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Original Article

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Variants in *STXBP3* are Associated with Very Early Onset Inflammatory Bowel Disease, Bilateral Sensorineural Hearing Loss and Immune Dysregulation

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Abstract

Background and Aims: Very early onset inflammatory bowel disease [VEOIBD] is characterized by intestinal inflammation affecting infants and children less than 6 years of age. To date, over 60 monogenic aetiologies of VEOIBD have been identified, many characterized by highly penetrant recessive or dominant variants in underlying immune and/or epithelial pathways. We sought to identify the genetic cause of VEOIBD in a subset of patients with a unique clinical presentation.

Methods: Whole exome sequencing was performed on five families with ten patients who presented with a similar constellation of symptoms including medically refractory infantile-onset IBD, bilateral sensorineural hearing loss and, in the majority, recurrent infections. Genetic aetiologies of VEOIBD were assessed and Sanger sequencing was performed to confirm novel genetic findings. Western analysis on peripheral blood mononuclear cells and functional studies with epithelial cell lines were employed.

Results: In each of the ten patients, we identified damaging heterozygous or biallelic variants in the Syntaxin-Binding Protein 3 gene [*STXBP3*], a protein known to regulate intracellular vesicular trafficking in the syntaxin-binding protein family of molecules, but not associated to date with either VEOIBD or sensorineural hearing loss. These mutations interfere with either intron splicing or protein stability and lead to reduced STXBP3 protein expression. Knock-down of *STXBP3* in CaCo2 cells resulted in defects in cell polarity.

Conclusion: Overall, we describe a novel genetic syndrome and identify a critical role for *STXBP3* in VEOIBD, sensorineural hearing loss and immune dysregulation.

Key Words: VEOIBD; sensorineural hearing loss; STXBP3

1. Introduction

Inflammatory bowel diseases [IBD] are chronic inflammatory diseases that result in a dysregulated immune response to microbes in the gastrointestinal tract in genetically susceptible individuals.[1–4] Very early onset IBD [VEOIBD] manifests prior to 6 years of age.[5] A growing number of genetic aetiologies underlie VEOIBD, with over 60 identified to date, including causal monogenic variants in genes associated with primary immunodeficiency [*IL10R*, *WAS*, *BTK*, *NCF1*],[6–13] inflammasomopathies [e.g. *NLRC4*, *MEFV*],[14–17] haemophagocytc lymphohistiocytosis [HLH – e.g. *SH2D1A*, *XIAP*][18–21] and epithelial homeostasis [e.g. *TTC7A*, *CASP8*].[22,23] We describe ten patients from five unrelated and non-consanguineous families with medically refractory VEOIBD and variants in *STXBP3*, encoding Syntaxin-Binding Protein 3, a gene not previously

associated with VEOIBD. Eight of these ten patients also have severe bilateral sensorineural hearing loss [BSNHL].

2. Materials and Methods

2.1. Sample collection

Written consent was obtained from patient families using a protocol approved by The Institutional Review Board at Boston Children's Hospital and/or The Children's Hospital of Philadelphia. Control and patient blood samples were collected in EDTA tubes and processed within 24 h of venipuncture. Peripheral blood mononuclear cells [PBMCs] were obtained by density gradient centrifugation [Lymphoprep, Axis-Shield] and re-suspended in complete medium (Roswell Park Memorial Institute [RPMI] 1640 supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin; all from Invitrogen/Life Technologies).

2.2. Cell lines and siRNA knockdown

Caco2-BBE cells, a human epithelial colorectal adenocarcinoma cell line from The American Type Culture Collection [ATCC], were cultured in DMEM [Dulbecco's modified Eagle's medium] containing 20% fetal bovine serum [FBS]. The cells were subcultured by partial digestion with 0.25% trypsin and 0.9 mmol/L EDTA in Ca^{2+}-free and Mg^{2+}-free PBS. Small interfering RNA [siRNA]-mediated knockdown of STXBP3 in Caco2 cells was carried out using individual siRNA as described below by using Lipofectamine 2000 reagent in Opti-MEM I medium [Thermofisher Scientific], according to the manufacturer's protocol, with a final siRNA concentration of 50 nM. STXBP3 siRNA#1 sequence: 5′-guaaaucggagaacaagua-3′ and non-targeting [NT] siRNA consist of a pool of siRNAs of catalogue number D-001206-13-20 sequences: 5′-uagcgacuaaacacaucaa-3′; 5′-uaaggcuaugaagagauac-3′; 5′-auguauuggccuguauuag-3′; 5′-augaacgugaauugcucaa-3′. Cells were cultured for 48 h in complete medium and then used for different experiments, assessed 3–4 days post-transfection.

2.3. WES processing and analysis

Reprocessing and alignment of the sequencing data were performed under protocol 00000529 approved by the Institutional Review Board at Boston Children's Hospital and 2002-07-2805 at The Children's Hospital of Philadelphia [CHOP]. Paired-end reads were aligned to the human reference genome GRCh37.p10 using Novoalign [V3.02.03; http://www.novocraft.com]. Variants were detected using The Broad Institute's GATK[24] best practices, then annotated using SNPEff[25] 1000 Genomes Project [www.1000genomes.org/], Exome Variant Server [EVS] [http://evs.gs.washington.edu/EVS/] and Genome Aggregation Database [gnomAD; http://gnomad.broadinstitute.org/], in addition to pathogenicity databases and deleteriousness prediction methods including the Human Gene Mutation Database [HGMD], ClinVar, Combined Annotation-Dependent Depletion tool [CADD][26] Polyphen-2, SIFT, GERP, Grantham score, phyloP and phastCons. Additional quality control filters were applied to select high-quality variants with a minimum depth of 20. Remaining variants were then filtered, retaining only non-synonymous missense, nonsense and small indel, rare [minor allele frequency <1% in all three databases] or novel mutations. Mode of inheritance analysis was conducted to identify candidate autosomal recessive, X-linked and *de novo* variants.

2.4. Antibodies

The following antibodies were used: rabbit anti-human STXBP3 and STXBP2 [Novus Biologicals], goat anti STXBP3 [R&D Systems], mouse anti-STXBP3 [Santa Cruz Biotechnology], mouse anti-STX4 [BD Pharmingen], rabbit anti-Rab27a [Synaptic System], goat anti-STXBP2 [Santa Cruz Biotechnology], mouse anti-actin [Sigma-Aldrich], rabbit anti-Rab11 [Cell Signaling], goat anti-rabbit Dylight-488 [Thermo Fisher Scientific] and CTxβ-Alexa594 [Invitrogen].

2.5. Immunostaining and microscopy

siRNA-treated Caco-2-BBE cells were seeded in 24-well plates containing glass coverslips and allowed to grow at 37°C. Cells were fixed for 10 min with PBS containing 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS buffer and then blocked with 3% bovine serum albumin [BSA] in PBS for 30 min at room temperature. Immunostaining was performed using the primary rabbit anti-Rab11a antibody with secondary Dylight-488 goat anti-rabbit antibodies and stained with CTxβ-Alexa594. Coverslips were extensively washed with PBS and mounted with Prolong Gold anti-fade reagent [Thermofisher Scientific]. Images were collected using a Leica SP5-STED-CW microscope [Leica Microsystems]. Apical fluorescence distribution ratio was determined by quantifying the total fluorescence intensity in single cells using rectangles of a fixed size near the apical membrane and at the basal membrane of x–z slice images using Image-J [US-NIH, https://imagej.nih.gov/ij/]. The ratio was calculated as apical fluorescence intensity/total fluorescence intensity [apical+basal].

