstable proteins denature and precipitate, permitting their isolation by high-speed centrifugation (16,000g, 20 minutes at 4 °C). Soluble protein fractions were run on Western blots for the protein melting curve analysis.

**Apoptosis**

A colorimetric caspase-3 activity assay was performed according to the manufacturer’s instructions, with some modifications (Sigma Aldrich, St Louis, MO). Briefly, cells were stimulated with 20 ng/mL of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) at the indicated time points and lysed in 30 μL of lysis buffer. Protein concentrations were determined by using the Pierce BCA Protein Assay Kit (Thermo Fisher) and 50 μg of each protein extract was assayed with the colorimetric caspase-3 substrate Ac-DEVD-pNA. The release of the yellow chromophore p-nitroanilide (pNA) was measured in an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT) at 405 nm. Caspase-3 activity was calculated in comparison to a pNA standard curve.

**Wnt Signaling**

FAF1 wild-type (FAF1WT) and FAF1 knockout (FAF1KO) cells were cotransfected with a pcDNA3-β-catenin expression vector together with the pcDNA3 control empty vector or one of the FAF1 vectors (pWT, pArg85Pro, pAsp371Asn), as previously described. When indicated, after cell selection and recovery, cells were treated with the proteasome inhibitor MG132 (10 μM) for 4 hours. Transfected cells were harvested to extract the

RNA extraction and quantitative real-time PCR. Total RNA extraction was performed with the RNeasy Mini Kit according to manufacturer’s instructions (Qiagen, Hilden, Germany). RNA retrotranscription and PCR amplification details are included in the Supplementary Material.

**Functional assays.** DLD-1 knockout clones for FAF1 were transiently transfected with wild-type (pWT) or mutated (pArg85Pro, pAsp371Asn) plasmids using X-tremeGENE HP DNA transfection reagent. Two days later, cells were subjected to selection with 1000 μg/mL of G418 (InvivoGen, San Diego, CA) for 72 hours, as the FAF1 expression pcDNA3.1 vector carries a neomycin resistance cassette. Neomycin-resistant cells were allowed to recover in complete RPMI medium for 2 additional days before performing functional tests.

**Cellular Thermal Shift Assay (CETSA)**

Cells expressing wild-type or missense variants were harvested, washed with phosphate-buffered saline, and equally distributed into PCR tubes. Cell suspensions were shortly heated to 6 different temperatures (from 53°C to 58°C, ±1°C, 3 minutes) and immediately snap-frozen in a dry ice/ethanol bath. Subsequently, cells were lysed by 3 freeze-thaw cycles. After heating, stable proteins remain soluble while unstable proteins denature and precipitate, permitting their isolation by high-speed centrifugation (16,000g, 20 minutes at 4 °C). Soluble protein fractions were run on Western blots for the protein melting curve analysis.
cytoplasmic protein fraction, and β-catenin was detected by Western blotting.

Cell proliferation, colony formation, and migration methods are detailed in the Supplementary Material.

Results

The variant prioritization strategy applied to whole-exome sequencing data obtained from the initial unaffiliated familial cohort (75 individuals from 40 families) selected a FAF1 (Fas-associated factor 1) missense variant (c.1111G>A p.Asp371Asn), predicted pathogenic by in silico algorithms. The FAF1 variant was noticeable among others due to the gene function as a mediator of apoptosis and a negative regulator of both NF-κB and Wnt signaling pathways. The variant was identified in an Amsterdam-positive family of Spanish origin with 5 CRC cases and 3 affected generations (FAM13; Figure 1). The identified variant was initially detected in 3 CRC-affected family members (ages at diagnosis 46–74) and in 1 relative diagnosed with advanced adenoma at age 47. An RAD52 truncation variant, c.590_593dupAACC (p.Ser199Thrfs*88), was also detected in the 3 CRC-affected family members but not in the relative with the advanced adenoma.15 The 3 CRC-affected members did not share additional genetic variants with a plausible role in cancer predisposition. Targeted gene sequencing of FAF1 in 473 additional genetically unexplained CRC families yielded the identification of another predicted pathogenic missense variant, c.254G>C (p.Arg85Pro). It was identified in a Bethesda-positive patient with CRC diagnosed at the age of 55 (F-0681-00; Figure 1). Additional samples for variant segregation and tumor sample were not available.

