

Mutations in Tetratricopeptide Repeat Domain 7A Result in a Severe Form of Very Early Onset Inflammatory Bowel Disease

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BACKGROUND & AIMS: Very early onset inflammatory bowel diseases (VEOIBD), including infant disorders, are a diverse group of diseases found in children younger than 6 years of age. They have been associated with several gene variants. Our aim was to identify the genes that cause VEOIBD. **METHODS:** We performed whole exome sequencing of DNA from 1 infant with severe enterocolitis and her parents. Candidate gene mutations were validated in 40 pediatric patients and functional studies were carried out using intestinal samples and human intestinal cell lines. **RESULTS:** We identified compound heterozygote mutations in the Tetratricopeptide repeat domain 7 (*TTC7A*) gene in an infant from non-consanguineous parents with severe exfoliative apoptotic enterocolitis; we also detected *TTC7A* mutations in 2 unrelated families, each with 2 affected siblings. *TTC7A* interacts with EFR3 homolog B to regulate phosphatidylinositol 4-kinase at the plasma membrane. Functional studies demonstrated that *TTC7A* is expressed in human

enterocytes. The mutations we identified in *TTC7A* result in either mislocalization or reduced expression of *TTC7A*. Phosphatidylinositol 4-kinase was found to co-immunoprecipitate with *TTC7A*; the identified *TTC7A* mutations reduced this binding. Knockdown of *TTC7A* in human intestinal-like cell lines reduced their adhesion, increased apoptosis, and decreased production of phosphatidylinositol 4-phosphate. **CONCLUSIONS:** In a genetic analysis, we identified loss of function mutations in *TTC7A* in 5 infants with VEOIBD.

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Abbreviations used in this paper: co-IP, co-immunoprecipitate; EFR3B, EFR3 homolog B; MIA, multiple intestinal atresia; PI4KIII α , phosphatidylinositol 4-kinase III α ; SCID, severe combined immunodeficiency; shRNA, short hairpin RNA; TPR, tetratricopeptide repeat; *TTC7A*, tetratricopeptide repeat domain 7; VEOIBD, very early onset inflammatory bowel diseases; WT, wild type.

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Functional studies demonstrated that the mutations cause defects in enterocytes and T cells that lead to severe apoptotic enterocolitis. Defects in the phosphatidylinositol 4-kinase–TTC7A–EFR3 homolog B pathway are involved in the pathogenesis of VEOIBD.

Keywords: IBD; Intestinal Atresia; Autoimmunity; Intestine.

Very early onset inflammatory bowel diseases (VEOIBD), including forms of infantile disease, are a diverse group of diseases that are diagnosed before 6 years of age.¹ In contrast to adult-onset IBD, VEOIBD frequently encompasses a unique clinical presentation with severe, colonic disease that often has a poor response to standard therapies, including biologic agents.^{2,3} Recently, several groups, including our own, demonstrated that mutations in *IL10RA/B* genes⁴ cause a severe form of VEOIBD, with symptoms consistently developing in infancy.⁵ Subsequently, causative variants in *IL10*,⁶ *XIAP*,⁷ *ADAM17*,⁸ and *NCF4*,⁹ and association variants in the nicotinamide adenine dinucleotide phosphate oxidase genes *NCF2/RAC2*¹⁰ were identified in VEOIBD patients, suggesting that severe infantile colitis frequently starting immediately after birth might represent a group of heterogeneous monogenetic diseases.

Recently, mutations in the tetratricopeptide repeat domain 7 (*TTC7A*) gene were found to cause multiple intestinal atresia (MIA) with severe combined immunodeficiency (SCID), although no details about the intestinal phenotype or function of the *TTC7A* gene were provided.^{11,12} In this report, we describe novel human mutations in the *TTC7A* gene (we termed *TTC7A deficiency*) identified independently by whole exome sequencing that result in severe infantile apoptotic enterocolitis with and without MIA and define the intestinal defects associated with this novel form of VEOIBD.

Materials and Methods

Whole Exome Sequencing

Genetic studies were carried out with approval from the research ethics board at the Hospital for Sick Children, University of Oxford, Cedars-Sinai Medical Center, and Dr von Hauner Children's Hospital, LMU Munich. In the index case, whole exome sequencing was performed using the SureSelect Human All Exon 50 Mb kit (Agilent, Santa Clara, CA) with high-throughput sequencing conducted using the Solid 4 System at The Center for Applied Genomics through the Hospital for Sick Children (Toronto, ON) on the complete parent–child trio set. Sanger sequencing was used to verify variant genotypes in the index patient and her family, and 40 infantile patients from the institutions named here were screened for *TTC7A* mutations.

Histologic methods are presented in the [Supplementary Material](#).

Tandem Mass Spectrometry

Detailed methods are presented in the [Supplementary Materials](#). Briefly, to identify potential interactors of TTC7A, M2 anti-FLAG-agarose FLAG-agarose FLAG-tagged wild type

(WT), E71K, or Q526X TTC7A were transiently overexpressed in HEK293T, immunoprecipitated with FLAG-agarose, and bound proteins were trypsin digested and analyzed by tandem mass spectrometry as described previously.¹³

Knockdown of Endogenous TTC7A by Short Hairpin RNA

GIPZ human TTC7A short hairpin RNA (shRNA) (green fluorescent protein tagged) targeting coding regions and green fluorescent protein tagged control shRNA (Thermo Scientific, Logan, UT) were transfected into Henle-407 cells with Lipofectamine 2000 (Life Technologies, Carlsbad, CA). Detailed methods are provided in the [Supplementary Materials](#).

Apoptosis Analysis

Confluent cells were starved for indicated time points. Apoptosis was assessed by both measured caspase-3 using Western blotting and cytoplasmic DNA fragments using flow cytometric analysis of Annexin V. Cells were stained with Annexin V-phycoerythrin and 7-aminoactinomycin D (BD Biosciences, San Jose, CA) according to manufacturer's instructions, and samples were run on a BD LSR II analyzer. Apoptotic cells were identified as Annexin V⁺ 7-aminoactinomycin D[−] cells.

Cell Adhesion Assay

To evaluate cellular adhesion, approximately 5×10^4 cells were seeded on 96-well plates precoated with fibronectin (20 μ g/mL; Sigma-Aldrich, St Louis, MO), collagen type I (50 μ g/mL; Life Technologies), or bovine serum albumin (5% in phosphate-buffered saline; Sigma) for 60 minutes at 37°C. The wells were subsequently washed with phosphate-buffered saline twice to remove nonadherent cells. After fixation with 4% paraformaldehyde, attached cells were visualized by staining with 1% crystal violet dissolved in 33% acetic acid and were quantified by measuring the absorbance at 570 nm on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA).

Constructs, Western Blot, Cell Culture, and Immunoprecipitation

Details of constructs, antibodies, and methods used can be found in the [Supplementary Materials](#).

Statistical Analysis

Data are presented as mean \pm SD. Experiments were performed with a minimum of 3 replications. Statistical significance between groups was established at $P < .05$ using a 2-tailed Student *t* test. *P* values are indicated in the figure legends and text.