2.6. Analysis of single cell RNA sequencing data

We analysed an existing single-cell RNA-sequencing [scRNA-seq] dataset collected from adult healthy [n = 12 individuals] and patients with ulcerative colitis [UC] [n = 18 individuals] totalling 365 492 single cells. Healthy individuals were sampled with two adjacent colonic biopsies during screening colonoscopies, and patients with UC were sampled from inflamed and adjacent non-inflamed colonic tissue.[27] This dataset consists of 51 cell subsets across epithelial cells, fibroblasts, endothelial cells, B cells, myeloid cells and T cells. To assess for enrichment of *STXBP3* in these 51 cell subsets, we performed differential expression as a 1 vs. rest comparison using FindMarkers implemented in Seurat, setting 'test.use' to 'bimod'. We provide all results of these tests as Supplementary Tables 16–18 including the log-fold change, Bonferroni-adjusted p-values and the fraction of expressing cells in the cluster. We also assessed differential expression between inflamed and healthy cells within each cluster but did not find STXBP3 as a significantly differentially expressed gene. Two of the clusters with greatest STXBP3 enrichment arise almost exclusively from UC biopsies, precluding differential expression analysis.

3. Results

3.1. Variants in STXBP3 are associated with VEOIBD and sensorineural hearing loss

We performed whole exome sequencing [WES] in 1517 VEOIBD patients from five centres. We identified five patients from three families with VEOIBD and BSNHL, with heterozygous variants in *STXBP3* [Figure 1 and Table 1]. A fourth family was identified by GeneMatcher[28] consisting of three patients with biallelic variants in *STXBP3*, VEOIBD, BSNHL, craniofacial defects and hypotonia [Figure 1 and Table 1]. A fifth family was later identified, including

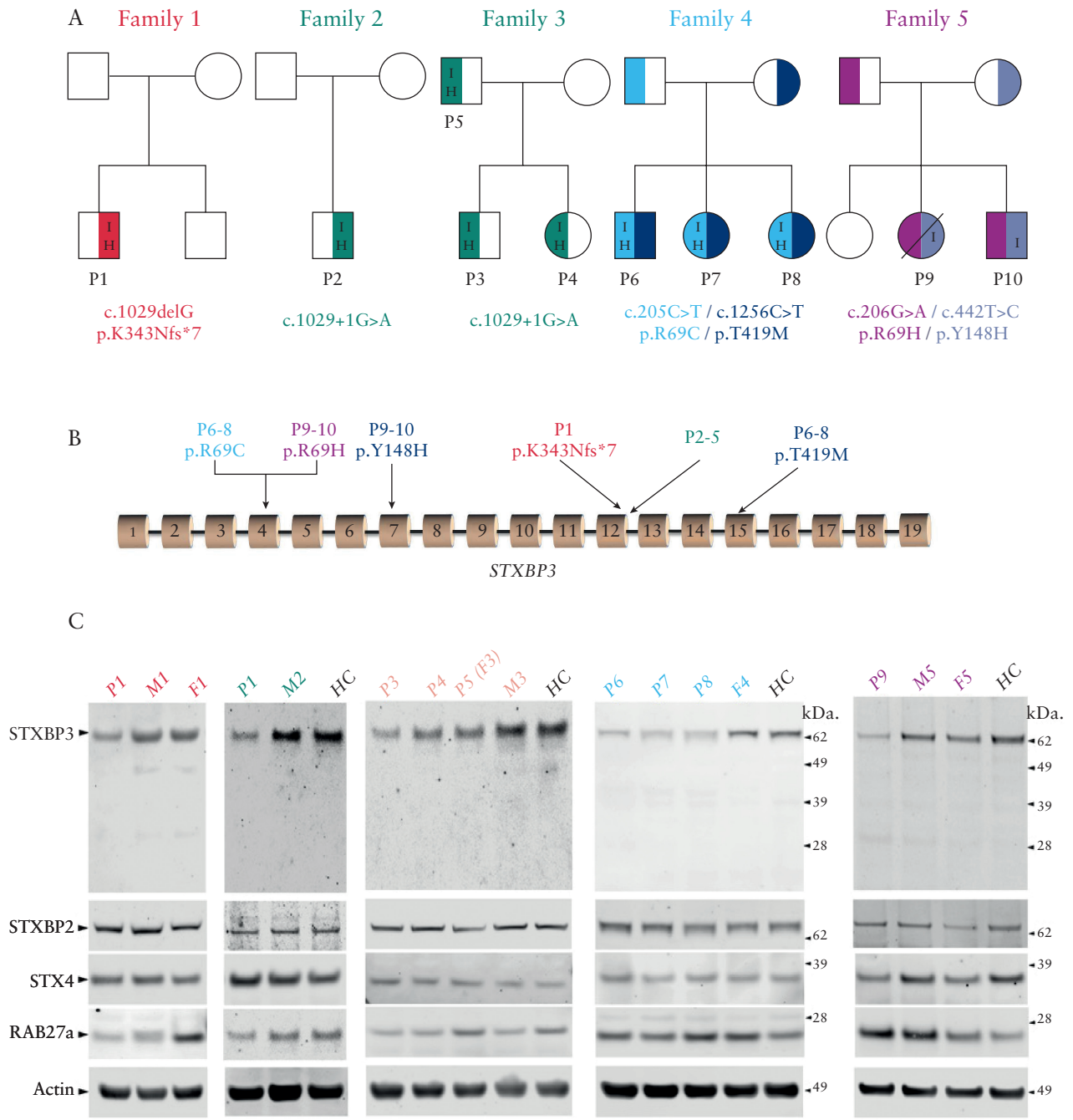

Figure 1. *STXBP3* variants in five families with very early onset IBD [VEOIBD]: [A] Pedigrees of five families illustrating ten patients with VEOIBD and variants in *STXBP3*. Patient 1 [P1] in Family 1 [red] has a *de novo* heterozygous *STXBP3* mutation c.1029delG, p.K343Nfs*7. Patient 2 [P2] in Family 2 [green] has a *de novo* heterozygous *STXBP3* mutation c.1029 + 1G>A. In Family 3, the two affected siblings [P3, P4] and affected father [P5] carry the same heterozygous variants in *STXBP3* as does P2 [c.1029 + 1G>A, green]. Patients 6–8 [P6–8] in Family 4 [blue] have compound heterozygous *STXBP3* variants c.205C>T, p.R69C [light blue] and c.1256C>T, p.T419M [royal blue]. Patients 9 and 10 of Family 5 [purple] have compound heterozygous *STXBP3* variants c.206G>A, p.R69H [purple] and c.442T>C, p.Y148H [lavender]. 'I' indicates patients with VEOIBD; 'H' indicates patients with bilateral sensorineural hearing loss. Where predictions can be made, corresponding amino acid changes are shown. [B] Schematic representation of the *STXBP3* gene showing the position of the identified *STXBP3* variants of each patient. This representation is not to scale. [C] Western blot analysis of protein expression levels in blood samples from Families 1–5 revealing reduced expression of STXBP3 in affected Patients 1–9 [P1–P9] as compared to healthy controls [HC] and mother/father of respective families [M1, F1, M2, M3, F4, M5 and F5].

two siblings with VEOIBD, hypotonia, and biallelic variants in *STXBP3* but no hearing loss [Figure 1 and Table 1].