The 2 identified missense variants are rare in large population datasets (ExAC database: allele count 5/121,210 for p.Asp371Asn and 19/121,270 for p.Arg85Pro) and are predicted to be pathogenic by most in silico tools (eg, CADD: 36 for p.Asp371Asn and 23.4 for p.Arg85Pro). The p.Arg85Pro variant is located on the FID domain (Fas-interacting domain) of the protein, and the p.Asp371Asn variant affects both the DEDID domain (death effector domain-interacting domain) and an ubiquitin-related domain (UAS). The p.Arg85Pro variant was predicted to...
alter the conformational structure of FAF1 (DAMpred), whereas the p.Asp371Asn amino acid change was predicted to impair the acetylation of the nearby K368 residue (MutPred2).

Tumor material from 2 p.Asp371Asn carriers (CRC diagnosed at 46 and 72 years of age, respectively; individuals IV-7 and III-5; Figure 1) was available to evaluate the expression of FAF1 by immunohistochemistry and the presence of LOH. FAF1 protein was expressed both in the cytoplasm and the nucleus of colon tumor cells, and LOH was evident, especially in III-5’s tumor (Supplementary Material).

**CRISPR/Cas9 FAF1 KO Modeling**

To further validate the suspected role of FAF1 genetic variants on CRC predisposition, we established an FAF1 KO cellular model by CRISPR/Cas9 gene editing on DLD-1 cells (Figure 2). According to bioinformatic CRISPR prediction tools, an sgRNA targeting the fourth exon was selected because of its favorable on-target and off-target scores. FAF1 gene editing was checked by Sanger sequencing (Figure 2B), and FAF1 depletion was confirmed by reverse-transcriptase PCR and Western blot (Figure 2C and D). Two FAF1 KO clones (#4 and #5) were selected for further expansion and characterization.

An initial screening of the tumor suppressor role of FAF1 was performed on CRISPR-generated knockout clones. The functional analysis demonstrated that permanent inactivation of FAF1 resulted in a more malignant phenotype of DLD-1 cells. Knockout clones showed a higher cell proliferation rate on the MTS assay in comparison with FAF1 WT cells (Figure 3A), as well as a subtle increase in cell migration (Figure 3B). Nevertheless, the most prominent effect was the sustained resistance to programmed cell death of FAF1-depleted cells. After exposing cells to the apoptosis-inducer TRAIL, both caspase-3 activity and cell death were noticeable in FAF1 WT cells, whereas FAF1 KO clones showed high resistance to apoptosis (Figure 3C and Supplementary Material). FAF1 has also been described as a negative regulator of the NF-κB signaling pathway, as it prevents the nuclear translocation of p65, a subunit of NF-κB. Depletion of FAF1 increased both the basal nuclear levels of p65 and the TNFα-induced NF-κB translocation.
(Figure 3D). Finally, as a ubiquitin-interacting protein, FAF1 has been reported to participate in the regulation of the Wnt signaling pathway. Lower levels of cytoplasmic β-catenin were detected in FAF1WT cells in comparison with FAF1KO cells, confirming the participation of FAF1 in β-catenin degradation (Figure 3E). Moreover, MG132 treatment promoted the accumulation of β-catenin in FAF1WT cells to levels comparable to FAF1KO cells, indicating its participation in the proteasomal degradation of β-catenin. These results supported the tumor suppressor role of FAF1, which was in line with the hypothesis that mutations affecting its correct functioning could lead to tumor development.

**Functional Characterization of Germline Variants**

To evaluate the functional effect of the identified FAF1 variants, site-directed mutagenesis was performed on a vector carrying the wild-type ORF of FAF1. Both c.254G>C and c.1111G>A mutations were PCR-generated and verified by Sanger sequencing (Figure 4A). None of them affected FAF1 RNA or protein expression (Figure 4B and C). In this line, a time-course expression analysis showed that wild-type and mutated FAF1 ORFs were equally expressed after transfection (Figure 4D), so the potential pathogenic mechanism of these variants appeared to be unrelated with their expression levels. Still, an amino acid exchange is an event that can disturb the conformational structure of a protein, thus compromising its stability and functionality. Therefore, we performed a CETSA assay to analyze the thermal stability of p.Arg85Pro and p.Asp371Asn variants within the cellular environment. The protein melting curve analysis revealed that both substitutions had reduced FAF1 protein stability in comparison with its wild-type form (Figure 4E). The instability of the p.Asp371Asn variant was already noticeable from lower point temperatures. Altogether, these results suggest that FAF1 missense alterations might contribute to protein dysfunction.