Results

Identification of Apoptotic Enterocolitis in a VEOIBD Patient

In Family 1 (index case), a female patient born at term to a Caucasian mother and Sudanese father presented with

high-output secretory diarrhea and hematochezia that started almost immediately after birth, requiring total parenteral nutrition. Colonoscopy demonstrated chronic inflammation with severe friability, exfoliative mucosal changes, and sloughed mucosa within the colonic lumen (Figure 1A). Biopsies taken from the duodenum showed villous atrophy and the duodenum and colon showed glandular dropout with crypt apoptosis and exploding crypts (Figure 1B and C). The severity of the epithelial injury was strikingly reminiscent of acute gastrointestinal graft-vs-host disease and intestine allograft rejection. There was no evidence of perianal disease or dermatological disease. The patient had clinical features of immunodeficiency, including

lymphopenia and hypogammaglobulinemia. The patient was treated with 2 mg/kg methylprednisone without significant response. At 11 months of age, she developed respiratory failure and succumbed shortly afterward (see [Supplementary Material](#) for details). Autopsy did not show any evidence of bowel atresia, but did confirm widespread severe apoptotic enterocolitis as identified previously by endoscopy.

In Family 2, an infant male was born at 36 weeks' gestation to non-consanguineous Caucasian parents. Shortly after birth, the infant presented with symptoms of small bowel obstruction due to short-segment jejunal atresia. Despite surgical resection, the intestinal disease progressed

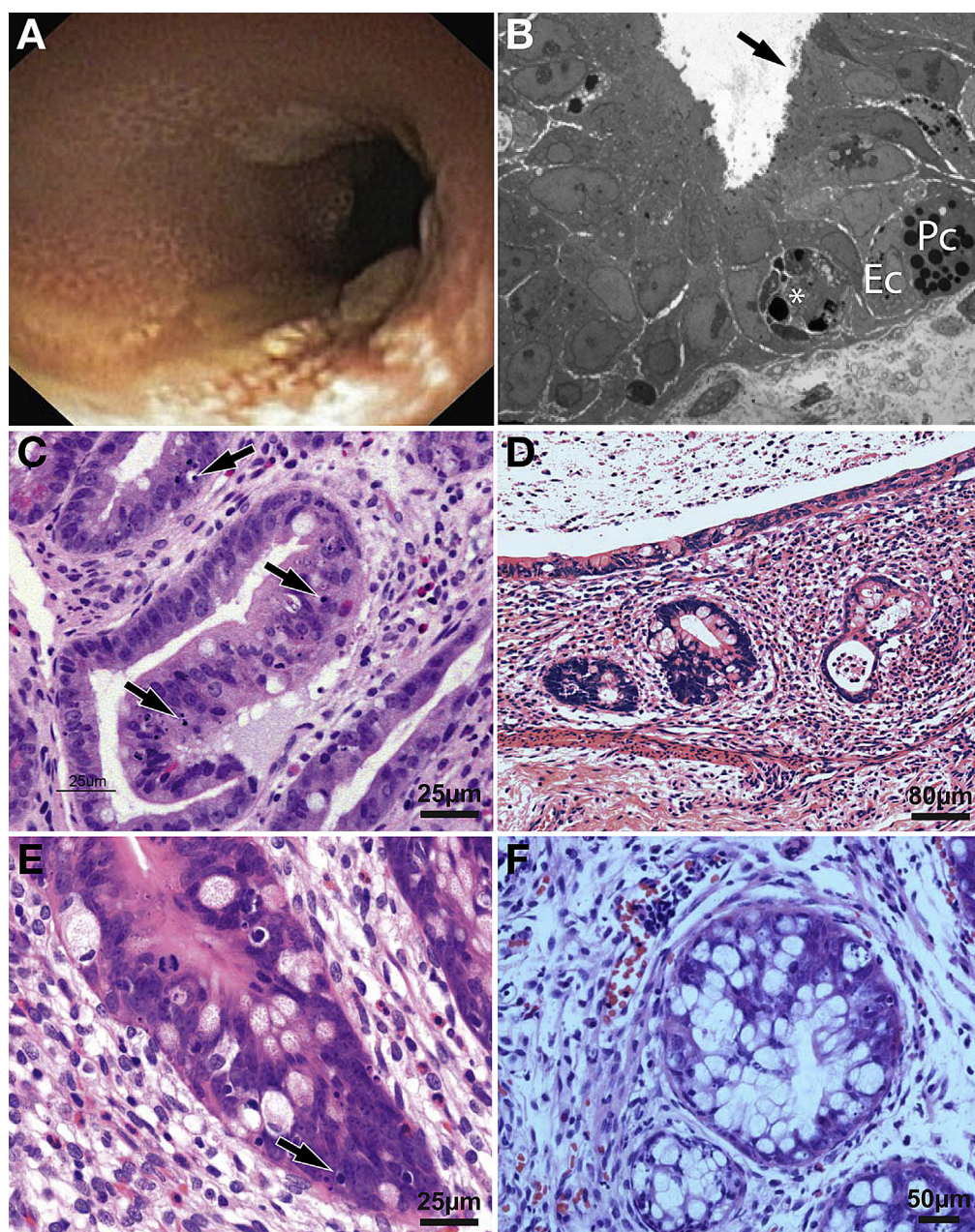


Figure 1. Histologic and endoscopic characteristics of intestine of patients with *TTC7A* mutations. (A) Colonoscopy showed severe inflammation characterized by continuous grade 2 colitis and multiple areas of exfoliation and sloughing of the surface epithelium from Family 1. (B) Low-magnification electron micrograph of crypt epithelium from the same biopsy shown in panel C from Family 1. Among regenerating crypt cells, apoptotic cell (white asterisk), enteroendocrine cell (EC), and Paneth cell (PC) are present. Crypt enterocytes showed sparse brush border microvilli (arrow). (C) High magnification of duodenal crypt epithelium showed extensive apoptosis from Family 1 (arrows) (H&E stain). (D) Low (D) and high (E) magnification of cecum epithelium showed extensive apoptosis from Family 2 (arrow) (H&E stain). (F) High magnification of cecum epithelium showed extensive apoptosis from Family 3 (H&E stain).

and the patient was found to have recurrent multiple atretic areas that also required resection. The disease continued to progress and the patient died of cardiac arrest before 3 months of age. A second child from the same family also had jejunal atresia at birth, which was initially resected. The intestinal disease progressed and ultimately resulted in the patient's death at the age of 19 months. Both children had evidence of immunodeficiency with lymphopenia and T-cell deficiency. Pathologic analysis showed loss of intestinal architecture, focal scarring, and severe inflammation with increased apoptosis reminiscent of graft-vs-host-disease, as described in Family 1 (Figure 1D and E).