3.2. Clinical manifestations

All patients presented within the first months of life with severe diarrhoea and in most cases, life-threatening hyponatraemia and hypoalbuminaemia, often requiring repeated intensive care unit admissions. Endoscopy and colonoscopy in most probands revealed erythema and haemorrhagic lesions in the small and large intestine, and ileal ulcerations [Figure 2A]. Histology revealed expanded lamina propria by lymphoplasmacytic inflammation, neutrophilic crypt abscesses, villous atrophy, eosinophilia, apoptosis,

Table 1. Summary of clinical case presentation

Clinical Phenotype	Family 1	Family 2	Family 3			Family 4			Family 5	
	P1	P2	P3	P4	P5 [F3]	P6	P7	P8	P9	P10
Sex	M	M	M	F	M	M	F	F	F	M
Status [alive/deceased]	A	A	A	A	A	A	A	A	D	A
Surgical intervention to gastrointestinal tract	+	+	+	+	+	–	–	–	–	+
Diarrhoeal episodes	+	+	+	+	+	+	+	+	+	+
Liver disease	–	–	–	–	–	–	–	–	+	–
Intestinal inflammation [SI/C]	+/+	+/+	+/+	+/+	+/+	–/+	?/?	?/?	?/?	?/?
Intestinal cell apoptosis [SI/C]	+/+	+/+	+/+	+/+	+/+	–/–	?/?	?/?	+/+	?/?
Intestinal eosinophilia [SI/C]	+/+	+/+	+/+	+/+	+/+	–/+	?/?	?/?	?/?	?/?
Fevers	+	–	–	+	+	?	?	?	?	?
NK cell numbers	L	L	N	?	?	?	?	?	?	N
CD8+ cell numbers	L	N	N	?	?	?	?	?	?	N
Hyponatraemia	+	+	+	+	?	?	?	?	–	–
Hypoalbuminaemia	+	+	+	+	?	+	+	+	+	+
LDH > 500 U/L	+	+	?	?	?	+	+	+	?	–
High arched palate	–	–	?	?	?	+	+	+	–	–
Microcephaly	–	–	?	?	?	+	+	+	–	–
Dolichocephaly	–	–	?	?	?	–	–	+	–	–
Central hypotonia at birth	–	–	–	–	–	+	+	+	+	+
Joint contractures	–	–	–	–	–	+	+	+	–	–
Syndactyly	–	–	–	–	–	+	+	+	–	–

Abbreviations: M, male; F, female; A, alive; D, deceased; SI/C, small intestine/colon; HSCT, haematopoietic stem cell transplant; L, low; N, normal; LDH, lactate dehydrogenase; + refers to present; – refers to absent; ? refers to unavailable.

minimal architectural distortion and crypt destruction [Figure 2B]. The patients all had a severe disease course that was refractory to conventional IBD therapy. Six patients also experienced recurrent infections. Two patients had low natural killer [NK] cell numbers. One patient had low cytotoxic T cell numbers [Table 1].

3.2.1. Detailed clinical presentations

A clinical summary of each patient is provided. P1 [Family 1] manifested diarrhoea within 2 weeks of life and had severe BSNHL. After failing various immunosuppressants, P1 underwent a diverting ileostomy, without improvement, and required total parenteral nutrition [TPN]. He had seven infections requiring admissions prior to 5 years of age: rhinovirus and enterovirus at 9 months old requiring supportive inpatient care; a central line infection with *Staphylococcus aureus* at 14 months of age for which he received antibiotics; respiratory syncytial virus [RSV] and coronavirus at 18 months requiring admission for supportive care; norovirus, rhinovirus and enterovirus at 3 years and 3 months old requiring admission for supportive care, followed by coronavirus infection ~1 month later requiring admission for supportive care. One and a half months later [at 3.6 years of age] he was admitted with an Epstein-Barr virus [EBV] infection. At 4 years old he developed a *Clostridium difficile* infection requiring admission and antibiotics. He was noted to have absent NK T cells at 4 months of age. With concern for impending HLH, based on absent NK cells and low CTLs, as well as the confirmed variant, he underwent haematopoietic stem cell transplant, with resolution of intestinal symptoms 4 years post-transplantation. His diverting ileostomy was taken down and repeat endoscopy/colonoscopy demonstrated mucosal healing [histologically confirmed]. P2 [Family 2] presented within weeks of life with BSNHL and medically refractory bloody diarrhoea. He was found to have low NK cells at 18 months in the context of azathioprine treatment, but normal CD8 T cell counts. He did not have any major viral or bacterial infections requiring hospitalization. By 3 years old, he exhibited several colonic foci of high-grade dysplasia and underwent a total colectomy but continued to have small intestinal disease. P3 [Family 3] was born with BSNHL and developed bloody diarrhoea by 6 months of age. He had 12 hospital admissions for vomiting and diarrhoea by 4 years of age, three of which had an infectious aetiology identified. Specifically, at 18 months he developed *Klebsiella pneumoniae* requiring antibiotics. At 3 years of age, he was admitted once for supportive care in the context of a rotaviral infection. He was hospitalized again at 3 years of age for vomiting and diarrhoea for which he received antibiotics and intravenous immunoglobulin (IVIG) despite no infection identified, and a third admission at 3 years old for diarrhoea secondary to *Klebsiella* and *Enterococcus* infection, for which he received antibiotics. He failed various medical interventions and required TPN. He underwent ileal diversion at 17 months old, with persistent symptoms and underwent a colectomy with resolution of symptoms. His sister, P4 [Family 3], developed bloody diarrhoea by 4 months of age and after failing several medications, underwent colectomy with ileostomy at 8 months old. She was hospitalized at 22 months of age for rotavirus for supportive management and again at 2 years old for acute gastroenteritis. Their father, P5 [Family 3], is also deaf since infancy and developed bloody diarrhoea within months of life and underwent partial colectomy at 18 months old but required a complete colectomy at 4 years old. After surgical revisions, he remains on no medications. P6–P8 [Family 4] have unaffected parents, but each exhibited reduced muscle tone at birth, a blistering skin disorder, multiple joint contractures, craniofacial dysmorphism and severe BSNHL. Each developed severe secretory diarrhoea with electrolyte imbalance and dehydration within 6 weeks of life. They were admitted multiple times for hypovolemic shock secondary to life-threatening diarrhoea. Specifically, P6 was admitted 11 times prior to 2 years of age with fever, vomiting and diarrhoea. Blood, urine and stool cultures were all negative except