Because both missense variants seemed to cause protein instability, we proceeded to functionally characterize them by targeting the main cellular and molecular pathways in which FAF1 participates. As FAF1 is a component of the Fas-death-inducing signaling complex (Fas-DISC), cell resistance to apoptosis was first analyzed by measuring caspase-3 activation. DLD-1 FAF1KO clones #4 and #5 were transiently transfected with pWT, pArg85Pro, or pAsp371Asn FAF1 vectors and exposed to TRAIL stimulation. When we rescued the expression of wild-type FAF1, cells were sensitive to TRAIL-induced apoptosis, whereas cells expressing both p.Arg85Pro and p.Asp371Asn FAF1 variants were more resistant to cell death (Figure 5A and Supplementary Material).

Another key feature of FAF1 is the regulation of NF-κB activity by preventing its nuclear translocation. After FAF1 variant transfection, the subcellular location of the p65 NF-κB subunit was assessed. Cells expressing either p.Arg85Pro or p.Asp371Asn variants subtly accumulated more p65 into the nucleus in comparison with those expressing wild-type FAF1. A similar trend was observed for TNFα-stimulated cells, on which the highest amount of nuclear p65 was detected in cells expressing the pAsp371Asn FAF1 variant (Figure 5B). To further confirm the contribution of FAF1 missense variants on NF-κB activation, we evaluated the expression of Cyclin D1 (CCND1), one of its downstream effector genes. Again, after TNFα treatment, the expression of pAsp371Asn FAF1 variant (Figure 5D). To further confirm the contribution of FAF1 missense variants on NF-κB activation, we evaluated the expression of Cyclin D1 (CCND1), one of its downstream effector genes. Again, after TNFα treatment, the expression of pAsp371Asn FAF1 variant was quite remarkable on clone KO #4 and, at a lower rate, the same trend was observed for clone FAF1KO #5. Regarding cell migration, the FAF1KO #4 clone expressing p.Arg85Pro and p.Asp371Asn FAF1 variants showed an increased wound-healing potential in comparison with cells transfected with the wild-type form of FAF1 (Figure 6D).

**Discussion**

We identified 2 rare genetic variants with plausible pathogenicity in the FAF1 gene in 2 independent cohorts of unaffiliated familial CRC. A thorough functional characterization of both variants was performed in a CRISPR/Cas9 cellular model to further confirm their pathogenicity and involvement in germline predisposition to CRC, including assays for proliferation, colony formation, cell migration, protein stability, resistance to apoptosis, and both NF-κB and Wnt signaling.
Figure 4. Assessment of c.254G>C and c.1111G>A FAF1 variants expression and protein stability. (A) Site-directed mutagenesis of the pcDNA3.1-FAF1-DYK vector. Sanger sequencing verification of the specific nucleotide substitutions (c.254G>C and c.1111G>A) are depicted in red. (B) Western blot analysis and (C) real-time PCR quantification of the expression levels of the indicated FLAG-tagged FAF1 transfected vectors at both protein and messenger RNA levels, respectively. (D) Time-course protein expression analysis on FAF1KO #4 clone revealing the pcDNA3.1 expression peak 72 hours after transfection. No remarkable expression differences were observed between FAF1 vectors at the indicated timepoint. (E) Representative Western blot for the CETSA assay of FLAG-tagged FAF1 transfected cells, either with wild-type or missense variants. On the right, band intensity quantification by densitometry. All data were normalized to the amount of protein detected for each condition at the lowest test temperature and fitted by a 4-parameter logistic (4PL) regression. Data represent mean ± standard deviation (n = 3).
Figure 5. FAF1 variants contribute to apoptosis resistance as well as deregulation of NF-κB and Wnt signaling pathways. 

(A) Determination of caspase-3 activity after TRAIL-induced apoptosis.