Family 3 had 2 infant daughters from consanguineous parents who presented with diarrhea and failure to thrive shortly after birth. Both children had no evidence of overt immunodeficiency and pathologic analysis of colonic biopsies showed similar loss of intestinal architecture, focal scarring, and severe inflammation with increased enterocyte apoptosis (Figure 1F) and areas where surface epithelium was detached, as described in Family 1 and Family 2. The younger girl died before the age of 1 year due to uncontrolled candida sepsis and the older girl is presently being partially treated with total parenteral nutrition (see Supplementary Material for details; summarized in Table 1).

Whole Exome Sequencing

Whole exome sequencing of Family 1 resulted in >80 times coverage of exomes and the subsequent identification of a nonsynonymous variant in exon 2 inherited from the father, and a nonsense mutation in exon 14 inherited from the mother in the *TTC7A* gene (Figure 2A). The nonsynonymous mutation in exon 2 at c.211 G>A resulted in a glutamic acid to lysine substitution at amino acid position 71 (p.E71K; rs147914967). The mutant allele is not found in either the National Center for Biotechnology Information database or the 1000 Genomes database, and is only found in 1 heterozygous allele from 6503 healthy individuals genotyped in the National Heart, Lung, and Blood Institute's Exome Sequencing Project (<http://evs.gs.washington.edu>). The mutation was predicted to be highly deleterious with a Polyphen¹⁴ score of 0.99 and is located in a highly conserved α -helical region (Figure 2D and Supplementary Figure 1). The second *TTC7A* mutation in exon 14 at c.1944 C>T transition resulted in a nonsense mutation causing the premature termination of the protein at amino acid 526 (p.Q526X). This nonsense mutation has not been described previously in the datasets mentioned.

In siblings from Family 2 with severe apoptotic enterocolitis, we also identified heterozygous *TTC7A* mutations. The c.844-1 G>T mutation in the splice acceptor site of exon 7 was inherited from the mother and a c.1204-2 A>G mutation in splice acceptor site of exon 10 was inherited from the father (Figure 2B). These *TTC7A* mutations were predicted to result in loss of the splice acceptor sites for both exons 7 and 10, leading to skipping of both exon 7 and 10, respectively, and causing premature stop codons that would disrupt tetratricopeptide repeat (TPR) domains (Figure 2D). These splice mutations have not been previously described

Table 1. Summary of *TTC7A* Mutations and Clinical Features

	Age at presentation	Sex	CS	Clinical features	Immune workup	Outcome	<i>TTC7A</i> mutation	<i>TTC7A</i> mutated protein
Patient 1 (Family 1)	At birth	Female	No	Bloody diarrhea, AE	Lymphopenia, hypogammaglobulinemia	Died at 11 mo of age	c.211 G>A	p.E71K
Patient 2 (Family 2)	At birth	Male	No	Obstruction, stricture, AE	Lymphopenia	Died at 3 mo of age	c.1944 C>T	p.Q526X
Patient 3 (Family 2)	At birth	Female	No	Obstruction, stricture, AE	Lymphopenia	Died at 19 mo of age	c.844-1 G>T	Loss of the splice acceptor sites for exons 7 and 10
Patient 4 (Family 3)	At birth	Female	Yes	Bloody diarrhea, AE	Normal	Died at 11 mo of age	c.1204-2 A>G	Loss of the splice acceptor sites for exons 7 and 10, respectively
Patient 5 (Family 3)	At birth	Female	Yes	Bloody diarrhea, AE	Normal	Alive, TPN for partial control	c.2494 G>A	p.A832T
							c.2494 G>A	p.A832T

AE, apoptotic enterocolitis; CS, consanguinity; TPN, total parenteral nutrition.

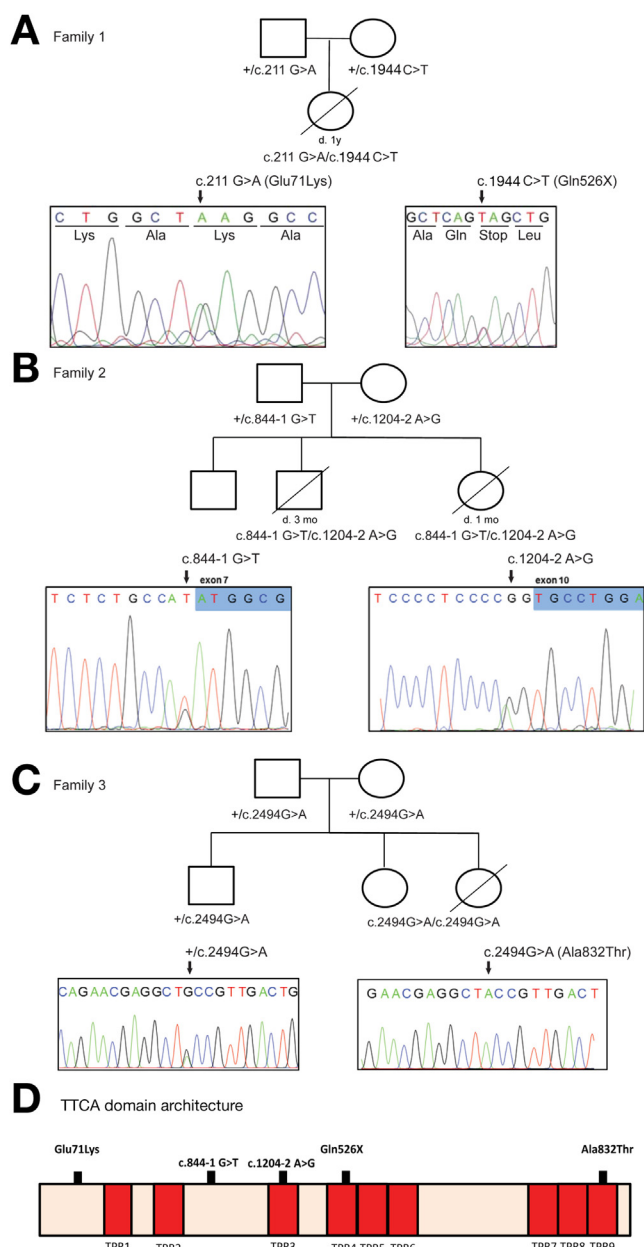


Figure 2. *TTC7A* genetic analysis. (A) Pedigree and *TTC7A* mutations in Family 1. Patient from Family 1 was heterozygous for 211G>A (p.E71K) inherited from her father and c.1944 C>T (p.Q526X) inherited from her mother. (B) Pedigree and *TTC7A* mutations in Family 2. Siblings from Family 2 were heterozygous for a novel c.844-1 G>T *TTC7A* mutation in splice acceptor site of exon 7 inherited from the mother and a novel c.1204-2 A>G *TTC7A* mutation in splice acceptor site of exon 10 inherited from the father. A third sibling was found to be negative for both mutations. (C) Pedigree and *TTC7A* mutations in Family 3. Siblings from Family 2 were homozygous for a nonsynonymous mutation in exon 20 at c.G2494A resulted in a alanine acid to threonine substitution at amino acid position 832 (p.A832T). (D) Location of *TTC7A* mutations in patients. Illustration of *TTC7A* protein with TPR domains in red and identified mutations are highlighted.

in the datasets mentioned and it is likely that these mutations will result in nonsense-mediated decay of the *TTC7A* messenger RNA.