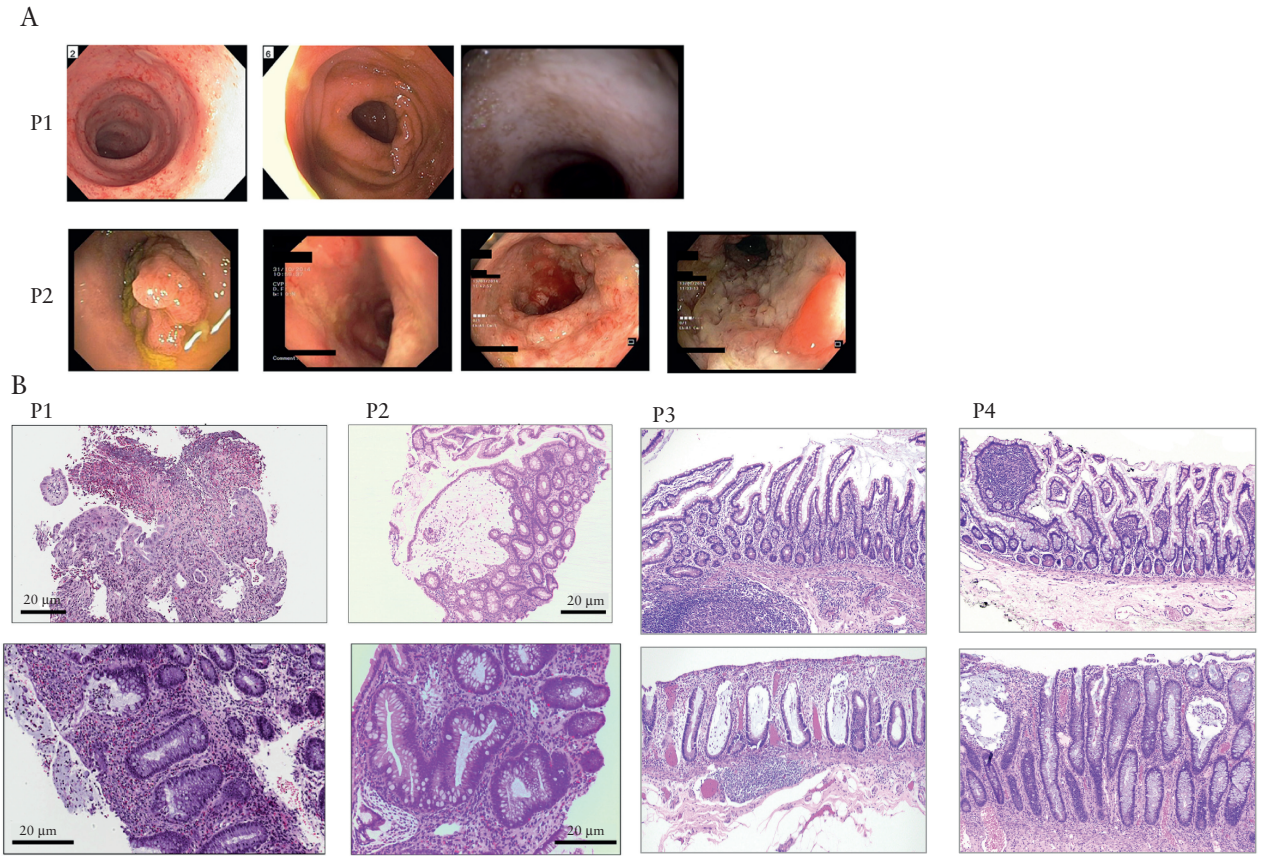

Figure 2. Abnormal colonoscopy and histology of biopsies from ileum and colon of patients with STXBP3 variants. [A] Colonoscopic images of Patient 1 [P1] and Patient 2 [P2] showing abnormal mucosa with oedema, pseudopolyps, exudate, ulcers and haemorrhage. [B] H&E-stained ileal [upper panel] and colonic [lower panel] biopsies from Patients 1, 2, 3 and 4 [P1–P4] revealing active colitis including neutrophilic crypt abscesses, minimal evidence of crypt architecture distortion with mildly increased lamina propria lymphoplasmocytosis; epithelial apoptosis is not identified. Mild lamina propria eosinophilia is noted.

for during an admission at 19 months of age which grew *Klebsiella* from his central venous line, requiring antibiotics. He was admitted again at 20 months of age for fever and diarrhoea with dehydration and shock. Despite lack of infectious aetiologies identified, he received antibiotics. P7 was admitted eight times by 8 years of age to manage fever, vomiting, and profuse diarrhoea with dehydration requiring supportive care. In addition, she was admitted at 3 days of life for 57 days for vomiting and diarrhoea and despite negative cultures she was started on antibiotics. She was hospitalized at 17 months of age for *Escherichia coli* growing from her central line, requiring antibiotics. At 24 months of age, she was admitted for bloody diarrhoea secondary to rotaviral infection requiring supportive care. At 4.5 years of age, she developed pneumonia, diarrhoea and vomiting and was started on antibiotic treatment despite negative cultures. At 8 years of age, she developed a pneumonia and bloody stools that progressed to shock and received antibiotics. P8 was admitted nine times by 2.5 years of age. At 1 month old she developed *Streptococcus pyogenes* in blood resulting in diarrhoea, hypovolaemic shock and respiratory distress necessitating supportive care and antibiotics. At 2 months of age, she was admitted for fever, diarrhoea, vomiting and respiratory difficulty. She was found to have RSV pneumonia as well as *C. difficile* toxin in her stools, for which she received antibiotics. At 16 months of age, she was admitted for fever, vomiting and diarrhoea and was found to have *Haemophilus influenzae* for which she received supportive care given allergies to available antibiotics. During the remaining six admissions, all cultures were negative. P6–8 required TPN and are now in young adulthood with significant improvement of symptoms. In Family 5, P9 and P10 both had congenital myopathy, contractures and enteropathy, requiring TPN within 6 months of life for P9 and at just over 1 year old for P10. Both were managed as autoimmune enteropathy given histological small bowel findings of cell apoptosis and lymphocyte infiltration. Neither developed excess infections. Following immunosuppression, P9 developed acute rapidly progressive hepatitis with cholestasis, fever and thrombocytopenia, progressing to fulminant hepatic failure and death at 8 years old. Post-mortem examination was not indicative of veno-occlusive disease or HLH. P10 required transient ventilation at birth to support his myopathy. He manifested diarrhoea at 6 weeks old and required TPN throughout childhood. At 1 year of age, he developed pneumonia and required bilevel positive airway pressure [BIPAP] ventilation. He responded to immunosuppression. Intriguingly, P10 developed episodes of constipation with recurrent sigmoid dilation and partial sigmoid volvulus. He required a sigmoid colectomy with end-to-end anastomosis. At the time of resection, inflammation was not present in his small or large intestine. Neither P9 nor P10 showed hearing loss.