(B) Western blot analysis of p65 nuclear translocation at basal levels (-TNFα) or after TNFα-stimulation and (C) real-time PCR quantification of CCND1 expression levels under the same experimental conditions, assayed in triplicate for FAF1KO #4. (D) β-catenin detection in cytoplasmic protein lysates on FAF1KO cells cotransfected with a pcDNA3 β-catenin expression vector together with the indicated FAF1 vectors. Unless otherwise indicated, data on bar graphs represent mean ± SD (n = 3). *P < .05, analysis of variance with Tukey post hoc test.
The FAF1 genetic variant was prioritized over an RAD52 genetic variant in family FAM13 due to additional segregation in an advanced adenoma case. Human RAD52 can be considered a nonessential gene, whereas FAF1 is necessary for correct embryonic development. Accordingly, when using the pLI score to estimate their tolerance of loss-of-function variation, RAD52 pLI score is 0 as compared with 1 for FAF1. Overall, these will be suggesting that the latter gene will be more intolerant to mutation and more likely to be involved in cancer predisposition.

FAF1 is an evolutionary conserved proapoptotic scaffolding protein. It contains 2 apoptosis-related domains (FID and DEDID), as well as several ubiquitination-associated functional domains. It is considered a component of the death-inducing signaling complex (DISC), and it participates in both receptor-dependent and independent apoptosis pathways. Moreover, FAF1 retains the NF-κB subunit p65 in the cytoplasm via physical interaction, thus inhibiting NF-κB nuclear translocation and activation. In addition, its ubiquitin-binding capacity antagonizes the canonical Wnt signaling pathway and participates in the DNA replication fork dynamics. Therefore, FAF1 has been contemplated as a tumor suppressor gene, whose downregulation may contribute to tumorigenesis. Our functional characterization of FAF1 knockout (KO) clones confirmed the involvement of this protein in apoptosis resistance and cell cycle deregulation.

The identified FAF1 missense mutations in the present study are located in well-defined functional domains. The c.254G>C variant (p.Arg85Pro) is included in the FID domain, whereas the c.1111G>A variant (p.Asp371Asn) is located inside both the helix-rich DEDID and the UAS domains. In silico pathogenicity tools predicted a possible pathogenic conformational change of FAF1 p.Arg85Pro, as well as a loss of acetylation of the K368 residue in FAF1 p.Asp371Asn. Acetylation usually works as a stabilizing mechanism, preventing the ubiquitination and subsequent proteasomal degradation of the protein. These results were reinforced by the CETSA assay, which confirmed that both amino acid substitutions altered protein stability, thus potentially modifying protein-protein interactions.

We then assessed whether p.Arg85Pro and p.Asp371Asn variants affected the main cellular processes in which FAF1 participates. Cells expressing p.Arg85Pro and p.Asp371Asn FAF1 variants were more resistant to TRAIL-induced apoptosis, which is consistent with the involvement of both FID and DEDID domains in the death signaling cascade. Higher NF-κB activity and cytoplasmic β-catenin accumulation were detected mainly in p.Asp371Asn FAF1 expressing cells, which may be explained by the location of this mutation inside the overlapping domains DEDID and UAS. The DEDID domain is the one that drives the p65-FAF1 interaction and its retention into the cytoplasm.

The balance between survival and programmed cell death is an important homeostatic process that, when disrupted, can facilitate the development of cancer. This equilibrium is specially regulated in normal colonic epithelial cells at the base of crypts, which are highly prone to apoptosis. Therefore, a special role can be hypothesized for those tumor suppressor genes with proapoptotic functions or negatively regulating both Wnt and NF-κB pathways. Accordingly, we can hypothesize that FAF1, when disrupted, will deviate the normal homeostatic process and lead to resistance to cell death. In this regard, several studies have already proposed new candidate CRC predisposing genes involved in apoptosis. The pathogenicity of a germline variant in the proapoptotic UNC5C gene was validated and suggested to increase the risk for CRC, although with controversy. A screening study of a Finnish cohort identified 2 truncating variants in the UACA gene, which was proposed as a novel candidate gene. Similarities can be found between UACA and FAF1, as both genes promote apoptosis and control the nuclear factor NF-κB activity. In addition, in a previous study from our research group, we postulated NFKBIZ as a novel CRC candidate gene, as it
interferes with the DNA binding capacity of both p50 and p65 NF-κB subunits.\textsuperscript{15} These biological processes can be relevant not only in CRC predisposition but also in germline susceptibility to other neoplasms. For example, germline mutations in the \textit{FAS} gene, one of the main sensors of the extrinsic apoptosis pathway, are associated with the development of autoimmune lymphoproliferative syndrome and a high risk for both Hodgkin and non-Hodgkin lymphoma.\textsuperscript{12,13}