In siblings from Family 3 with severe apoptotic enterocolitis, we identified a homozygous nonsynonymous mutation in exon 20 at c.2494 G>A (Figure 2C) that resulted in an alanine to threonine substitution at amino acid position 832 (p.A832T). The mutation was predicted to be highly deleterious with a Polyphen¹⁴ score of 0.99 and is located in a highly conserved region of the ninth TPR domain (Figure 2D; Supplementary Figure 1) and has not been described previously in the datasets mentioned (see Table 1 for a summary).

Functional Analysis of *TTC7A* Mutations in Enterocytes

Immunostaining of *TTC7A* from healthy human control intestinal tissue (duodenum, ileum, and colon) showed that *TTC7A* was strongly expressed in enterocytes with areas of discreet localization at the plasma membrane and only few lamina propria cells stained positive (Figure 3A). This observed pattern of intestinal expression suggests a primary role for *TTC7A* in enterocyte homeostasis. To determine if the mutation identified in Family 1 and Family 3 resulted in abnormal *TTC7A* cellular localization, we transiently transfected Caco-2 cells with Myc-tagged WT, E71K, Q526X, and A832T *TTC7A*. Immunostaining using anti-Myc antibody demonstrated that E71K, Q526X, and A832T *TTC7A* mutants appeared to accumulate in cytoplasmic puncta, and the WT-*TTC7A* localized diffusely in the cytoplasm (Figure 3B). In addition, biopsies from the Family 2 patient with the *TTC7A* splice acceptor site mutations predicted to result in complete loss of the protein showed loss of *TTC7A* in enterocytes, as expected, indicating that these mutations might result in nonsense-mediated decay of the *TTC7A* messenger RNA (Figure 3C).

Knockdown of *TTC7A* by shRNA resulted in loss of cobblestone morphology, typical of human Henle-407 cells with the development of fibroblastoid morphology with spindle-like features (Figure 3D and Supplementary Figure 2). In addition, overexpression of E71K, A832T, and Q526X *TTC7A* in Caco-2 cells demonstrated cytoplasmic accumulations of Myc-*TTC7A* in addition to disrupted cortical actin staining suggestive of adhesion defects or loss of cellular polarity (Supplementary Figure 3). Reduced expression of *TTC7A* in the enterocytes also resulted in detachment during trypsinization (Figure 4A), impaired adhesion to collagen and fibronectin (Figure 4B), and increased apoptosis, as measured by caspase-3 (Figure 4C) and Annexin V (Figure 4D). These cellular changes are reminiscent of the apoptosis and mucosal exfoliation described in our patient.

TTC7A Binding Partners

Tandem mass spectrometry was performed on proteins co-immunoprecipitated (co-IP) from HEK293T cells expressing human *TTC7A*, with the aim of identifying *TTC7A* binding partners. Isolated proteins were digested with trypsin to generate peptide fragments and analyzed by tandem mass spectrometry (Supplementary Figure 4). To

refine this list to TTC7A binding partners, spectral hit counts were compared between WT TTC7A and the E71K mutation samples, and determined that phosphatidylinositol 4-kinase III α (PI4KIII α) protein fragments were able to co-IP with WT TTC7A, but were significantly reduced with E71K TTC7A mutation (Figure 5A and Supplementary Figure 4). This PI4KIII α and TTC7A interaction was supported by a weighted coexpression network¹⁵ from small bowel gene expression data demonstrating that *Ttc7a* falls within a subnetwork (module) of the mouse small bowel network that included *Pi4kca* (murine form of PI4KIII α) (Supplementary Tables 1–4; Supplementary Figure 5). The additional hits identified in the tandem mass spectrometry screen (Supplementary Figure 4) implicated several proteins associated with ubiquitination pathways, including E3 ligases (HUWE1, HECTD1, UBR5), and proteins that function in the ubiquitin-proteasome system (USP9X, PSMD1, VCP).

Loss of TTC7A Results in PI4KIII α Dysfunction

As TTC7A has been implicated previously in PI4KIII α regulation in yeast,^{16,17} and confirmed through the tandem mass spectrometry and network analysis of mouse small bowel, we next confirmed through co-IP experiments that TTC7A and PI4KIII α interacted in human cell lines. We found that Myc-Flag-tagged WT TTC7A was able to co-IP PI4KIII α , indicating that these proteins interact either directly or indirectly in a larger complex (Figure 5B). We also observed reduced co-IP of PI4KIII α with the TTC7A Q526X and E71K mutated proteins identified in Family 1 and the A832T TTC7 mutation identified in Family 3 (Figure 5B). As the splice variants identified in Family 2 were assumed to be unstable, we would predict that the gene product of these *TTC7A* mutations would also not bind to PI4KIII α .

We next examined human PI4KIII α in intestinal tissue of healthy controls and found that PI4KIII α was abundantly expressed in both enterocytes and immune cells, including lymphocytes (Figure 5C, left panel). In our patients with TTC7A deficiency, the severe disruption of the bowel architecture with sloughing of the majority of enterocytes made interpretation of PI4KIII α localization difficult; however, in areas with relatively preserved epithelial architecture, we observed overall reduced PI4KIII α expression in a patient from Family 2 (while lamina propria expression was preserved; Figure 5C). To confirm these results, we transiently co-transfected TTC7A and TTC7A shRNA into Henle-407 cells and observed a reduction in PI4KIII α (Figure 5D and E). These results indicate that loss of TTC7A resulted in aberrant subcellular localization of PI4KIII α in enterocytes.

Finally, we determined that knockdown of TTC7A in human Henle-407 cell lines resulted in decreased phosphatidylinositol 4-phosphate, the end product of PI4KIII α enzyme, in both the cytoplasm (Figure 5E) and at the plasma membrane (Figure 5F). Together these results indicate that TTC7A is required for PI4KIII α localization to the plasma membrane and that TTC7A deficiency results in loss of PI4KIII α signaling in enterocytes.