3.3. Variant analysis

WES revealed *de novo* heterozygous variants in *STXBP3* in P1 and P2 from Families 1 and 2, respectively. In Family 3, affected siblings [P3, P4] and an affected father [P5] have a heterozygous variant in *STXBP3*. P1 has a deletion at position 1029, the last nucleotide

Table 2. Variants reported in *STXBP3* and related pertinent genes [either in vesicular pathway or known VEOIBD genes]

Affected individual	Gene	Genomic location hg19 [Chr:Pos]	Allele frequency	Allele count	Variant cDNA change	Amino acid change	Segregation	CADD score	PHRED-scaled score
P1	*STXBP3*	1:109336269	NR	NR	c.1029delG	p.K343Nfs*7	*De novo*	5.412	26.0
	RAB27A	15:55522599	NR	NR	c.239C>T	p.R80K	Maternal	7.014	33.0
P2	*STXBP3*	1:109336270	NR[a]	NR[a]	c.1029 + 1G>A		*De novo*	5.818	27.2
	PRF1	10:72360387	2.9×10^{-2}	8191	c.272C>T	p.A91V	Homozygous	5.407	26.0
P3	*STXBP3*	1:109336270	NR[a]	NR[#]	c.1029 + 1G>A		Paternal	5.818	27.2
	LRBA	4:151793903	2.1×10^{-2}	5728	c.2170A>G	p.I724V	Paternal	1.362	12.6
P4	*STXBP3*	1:109336270	NR[a]	NR[#]	c.1029 + 1G>A		Paternal	5.818	27.2
	LRBA	4:151793903	2.1×10^{-2}	5728	c.2170A>G	p.I724V	Paternal	1.362	12.6
P5 [F3]	*STXBP3*	1:109336270	NR[a]	NR[#]	c.1029 + 1G>A		Unknown	5.818	27.2
	LRBA	4:151793903	2.1×10^{-2}	5728	c.2170A>G	p.I724V	Unknown	1.362	12.6
P6	*STXBP3*	1:109339248	1.4×10^{-5}	4	c.1256C>T	p.T419M	Maternal	3.967	27.7
		1:109299335	NR	NR	c.205C>T	p.R69C	Paternal	4.267	32.0
P7	*STXBP3*	1:109339248	1.4×10^{-5}	4	c.1256C>T	p.T419M	Maternal	3.967	27.7
		1:109299335	NR	NR	c.205C>T	p.R69C	Paternal	4.267	32.0
P8	*STXBP3*	1:109339248	1.4×10^{-5}	4	c.1256C>T	p.T419M	Maternal	3.967	27.7
		1:109299335	NR	NR	c.205C>T	p.R69C	Paternal	4.267	32.0
P9	*STXBP3*	1:109315290	NR	NR	c.442T>C	p.Y148H	Maternal	5.633	26.6
		1:109299336	4.1×10^{-6}	1	c.206G>A	p.R69H	Paternal	5.502	26.2
P10	*STXBP3*	1:109315290	NR	NR	c.442T>C	p.Y148H	Maternal	5.634	26.6
		1:109299336	4.1×10^{-6}	1	c.206G>A	p.R69H	Paternal	5.502	26.2

MAF, minor allele frequency in gnomAD; allele count in gnomAD; NR, not reported; . fs, frameshift.

[a]There is a mutation reported a single time in gnomAD at position c.1029 + 1 with a different insertion of G>T.

of exon 12, predicted to result in a frameshift mutation. P2–P5 inherited an adenine instead of a guanine at position 1029 + 1 in the first nucleotide of intron 12, predicted to impair splicing at the same location in two unrelated families. Among 138 632 adults described without severe paediatric-onset disease in the gnomAD database [http://gnomad.broadinstitute.org/gene/ENSG00000116266], 22 heterozygous loss of function variants of high confidence and sequencing depth are reported in STXBP3. However, additional variants not thought to be independently deleterious,[26] but in pathways of interest to syntaxin binding proteins or in known VEOIBD genes, were identified [Table 2]. Specifically, P1 inherited a maternally transmitted heterozygous missense variant in *RAB27A* [c.239C>T, p.R80K, not previously reported]. P2 inherited a homozygous variant in *PRF1* [c.272C>T, p.A91V] with a frequency of 0.03 [8444/275 566 alleles; 165 homozygous individuals] in gnomAD. When present alone, these are considered normal polymorphisms, but they have been described in familial HLH [F-HLH] patients carrying variants in other cytolytic pathway genes or as one of the alleles in F-HLH patients with compound heterozygosity in *PRF1*[29]. P3–5 have a heterozygous missense variant in *LRBA* [c.2170A>G, p.I724V]. Although homozygous recessive damaging *LRBA* variants lead to common variable immunodeficiency with enteropathy,[30] p.I724V is reported to be 94 times in a homozygous fashion in gnomAD, so is unlikely to cause VEOIBD alone.

In contrast to P1–P5 with heterozygous STXBP3 variants, P6–P10 have biallelic rare missense variants in *STXBP3*. Specifically, P6–P8 inherited c.1256C>T, p.T419M (minor allele frequency [MAF] in ExAC of 8.34×10^{-6}) along with c.205C>T, p.R69C [not previously reported], while P9 and P10 inherited c.442T>C, p.Y148H [not previously reported] and c.206G>A, p.R69H [MAF in gnomAD of 4.19×10^{-6}]. P10 also inherited a compound heterozygous variant in *RTEL1*. He and his unaffected family members have heterozygous variants in other genes associated with VEOIBD [*TTC7A*, *TRIM22*, *SKIV2L*, *RTEL1*, *STAT1]*.

3.4. Variants in *STXBP3* are associated with a reduction in protein expression

Western blot analysis was performed on blood samples from Families 1–5. Reduced expression of STXBP3 of approximately 50–80% [by comparative densitometry] was seen in all affected patients, including those with homozygous recessive as well as heterozygous variants [Figure 1C]. Importantly, expression of the related protein STXBP2 [known to be causative for HLH, VEOIBD and hearing loss][31,32] was not affected. Also, the expression level of Syntaxin-4 [STX4], which is known to interact with STXBP3, was not affected. However, Patient 1 and his mother, who both carried a variant in *RAB27A*, had reduced RAB27A protein expression compared with healthy controls.

3.5. Reduced expression of STXBP3 in intestinal epithelial cells [IECs] affects cell polarization and growth

All patients presented with severe diarrhoea and intestinal inflammation, and most manifested sensorineural hearing loss, which resembles clinical features described in patients with mutations in the related gene *STXBP2*.[31,32] *STXBP2* is expressed in epithelial cells and is involved in membrane trafficking[33–35]. Therefore, we sought to determine whether STXBP3 may exhibit similar functions. To assess this possibility, we treated Caco2 cells, a human colorectal cancer cell line,[36] with control or *STXBP3*-specific siRNAs. Western blot analysis showed that siRNA treatment led to nearly 60% reduction in STXBP3 expression compared with control cells [Figure 3A]. To assess whether STXBP3 depletion impacts trafficking to the apical plasma membrane [PM], polarized cells cultured for 72 h on glass coverslips were fixed and stained with antibodies against RAB11A—a protein known to regulate vesical transport[37,38]—and cholera toxin β-subunit conjugated with Alexa-594 [CTxβ] to visualize GM1 ganglioside, an apical marker.[39] Non-targeting [NT]