Taking into account the variants found in our series, the \textit{FAF1} frequency in familial CRC could be considered approximately 0.4% (2/513). When adding other genes involved in apoptosis (\textit{UNC5C}, \textit{UACA}, \textit{NFKBIZ}) with reported variants in familial CRC cohorts, the frequency of apoptosis defects could rise to >1%.\textsuperscript{39-41,44} Undoubtedly, analysis of additional larger familial CRC cohorts is needed to provide further information about the prevalence and implication of mutations in \textit{FAF1} and other apoptosis-related genes in hereditary CRC.

In summary, our findings suggest germline \textit{FAF1} mutations may be implicated in inherited susceptibility to CRC, and postulate resistance to apoptosis as the plausible underlying mechanism.

### Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of \textit{Gastroenterology} at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2020.03.015.

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Conflict of interest
The authors disclose no conflicts.

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Supplementary Material.
Exome Sequencing and Variant Identification

Exome Sequencing and Variant Prioritization in Initial Cohort

Germline DNA samples used for exome sequencing were isolated from peripheral blood using the QIAamp DNA Blood (Qiagen, Hilden, Germany) following the manufacturer’s instructions.

Whole-exome sequencing (WES) was performed in samples of selected patients using the HiSeq2000 platform (Illumina, San Diego, CA) and SureSelectXT Human All Exon v5 kit (Agilent, Santa Clara, CA) for exon enrichment. WES cannot reliably detect copy-number variants as in large deletions/duplication/insertions. Indexed libraries were pooled and massively parallel sequenced using a paired-end 2 × 75-base pair read length protocol. Quality control of sequencing data was made in all samples previously to their analysis using the Real-Time Analysis software sequence pipeline (Illumina, San Diego, CA). Burrows-Wheeler Aligner (BWA-MEM algorithm) was used for read mapping to the human reference genome (build hs37d5, based on NCBI GRCh37).1 PCR duplicates were discarded using MarkDuplicates tool from Picard and then indel realignment and base quality score recalibration were performed with the Genome Analysis Toolkit (GATK).2 The GATK tools HaplotypeCaller and MuTect2 were used for SNV and short indels calling for germline and tumor samples, respectively.2 Regarding variant annotation, different databases were considered, including SnpEff, ANNOVAR, and dbNSFP, for pathogenicity and variant position annotation. PhyloP (phyloP46way_placental score ≥ 1.6), SIFT (prediction of damaging), PolyPhen2 (HumVar prediction of probably damaging or possibly damaging), MutationTaster (prediction of disease-causing or disease-causing-automatic), LRT (prediction of deleterious), and CADD (Phred score ≥ 15) were used for pathogenicity prediction of missense variants. Germline WES data were analyzed through an in-house R language pipeline described in previous studies.3–5 Functions related with CRC or cancer were prioritized. DNA repair, apoptosis, autophagy, cell growth, cell proliferation, inflammatory response, cell cycle, angiogenesis, cell differentiation, cell adhesion, and chromatin modification, among others, were included.

Variant Identification in Pooled Samples in Replication Cohort

Patients were screened for genetic variants in FAF1 using a combination of PCR amplification in pooled DNAs and targeted massively parallel sequencing, as previously described.5 Primers are listed in Supplementary Figure 6. Again, variant prioritization was performed as previously described.3–5

Variant Validation, Segregation Analysis, and Tumor LOH

Results for the prioritized variants in initial and replication cohorts were validated by using specific primers for PCR amplification designed using Primer3Plus1 and Sanger sequencing (GATC Biotech, Cologne, Germany). Segregation analysis of the prioritized variants was performed when possible in additional family members affected with CRC or advanced adenoma (Supplementary Material). LOH was tested by comparing Sanger sequencing results including the identified variant from germline and tumor DNA of the same individual. Primers are listed in Supplementary Figure 6.