Discussion

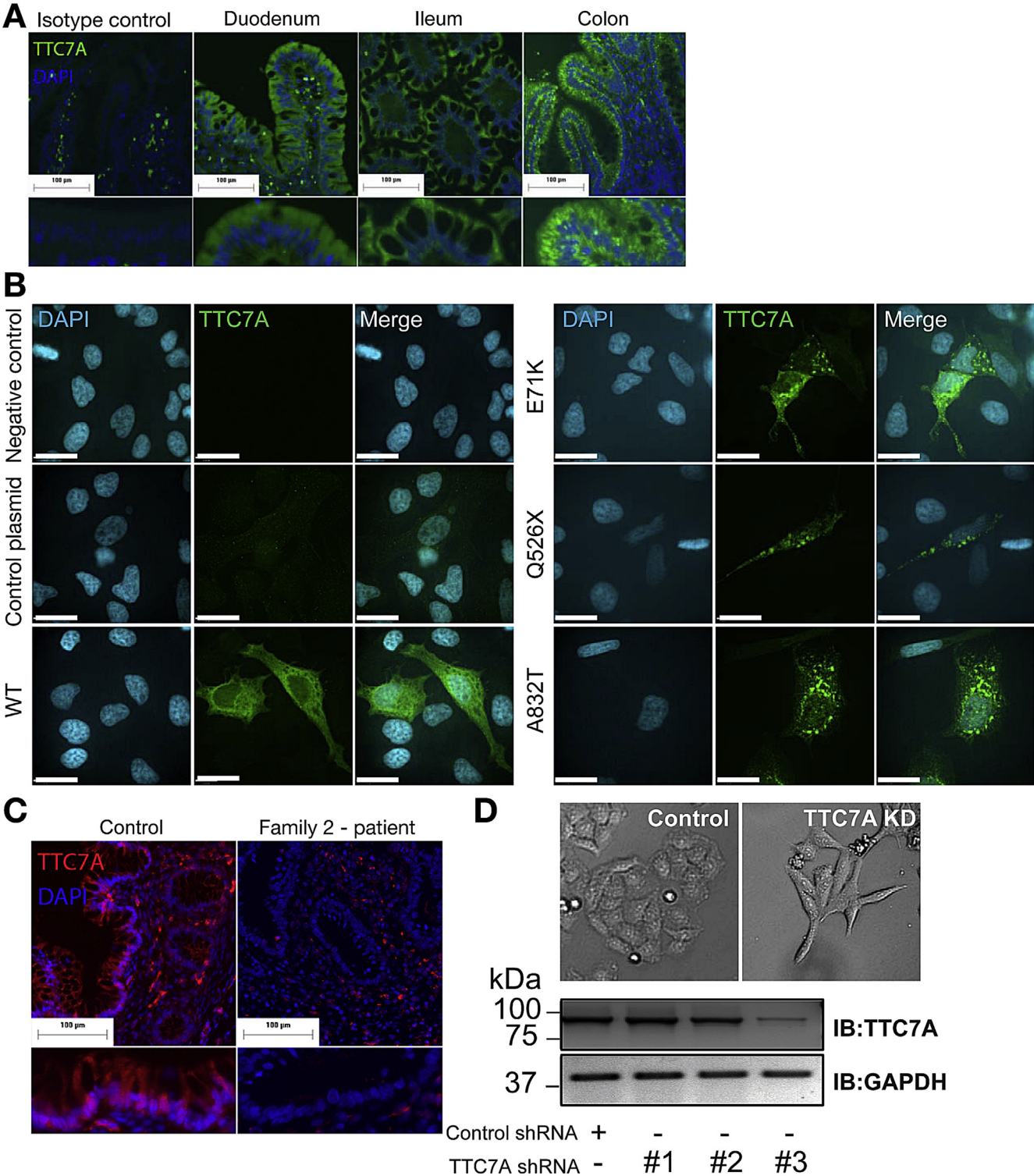
Our index case (Family 1) had severe infantile apoptotic enterocolitis with a presentation significantly different from previously described cases of VEOIBD with *IL10* and *IL10R* mutations that are invariably present with colitis and perianal disease.^{4,5,18–20} The severe enterocolitis with friability and exfoliative mucosal changes along with villous atrophy, gland dropout, and crypt apoptosis led to our genetic exploration through whole exome sequencing and identification of *TTC7A* as the causative gene.

The TPR domain is defined by a degenerate consensus sequence of 34 amino acids²¹ and 4 of the 5 *TTC7A* mutations found in our patients resulted in disruption of these TPR domains. TPR domains mediate protein–protein interactions and the assembly of multi-protein complexes that are involved in the regulation of cell cycle, transcription, and protein transport.²² Our tandem mass spectrometry and intestinal network experiments demonstrated an association between TTC7A and PI4KIII α that was previously only described in yeast.^{16,17} In yeast, the *TTC7* ortholog, *YPP1*, is essential and has been shown to rescue a lethal α -synuclein (α Syn-A53T) yeast mutant.²³ Ypp1 (TTC7) directly binds to Stt4 (PI4KIII α), and this binding is critical to maintaining phosphatidylinositol 4-phosphate levels and PI4KIII α stability at the plasma membrane.^{16,17} In addition, in yeast, the phenotypes of *YPP1* (TTC7) and *STT4* (PI4KIII α) conditional mutants are identical and both mutants result in cell wall destabilization and defective organization of actin. Overexpression of *STT4* (PI4KIII α) also suppresses the temperature-sensitive growth defect observed in *YPP1* (TTC7) mutants.¹⁷ The role of TTC7A in PI4KIII α recruitment to the plasma membrane was also recently confirmed in mammalian cell lines²⁴ and we demonstrate for the first time that TTC7A and PI4KIII α directly interact in human cell lines. Because TTC7A is required for proper localization of PI4KIII α at the plasma membrane,²⁴ we propose that TTC7A mutations result in disease through loss of PI4KIII α at the plasma membrane and subsequent reduction of phosphatidylinositol 4-phosphate that is required for cell polarity and survival. In support of this model, down-regulation of PI4KIII α results in increased apoptosis²⁵ and, in addition, intestinal-specific murine knockout of *Pi4kca* (PI4KIII α) results in a strikingly severe intestinal phenotype with widespread mucosal epithelial degeneration²⁶ reminiscent of our patients with *TTC7A* mutations. Therefore, our results demonstrate a direct interaction between PI4KIII α and TTC7A. And similar to the phenotype observed in TTC7A-deficient patients, TTC7A knockdown in human intestinal-like cell lines resulted in decreased adhesion and increased apoptosis. These results indicate that disruption of the PI4KIII α –TTC7A pathway results in a combined T-cell and enterocyte defect that results in the intestinal phenotype described here (Figure 6).

The EFR3 homolog B (*EFR3B*; ENSG00000084710) gene product EFR3B tethers TTC7A (and TTC7B) to the plasma membrane and is essential for both TTC7A and PI4KIII α function.²⁴ In addition, knockdown of EFR3B results in the loss of both TTC7A and PI4KIII α at the plasma membrane

and is critical for PI4KIII α signaling.²⁴ Interesting, several *EFR3B* single nucleotide polymorphisms located both in the *EFR3B* gene and its flanking regions were reported to be associated with Crohn's disease (<http://www.ibdgenetics.org>; Supplementary Table 5; lead single nucleotide polymorphism rs1077492; $P = 1.9 \times 10^{-14}$, odds ratio, 1.11). This locus on chromosome 2 at 25.12 Mb was recently

reported in the International Inflammatory Bowel Disease Genetics Consortium meta-analysis as an IBD locus.²⁷ In silico analyses carried out by the International Inflammatory Bowel Disease Genetics Consortium suggested ADCY3 as a potential candidate at this locus²⁷; however, *EFR3B*'s role in regulating PI4KIII α -TTC7A implicates *EFR3B* as a plausible causative IBD gene at this locus and that this