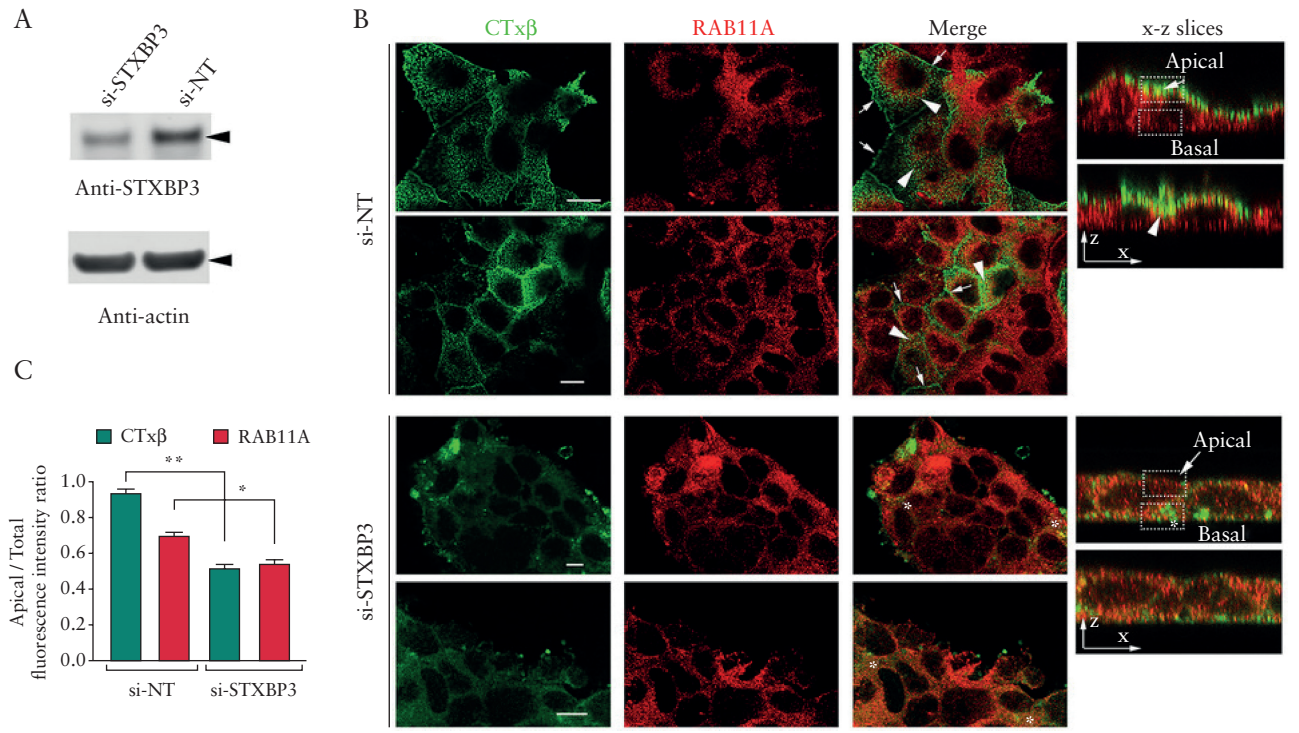

Figure 3. *STXBP3* knock-down disrupts intestinal epithelial cell polarization and delay monolayer formation. [A] Caco2 cells transfected with either non-targeting [NT], or STXBP3 siRNAs and cultured for 72 h were analysed by Western blot for the indicated proteins. STXBP3 knock-down efficiency was about 50%. Blots are representative of three independent experiments. [B] siRNA-treated cells were seeded onto glass coverslips, incubated for 72 h, fixed, permeabilized and stained using RAB11A antibody and CTxβ-Alexa594. The *x–z* slices show 3-D views of confluent Caco2 cells. Arrows show GM1 localization at the plasma membrane. Arrowheads show GM1 localization in intracellular vesicles in the apical region. Asterisk shows abnormal GM1 distribution in intracellular vesicles close to the basal membrane region. Scale bars = 5 μm. [C] Quantification of the fluorescence intensity ratio of apical over total fluorescence [apical + basal] distribution of CTxβ-Alexa594 and RAB11A in siRNA-treated Caco-2 cells as shown in B [*x–z* slices, dashes rectangles]. Results are the mean ± SEM of two independent measurements; $n = 50$ cells. $^{*}p < 0.05$, $^{**}p < 0.01$.

siRNA-treated cells were almost confluent after 72 h and formed a continuous polarized cell monolayer. CTxβ was detected on the apical PM [Figure 3B, top panel: *x–z* slices, arrows] and on intracellular vesicles [Figure 3B, top panel: merge, arrowheads]. RAB11A vesicles were present throughout the cell with slight enrichment toward the apical membrane [*x–z* slices]. In contrast, *STXBP3* siRNA-treated cells were delayed in forming monolayers, forming mostly cell islands with an abnormal distribution of GM1 [Figure 3B, bottom panel asterisk] that accumulated in intracellular vesicles close to the basal membrane. RAB11A vesicles were scattered throughout the cytoplasm. We compared the ratio of fluorescence intensity for GM1 and RAB11A in the apical region to the total fluorescence intensity and found that si-*STXBP3*-treated Caco2 cells had a significantly lower ratio of apical to total GM1 fluorescence than si-NT-treated cells and a similar trend was noted for RAB11A [Figure 3C]. Therefore, STXBP3 is necessary for proper localization of GM1 to the apical cell membrane, suggesting that STXBP3 deficiency might disrupt transport in IECs.

3.6. STXBP3 RNA expression analysis

STXBP3 is reported as being ubiquitously expressed [Human Protein Atlas, https://www.proteinatlas.org/ENSG00000116266-STXBP3]. Based on the inflammatory manifestations in colonic tissue, we further investigated this by analysing our recently published scRNA-seq from adult colonic tissue in order to assess the expression of *STXBP3* in epithelial, stromal and immune cells in a reference single-cell atlas of healthy control screening colonoscopies [control cohort] and the colon of patients with UC.[27] Jointly assessing for enrichment across all clusters agnostic to disease state, we found STXBP3 enriched in M-like cells, cycling amplifying cells, and stem cells in the epithelial compartment [Figure 4A, Supplementary Table 16]. In the stromal compartment, *STXBP3* was enriched in inflammatory fibroblasts; a cell subset only captured from patients with ongoing intestinal inflammation [Figure 4B, Supplementary Table 17]. In the immune compartment, *STXBP3* expression is enriched in cycling monocytes, dendritic cells [DC1], cycling B cells and cycling T cells [Figure 4C, Supplementary Table 18]. Taken together, we identify that *STXBP3* is expressed in epithelial, stromal and immune cells. Intriguingly, two of the cell subsets with the highest frequency of expressing cells were inflammatory fibroblasts and M-like cells: subsets detected almost exclusively in UC biopsies.[27] Further studies evaluating STXBP3 expression within intestinal cells isolated from patients with VEOIBD will be required to further confirm and extend these findings.