Additional Pathogenicity Prediction Tools

Besides the bioinformatic pathogenicity tools used in the variant prioritization process, 2 additional in silico tools were used to predict protein structural alterations (DAMpred, https://zhanglab.ccmb.med.umich.edu/DAMpred/) and molecular mechanisms that could become altered and lead to protein misfuction (MutPred2, http://mutpred.mutdb.org/).3

Immunohistochemistry

Immunostains were performed on histological 2-μm sections from colon tumor and normal mucosa from patient III-5 of family FAM13. After deparaffination, antigen retrieval was performed with citrate buffer 10 mM, and tissue was permeabilized with 1% Triton X-100. Peroxidase activity was blocked with 3% hydrogen peroxide. Sections were treated for 2 hours with Dako serum-free protein blocker (Agilent, Santa Clara, CA), incubated for 16 hours with monoclonal rabbit anti-FAF1 antibody at 1/700 dilution (ab183045, Abcam, Cambridge, UK), and for 1 hour with goat anti-rabbit secondary antibody at 37°C (Dako REAL EnVision HRP Rabbit; Agilent). Sections were counterstained with hematoxylin, mounted. An Olympus BX41 microscope (Olympus, Tokyo, Japan) was used to visualize the immunostains.

Functional Characterization of Genetic Variants

CRISPR/Cas9-mediated FAF1 Knockout Generation

The Benchling (http://benchling.com) and the MIT (http://crispr.mit.edu) CRISPR tools were used to design the sgRNA against the coding region of the FAF1 gene. The top and bottom strands of the sgRNA were purchased from IDT (Coralville, IA) and cloned into the Lentiviral vector (which also packages the Cas9 coding sequence) as follows. Briefly, LentiCRISPRv2-Puro vector was digested with Esp3I (Thermo Fisher,
Waltham, MA) and run on 0.8% agarose gel. The 12-kb band corresponding to the plasmid backbone was extracted with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The sgRNA-encoding oligonucleotides were annealed, phosphorylated with the T4 Poly
ucleotide Kinase (NEB, Ipswich, MA), and ligated into the LentICRISPR backbone with T4 ligase (NEB, Ipswich, MA).

The vector was transiently transfected into the DLD-1 CRC cell line using X-tremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland), thus avoiding the stable expression of Cas9 and reducing its subsequent undesired effects, such as off-target activity and cytotoxicity.

Two days later, transfected cells were puromycin-selected (4 μg/mL) and CRISPR-editing efficacy on the targeted locus was verified by Sanger sequencing and TIDE webtool analysis. For single-cell cloning, puromycin-resistant cells were seeded into a 96-well plate at a density of 1 cell/well. After 3 weeks, several clones were characterized and selected for further analysis. FAF1 editing was validated by Sanger sequencing, and gene inactivation was checked by quantitative real-time PCR and Western blot. Primers are listed in Supplementary Tables 1–4.

**Protein Extraction and Western Blot**

To obtain whole-cell protein extracts, cells were detached from cell culture plates with Accutase (Sigma Aldrich, St Louis, MO) and lysed with RIPA buffer supplemented with Complete Protease Inhibitor Cocktail and PhosSTOP (Roche). To assess NF-κB translocation and β-catenin accumulation, cytoplasmic and nuclear protein fractions were separated as follows. Cells were resuspended in ice-cold hypotonic buffer (Tris 10 mM, NaCl 10 mM, MgCl2 10 mM) and lysed with 1% NP-40. Cytoplasmic proteins were separated from nuclei by high-speed centrifugation, and the nuclear protein fraction was lysed by pulse sonication and RIPA extraction. Sample protein concentrations were determined by using the Pierce BCA Protein Assay Kit (Thermo Fisher, Waltham, MA). Equal amounts of protein lysates were resolved in NuPAGE Bis-Tris protein gel electrophoresis, followed by protein transfer onto Immobilon PVDF membranes (Millipore, Bedford, MA), according to manufacturer’s protocols (Thermo Fisher). Proteins were blotted with the indicated primary and secondary DyLight antibodies and detected by using the Odyssey Imaging System (LI-COR, Lincoln, NE).