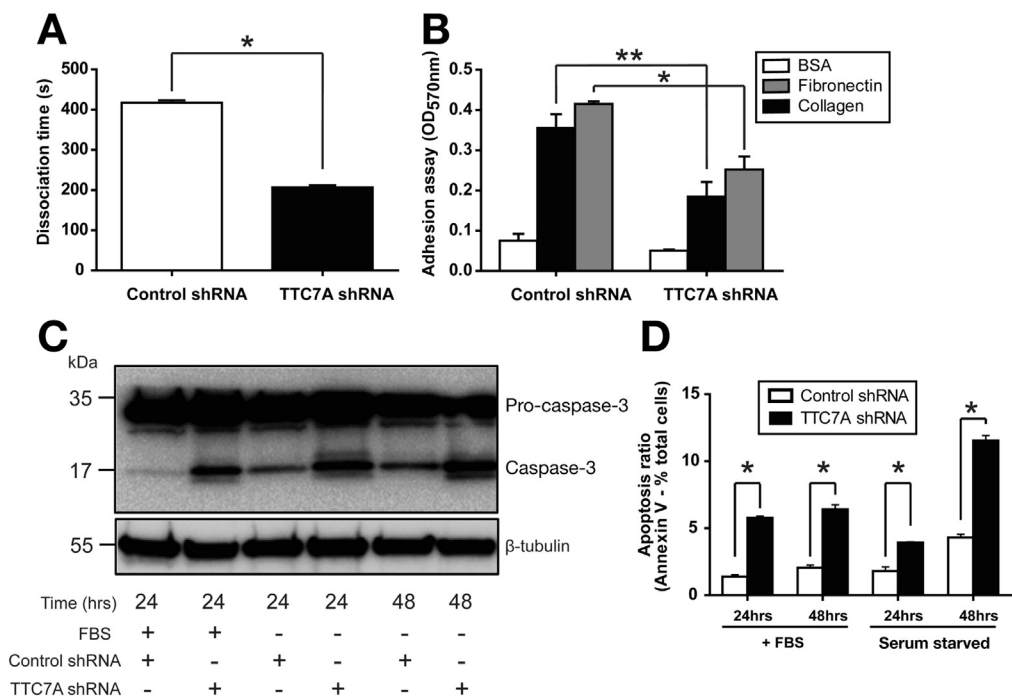


Figure 4. (A) Impaired cell adhesion in TTC7A-depleted cells. The total dissociation time, defined as the time required for complete dissociation of all cells from the tissue culture plate, was markedly reduced in TTC7A-depleted Henle-407 cells compared with control cells. Dissociation assay ($n = 6$, biological replicates; Student t test, $*P = .0022$). (B) Impaired cell adhesion to collagen and fibronectin in TTC7A-depleted cells. Cell adhesion assays were performed using crystal violet staining. Control and TTC7A-depleted Henle-407 cells were seeded on 96-well plates coated with either collagen or fibronectin. Adhesion was assessed on the basis of optical density (OD) at 570 nm. $n = 3$; adhesion assay ($n = 3$) biological replicates, Student t test, $**$ collagen: $P = .027$ and $*$ fibronectin: $P = .0077$. (C) TTC7A-depletion in Henle-407 cells results in greater caspase-dependent apoptosis. To investigate the impact of TTC7A suppression on the induction of apoptosis, the activation of caspase-3 was measured by Western blot. Specific cleavage of pro-caspase 3 (32 kDa) into the active caspase-3 fragments (17 kDa) was increased in cells serum starved for 24 and 48 hours. $n = 3$; $P = .012$, analysis of variance. (D) TTC7A-depletion in Henle-407 cells results in greater apoptosis measured by flow cytometric analysis of Annexin V. To examine the significance of TTC7A suppression, after loss of attachment, flow cytometric analysis was conducted to quantify the extent of apoptosis in cells starved for 24 and 48 hours. Cells were stained with Annexin V-phycoerythrin and 7-aminoactinomycin D (viability marker); apoptotic cells were identified as Annexin V⁺ 7-aminoactinomycin D⁻ cells. In TTC7A-depleted cells, the proportion of cells in early apoptosis increased to approximately 4.5% at 24 hours and 11.2% at 48 hours of serum-starvation compared to 1.3% (24 hours) and 4.1% (48 hours) in control cells. Annexin V apoptosis assay $n = 3$ biological replicates, Student t test, fetal bovine serum (FBS), 24 hours: $*P = .0018$; FBS, 48 hours: $*P = .0076$; serum starved, 24 hours: $*P = .021$; serum starved, 48 hours: $*P = .0034$. BSA, bovine serum albumin.

PI4KIII α -TTC7A-EFRB3 pathway plays a broader role in adult-onset IBD.

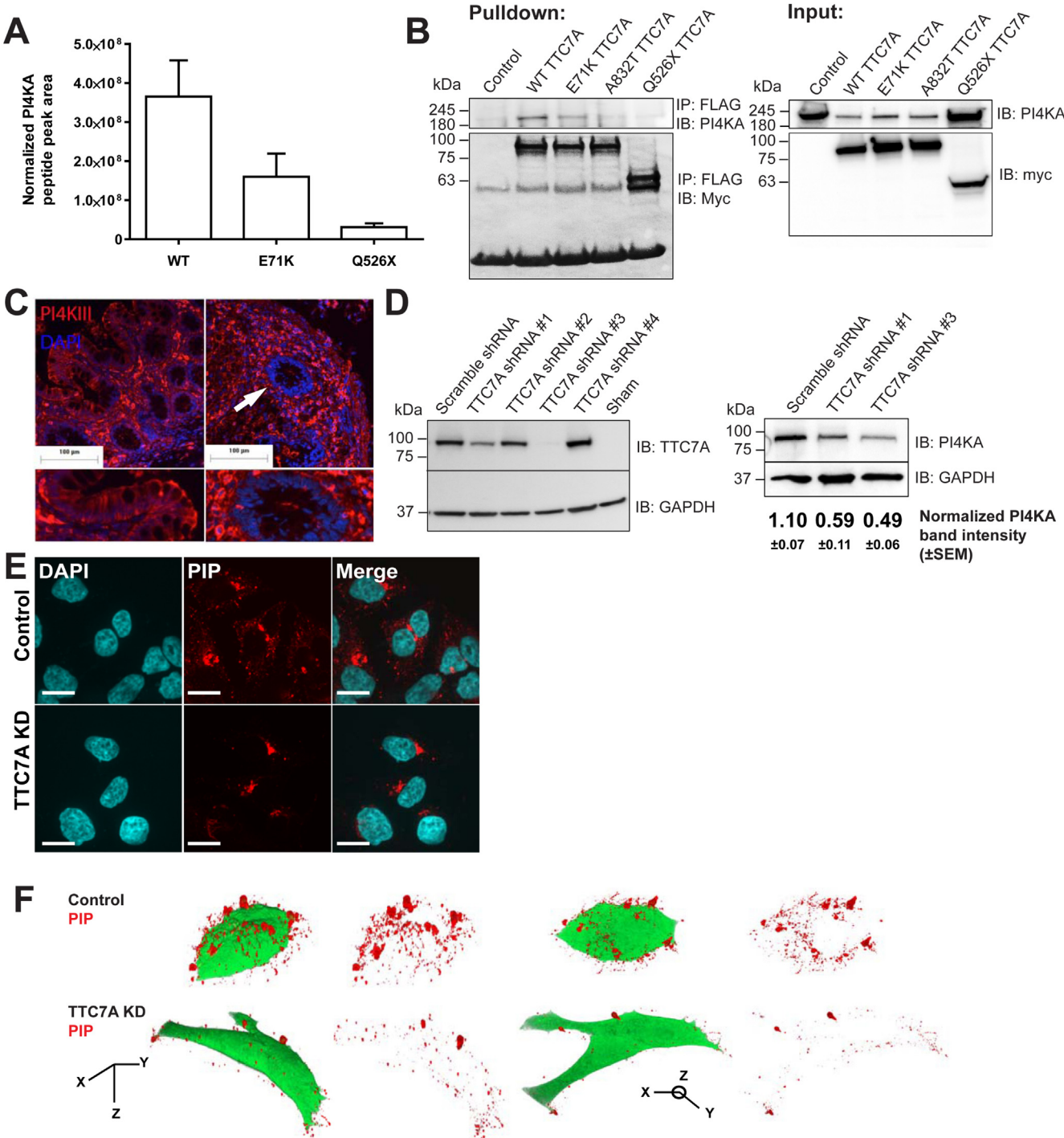
Mutations in a *Ttc7a* TPR domain^{28–30} result in flaky skin (*fsn*) mice and the associated pleiotropic abnormalities,