3.7. Comparative analysis of *STXBP3* variants in VEOIBD and control cohorts

To measure the potential significance of the variants observed in *STXBP3* or in both *STXBP3* and one of the additional variants identified [*RAB27A*, *PRF1* or *LRBA*], we made a null expectation model for these observations, as done previously for single gene defects generated from large population cohorts.[40] Among the 1517 VEOIBD patients sequenced, we identified the ten individuals described above with rare [MAF ≤ 0.1%] non-synonymous coding or splice site variants in *STXBP3*. Among these, five affected individuals [Families 4

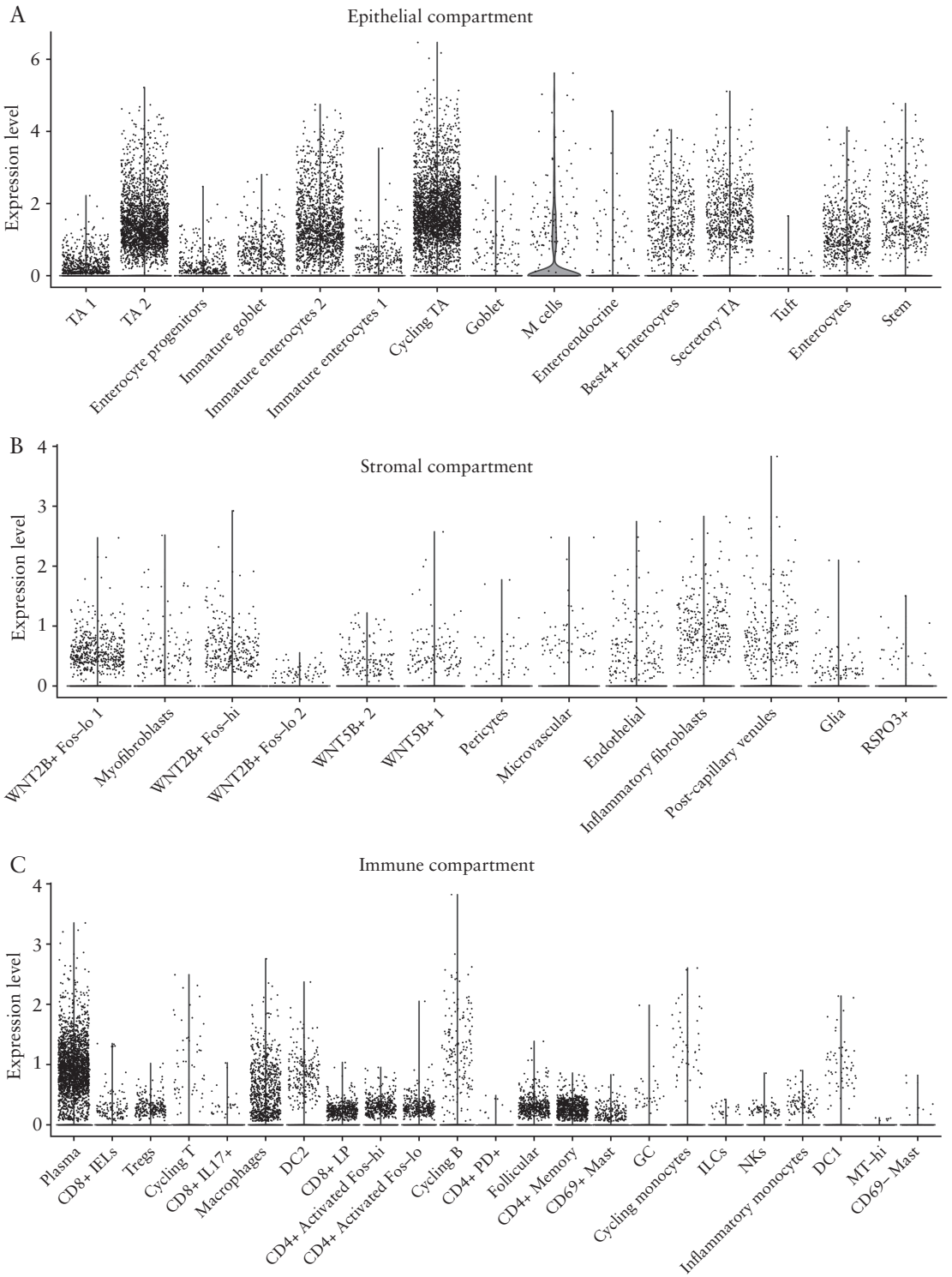

Figure 4. Analysis of single-cell RNA-sequencing from adult colonic tissue of healthy controls and UC patients. Violin plots for *STXBP3* expression in cell subsets from colonic samples analysed as [A] 123 006 cells from the epithelial compartment, [B] 31 872 cells from the stromal compartment and [C] 210 614 cells from the immune compartment. All violin plots were generated using standard Seurat code without modification to smoothing or density. Violin points represent individual cells and density only generated when >25% of cells in the indicated sample have a non-zero measurement for gene; the widest aspect represents the centre of positive measures, and minima and maxima are represented within the scale with minima at 0 and maxima encompassing all points for the count-based expression level (log[scaled UMI + 1]) of each gene. Full statistical results are available in Supplementary Tables 16–18.

and 5] had rare non-synonymous compound heterozygous variants in *STXBP3*. The number of such variant observations in the VEOIBD cases is significantly higher than in gnomAD [$p < 1 \times 10^{-16}$], where the expected frequency is 1.17×10^{-5} [or ~1.2/100 000 individuals]. The cases in Families 1, 2 and 3 include three probands and two additional affected first-degree relatives, each inheriting a rare

[MAF ≤ 0.1%] non-synonymous heterozygous variant in *STXBP3* and a heterozygous variant [MAF ≤ 3%] in any one of *RAB27A*, *PRF1* or *LRBA*. The expected rate of identifying such a combination of variants in gnomAD is 5.64×10^{-4} [or ~5.6/10 000 individuals]. The number of such variant observations in the VEOIBD cases is significantly higher than in gnomAD [$p = 8.67 \times 10^{-3}$]. When combined, we find that ten affected individuals in five families inherited a rare variant in *STXBP3*, some of whom also inherited another variant in *RAB27A*, *PRF1* or *LRBA* which is significantly enriched in VEOIBD cases over gnomAD [$p = 3.10 \times 10^{-4}$]. Taken together, the occurrence of presumptive *STXBP3* loss-of-function variants in five independent families with life-threatening VEOIBD and sensorineural hearing loss strongly supports a strong association of variants in *STXBP3* in this clinical condition.

3.8. *STXBP3* and adult-onset IBD

To examine whether variants in *STXBP3* are also associated with altered risk in adult IBD, we analysed variants in the *STXBP3* gene in patients with IBD (UC, Crohn's disease [CD] or both) in the NIDDK ExomeChip cohort, which is a cohort of European ancestry individuals recruited at IBD centres across North America comprising 12 467 IBD cases and 8352 non-IBD controls as previously described.[41] None of the six *STXBP3* variants genotyped on ExomeChip were observed to be associated with IBD, CD or UC in these cohorts. Similarly, there are also no significant variants in STXBP3 in the most recent list of significant loci identified from Genome Wide Association Studies [https://www.ebi.ac.uk/gwas/efotraits/EFO_0003767].