**RNA Extraction and Quantitative Real-Time PCR**

Total RNA extraction was performed with the RNeasy Mini Kit according to manufacturer’s instructions (Qiagen). RNA was retrotranscribed using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Multiplex quantitative PCR was performed with the Applied Biosystems (Foster City, CA) 7300 PCR System by using specific TaqMan assays for FAF1-FAM (hs00169544_m1) and CCND1-FAM (hs00765553_m1). The endogenous control gene was GAPDH-VIC (4326317E). Relative expression levels of each target gene were calculated for each sample as −ΔCt values (−ΔCt= − [Ct target gene – Ct endogenous control]).

**Cell Proliferation**

The proliferative capacity of cells was determined using the colorimetric CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). Transfected cells were seeded at a density of 5000 cells per well in a 96-well plate, in quintuplicate. After 72 hours, 20 μL of CellTiter 96 aqueous reagent was added to each well. Plates were incubated at 37°C for 3 hours and absorbance was read at 490 nm using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT).

**Colony Formation Assay**

Single-cell suspensions were prepared with Accutase and by filtration through a 40 μm cell strainer when needed. A total of 200 cells per well were seeded into a 6-well plate in complete growth medium. After 14 days, colonies were fixed in methanol and stained with 0.5% crystal violet (Sigma Aldrich). Colonies were imaged on an EliSpot Reader System (AID GmbH, Strassberg, Germany) and analyzed by ImageJ (National Institutes of Health, Bethesda, MD).

**Migration Assay**

The wound-healing assay was performed by seeding transfected cells until confluence in 12-well plates. Cells were treated with 0.2 μg/mL of mitomycin C to arrest proliferation before scratching the confluent cell monolayer with a sterile pipette tip. Migration was monitored every 24 hours until scratch closure. Images were acquired by using the cell-R software on an Olympus IX51 microscope (Tokyo, Japan). ImageJ software was used to quantify the scratch closure area at each time point.
References


Supplementary Figure 1. Immunohistochemistry analysis. FAF1 immunohistochemical staining in normal colonic mucosa of a control individual and tumor tissue of a FAF1 c.111G>A mutation carrier (FAM13, individual III-5). A general FAF1 intracellular staining is detected.

(A) c.111G>A (p.Asp371Asn) FAF1 variant in FAM13.

(B) c.254G>C (p.Arg85Pro) FAF1 variant in F-0681-00.

Supplementary Figure 2. Variant validation and segregation analysis. (A) c.1111G>A (p.Asp371Asn) FAF1 variant in FAM13. Sequencing results show the heterozygous change in individuals III-3, III-5, IV-5, and IV-7. Sequencing results are shown using the reverse primer. The observed C>T heterozygous change corresponds to G>A in the forward orientation. (B) c.254G>C (p.Arg85Pro) FAF1 variant in F-0681-00. Sequencing results show the G>C heterozygous change in the patient’s sample. A reference sequence from a noncarrier is also presented.
Supplementary Figure 3. LOH results. LOH tested for 2 c.1111G>A (p.Asp371Asn) FAF1 variant carriers (FAM13, individuals IV-7 and III-5). Results show a slight LOH in the tumor from patient IV-7 and a complete depletion of the wild-type allele in the case of the patient III-5.

Supplementary Figure 4. CRISPR/Cas9 FAF1^KO modeling results. Time-course development of TRAIL-induced apoptosis, measured as caspase-3 activation (n = 1). On the right, representative images of treated cells at the indicated timepoints. Scale bar, 200 μm.
Supplementary Figure 5. Functional characterization of germline variants results. Determination of caspase-3 activity after TRAIL-induced apoptosis on FAF1\(^{\text{KO}}\). Cells expressing both p.Arg85Pro and p.Asp371Asn FAF1 variants were more resistant to cell death in comparison to those expressing the wild-type form of FAF1.

Supplementary Table 1. PCR primers used in the study

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NOTE. Sanger validation of FAF1 missense variants on patients’ samples.
### Supplementary Table 2. Mutation identification in replication cohort

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### Supplementary Table 3. CRISPR Gene Editing

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### Supplementary Table 4. Site-Directed Mutagenesis

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<td>FAF1 ORF c.254G-&gt;C</td>
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