including severe weight loss with diarrhea and intestinal apoptosis, being reported infrequently.^{31,32} In addition, the intestinal-specific knockout of *Pi4kca* (PI4KIII α) in mice resulted in a severe intestinal phenotype with widespread

Figure 3. Functional TTC7A enterocyte studies. (A) TTC7A expression in intestinal enterocytes. Immunofluorescence microscopy performed on human tissue sections immunostained using anti-TTC7A antibody (and 4',6-diamidino-2-phenylindole [DAPI]) demonstrates TTC7A expression in enterocytes within the duodenum, ileum, and colon. Lower inset panels represent zoomed images of the corresponding panel above. Scale bars = 100 μ m. (B) E71K, Q526X, and A832T mutations in TTC7A. Caco-2 cells were transiently transfected with Myc-tagged WT, E71K, Q526X, and A832T TTC7A, immunostained using anti-Myc antibody and visualized using confocal microscopy. Negative control panels represent staining with secondary antibody only. The control plasmid represents an empty vector sham transfection. Scale bars = 25 μ m. (C) TTC7A in enterocytes from patient cecum (Family 2). Immunofluorescence microscopy was performed on TTC7A-immunostained (and DAPI) cecal tissues sections from both control and patient (Family 2) biopsies. Compared to control staining (left panel), TTC7A expression is reduced in the patient sample (right panel). Scale bar = 100 μ m. (D) Stable knockdown of TTC7A resulted in morphological changes in Henle-407 cells. Expression of TTC7A in stably transfected Henle-407 cells was reduced (70%–80%) compared with Henle-407 cells stably transfected with control shRNA. The impact on cellular morphology of control and TTC7A shRNA knockdown was examined in Henle-407 cells by contrast microscopy (100 \times magnification) under normal culture conditions. Knockdown of TTC7A resulted in a loss of cobblestone morphology and development of fibroblastoid morphology with spindle-like features. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

mucosal epithelial degeneration.²⁶ The intestinal phenotype observed in these 2 mouse models is reminiscent of the phenotype seen in our infantile patients who had massive shedding of enterocytes with increased apoptosis; however, none of the patients developed psoriasis or other skin abnormalities like the *fsn* mice. TTC7A has been investigated in human psoriasis and found not to be associated with human dermatological disease.³⁰ Therefore, psoriasis might only be observed in the *fsn* mice and might not be part of the human disorder.

We have shown that TTC7A is expressed in enterocytes and has a role in enterocyte survival and function, suggesting that the physiological abnormalities observed in both mice and humans with TTC7A mutations result, at least in part, from epithelial dysfunction. However, as Chen et al¹¹ also demonstrated, TTC7A is expressed in the thymus with a marked reduction of thymocytes and lymphoid depletion in 1 patient with TTC7A deficiency, TTC7A plays a critical role in modulating immune homeostasis and the immunodeficiency also contributes to the pathogenesis of *TTC7A*



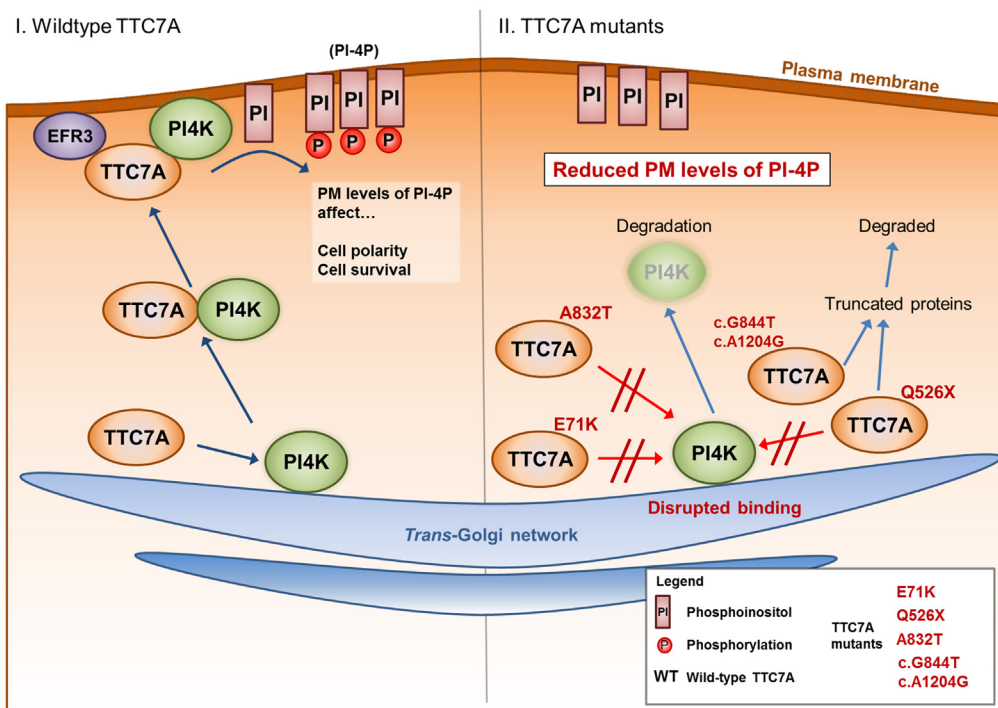


Figure 6. Summary of TTC7 mutations. Schematic representation of the role of TTC7A in the trafficking of PI4KIII α to the plasma membrane from the *trans*-Golgi network. The *left panel* represents WT TTC7A in enterocytes wherein TTC7A binds to and facilitates the transport of PI4KIII α from the *trans*-Golgi to the plasma membrane. At the membrane, PI4KIII α can catalyze the production of PtdIns-4P(PI-4P). PI-4P levels at the plasma membrane have been implicated in cell survival and the maintenance of cell polarity. In the *right panel*, the various TTC7A mutations identified in the patients are depicted. E71K, Q526X, and A832T TTC7A all demonstrated reduced binding to PI4KIII α , which could reduce the interaction between TTC7A and PI4KIII α , hindering transport to the plasma membrane (PM). Consequently, this will lead to reduced plasma membrane levels of PI-4P, a dysregulation that would affect downstream signaling pathways.