4. Discussion

We show that *STXBP3* is associated with VEOIBD, immune dysregulation and BSNHL in eight patients from four unrelated families, and VEOIBD in two patients from a fifth family. P1–P5, who are phenotypically similar, carry heterozygous variants within the intron 12 splice donor site, and are associated with a reduction in WT STXBP3 protein expression. Thus, it appears likely that these mutations interfere with protein expression by interfering with efficient splicing. In contrast, P6–P10 inherited biallelic *STXBP3* missense variants, and these patients exhibit phenotypic differences compared to patients P1–P5. With the important proviso that P1–P5 carry potentially contributory variants in *RAB27A*, *PRF1* and *LRBA*, and possibly in unidentified loci, their common VEOIBD and BSNHL probably require heterozygous loss-of-function of *STXBP3*. Although loss-of-function *STXBP3* mutations are reported in a heterozygous fashion in adults without significant paediatric-onset disease in gnomAD, clinical differences may result from variations in their specific location, the base pair change and incomplete penetrance. P6–P10 have biallelic missense variants in STXBP3, sharing variants in position 69 with unique variants in either position Y148H or T419M. They exhibit additional features including myopathy and hypotonia, limiting the possibility that these phenotypes reflect co-segregating modifier genes. Intriguingly, P9 and P10 from Family 5 did not demonstrate hearing loss. R69 is highly conserved among all members of the Sec/Mun18c protein family. The crystal structure of STXBP3[42] shows that R69 and T419 reside in critical regions that interact with other membrane trafficking proteins [e.g. STX4, VAMPs and DOC2B]-.[43–45] Y148 and T419 are highly conserved.[46]

Some STXBP3 heterozygous hypomorphic or loss-of-function variants alone may be insufficient to confer VEOIBD and hearing loss, given lack of disease in parents of Families 4 and 5 and heterozygous variants reported in gnomAD. Nonetheless, we identify reduced STXBP3 expression in all patients studied, regardless of the mode of inheritance. We propose two potential mechanisms whereby *STXBP3* loss-of-function is associated with disease. One, exemplified by Families 4 and 5, involves monogenic biallelic variants for *STXBP3*. A second involves digenic inheritance where *STXBP3* heterozygous loss-of-function is compounded with variants in exocytosis pathway genes such as *RAB27A*, *PRF1* or genes involved in VEOIBD such as *LRBA* in P1, P2 and P3–P5, respectively.

Damaging recessive or dominant negative monoallelic variants in *STXBP2* can cause F-HLH, VEOIBD and hearing loss.[32,47] Furthermore, defective protein trafficking and disruption of epithelial cell polarity associated with mutations in *STXBP2*, *STX3* and *MYO5B* present with microvillous inclusion disease [MVID].[34,48] Defective polarity and integrity of the epithelial barrier and VEOIBD is associated with mutations in *TTC7A*, *EPCAM* and *SPINT2*.[49–51] Our observation that STXBP3 may also function in maintaining epithelial polarity suggests that this appropriate maintenance of epithelial polarity may be essential to preventing VEOIBD. This observation raises the possibility that developing therapeutics with the ability to stabilize epithelial polarity may be an effective strategy for several genetic defects associated with VEOIBD.

Our data support previously published data that indicate STXBP2 and STXBP3 have non-redundant functions. STXBP3 regulates glucose uptake in adipose and muscle cells by controlling the trafficking of the GLUT4 glucose transporter to the plasma membranes.[52] In addition, *Stxbp3* heterozygous knockout mice develop glucose intolerance upon challenge with a high-fat diet.[53] Finally, a zebrafish insertional mutant in *stxbp3* displays smaller ears and otoliths, pericardial oedema and an underdeveloped gut [http://zfin.org/ZDB-FIG-070117-940], paralleling features in the patients.

STXBP3 RNA expression is ubiquitous across most cell types and may be enhanced in the setting of cell activation/proliferation, as evidenced by scRNA-seq data analysis of adult colonic tissue. Moreover, *STXBP3* RNA expression does not appear to be differentially expressed between UC and the control state, except for enhanced expression in inflammatory fibroblasts in UC patients.

Given two patients had defects in immune cell homeostasis [decreased NK and CD8 cell numbers in P1 and decreased NK cells in P2] and STXBP3 appears critical for epithelial cell polarization and growth, it remains unclear what cellular compartments of STXBP3 have critical non-redundant functions. Of note, bone marrow transplant appeared curative in P1 suggesting that, at least in some circumstances, haematopoietic expression of STXBP3 may most critical. Interestingly, however, colectomies appeared curative in P2–P5, which suggests that STXBP3 expression in other intestinal immune and/or epithelial compartments may be less essential. As larger cohorts of VEOIBD patients with and without BSNHL are identified and analysed, considerations regarding the specific tissue and cellular compartments of most importance will be better understood.

In conclusion, we have identified damaging variants in *STXBP3* associated with VEOIBD and BSNHL. Patients with *STXBP3* variants had reduced STXBP3 expression, regardless of the mode of inheritance, and importantly, STXBP2 expression was not affected. Our data also demonstrate an important role for STXBP3 in epithelial cell function. Overall, we describe a novel genetic syndrome identifying a critical role for the association of *STXBP3* in VEOIBD and BSNHL.

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Conflict of Interest

J.O. declares the following interest: independent contractor as 'Speaker' for Janssen and a consultant for Skygenics. S.B.S. declares the following interests: Scientific advisory board participation for Pfizer, Janssen, Celgene, Lilly, IFM therapeutics and Pandion Inc. Grant support from Pfizer, Janssen, Novartis. Consulting for Hoffman La Roche and Amgen. J.O.M. reports compensation for consulting services with Cellarity and Hovione. W.K.C. is on the scientific advisory board for Regeneron Genetics Center.

Author Contributions

J.O., J.R.K.: study plan, patient identification, sample acquisition, genetic analysis, manuscript and figure preparation. W.A.S., K.K., M.L.S., K.E.S., K.E.H., K.A.-Y., K.W., M.H.D., D.K., C.K., N.W.: study plan. N.D., M.D.: genetic analysis. V.S., S.N., F.N., H.F., J.E., A.E., A.M.M.: study plan and patient identification. W.K.C.: study plan, patient identification, genetic analysis, manuscript preparation. P.J., M.F., D.L., T.N., D.P.B.M., A.T., C.B.: study plan, genetic analysis. A.W.: study plan, sample acquisition. S.T.: patient identification, sample acquisition. A.C.K., F.T., J.O.M.: single-cell transcriptomic analysis. B.H.H.: study plan, manuscript review. J.D.: clinical information of patients. J.G.: histology preparation. B.T., K.R.E., A.J.C., S.H., A.B., D.V., N.C.: study plan, patient identification, genetic analysis. C.A.C., R.L.M., S.B.S.: study plan, patient identification, genetic analysis, manuscript preparation. E.M.B.: study plan, genetic analysis, manuscript preparation. C.G.G.: study plan, manuscript preparation.

Data Availability Statement

The data underlying this article are available in the article and in its online supplementary material.

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Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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