Figure 5. (A) Tandem mass spectrometry. E71K and Q526X mutations reduce the ability of TTC7A to immunoprecipitate PI4KA. Selected peptides from PI4KA and TTC7A were analyzed to determine the area under their MS1 peaks to assess the relative abundance of each peptide. The PI4KA present in each sample was normalized to the total TTC7A in each technical replicate to allow comparisons among biological replicates ($n = 3$). These normalized values were averaged over all experiments. Error bars represent the standard error. (B) PI4KIII α -TTC7A co-immunoprecipitate. HEK293T cells were transiently transfected with Myc-tagged WT (WT-TTC7A), E71K, Q526X, and A832T TTC7A constructs. Lysates were immunoprecipitated with anti-Myc antibody, and then immunoblotted using anti-PI4KIII α and anti-Myc (for TTC7A) antibodies. The control lane represents transfection with an empty vector. (C) Expression and localization of PI4KIII α is altered in patients with TTC7A deficiency. Immunofluorescence microscopy was performed on both control and patient colonic tissue sections immunostained with anti-PI4KIII α antibodies. In the *left panel*, immunohistochemistry demonstrated that PI4KIII α is highly expressed in enterocytes and immune cells from healthy human intestine. *Inset panel* depicts zoomed view of *left panel*, demonstrating PI4KIII α expression at the plasma membrane of enterocytes. In the patient tissues, immunohistochemistry demonstrated that PI4KIII α is dysregulated in enterocytes. *Inset panel* (representing region indicated by white arrow) demonstrates loss of PI4KIII α at the plasma membrane of enterocytes bordering the intestinal crypt. Scale bar = 100 μ m. (D) shRNA-mediated knockdown of TTC7A expression leads to decreased PI4KIII α levels. To test the efficacy of the TTC7A shRNA, Henle-407 cells were transiently co-transfected with WT TTC7A and the various knock-down constructs, labeled #1 through #4, including a scrambled shRNA control and sham transfection. shRNA #1 and #3 showed reduction in TTC7A expression (*left panels*). shRNA containing the same targeting sequences were used to lentivirally infect Henle-407 cells where expression of PI4KIII α was assessed in cell lysates by Western blot (*right panels*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was stained as loading control for all blots. The intensity of each PI4KIII α band was normalized to the GAPDH loading control by densitometry. Quantitation of band intensities (listed below each lane) demonstrates a statistically significant reduction in PI4KIII α expression after TTC7A knockdown (Student t test, $n = 3$, $P = .0234$). Each normalized band intensity is presented as \pm SEM. (E) TTC7A depletion results in decreased cytoplasmic phosphatidylinositol 4-phosphate (PI-4P) production. TTC7A knockdown and control Henle-407 cells were stained with antibodies against PtdIns4P (*in red*; Z-P004, IgM, Cedarlane, Burlington, NC) and 4',6-diamidino-2-phenylindole (DAPI) (*in blue*) to visualize nuclei. (F) TTC7A knockdown Henle-407 cells have reduced plasma membrane immunostaining for PI-4P compared with controls. For control and TTC7A knockdown (KD), Henle-407 cells Z-stack images were generated at 0.2-mm intervals and recapitulated using Volocity to generate a 3-dimensional model to illustrate cell surface levels of PIP. Unconjugated green fluorescent protein, expressed from the control and knockdown plasmids, was visualized and used to approximate the morphology of the cells. Each pair of images represents 2 views of the same cell according to axes depicted.

deficiency, as seen in Family 1 and Family 2. These results are consistent with those seen in the MIA patients described with SCID,^{11,12,33} and points to a severe defect in both enterocyte and T-cell function; however, patients from Family 3 did not have an overt T-cell defect and patients with MIA described previously^{11,12,33} had varying degrees of immunodeficiency, with some patients exhibiting mild T-cell lymphopenia in Chen et al,¹¹ who also suggested an enterocyte defect based on the high frequency of bloodstream infections with intestinal microbes.

Therefore, our studies also suggest that mutations in the *TTC7A* gene can result in a spectrum of intestinal disease ranging from VEOIBD with apoptotic enterocolitis, as first described here, to MIA with SCID, as described here and previously.^{11,12,33} In support of this, *TTC7A* mutations were found to cause hereditary MIA with SCID^{11,12,33}; however, apoptotic enterocolitis has not been reported. The patients from Family 1 and Family 3, with apoptotic enterocolitis with no evidence of MIA or stricturing disease on autopsy, had mutations that would be predicted to reduce *TTC7A* expression but not completely abolish function. In support, we also demonstrated that the mutations identified in Family 1 and Family 3 reduced *TTC7A* binding to PI4KIII α . Therefore, it is possible that the disease observed in patients from Family 1 and Family 3 represents a hypomorphic state, where some residual *TTC7A* activity in both enterocytes and the thymus results in severe enterocolitis without MIA and with or without lymphopenia.

All *TTC7A*-deficiency patients, including the patients described here, died in infancy due to their progressive bowel disease, failed allogeneic hematopoietic stem cell transplantation, or survived with short gut and total parenteral nutrition.^{11,12} Interestingly, both Chen et al¹¹ and Samuels et al¹² described an MIA *TTC7A*-deficiency patient who had hematopoietic stem cell transplantation and developed severe recurrence of MIA post transplantation. The recurrence of MIA after resection in our Family 2 patients and those presented by Chen et al and Samuels et al,^{11,12} suggests that *TTC7A* deficiency results in a severe intestinal inflammatory process driven by a combined epithelial and T-cell defect that continues post resection of atretic regions, and the enterocyte defect will not respond to hematopoietic stem cell transplant. Therefore, as we have demonstrated that an enterocyte defect is also found in patients with *TTC7A* deficiency, transplantation of allogeneic hematopoietic stem cells might not be warranted in *TTC7A*-deficient patients. However, our study opens the possibility of pharmacologically targeting the PI4KIII α –*TTC7A*–EFR3B pathway as a potential therapeutic approach. The identification of *TTC7A* as a candidate gene for a unique and unrecognized variant of severe apoptotic enterocolitis expands the genetic diversity of VEOIBD and the need to tailor therapeutic approaches for individual subtypes.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2014.01.015>.

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Conflicts of interest

The authors disclose no conflicts.

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