Neonatal-Onset Chronic Diarrhea Caused by Homozygous Nonsense *WNT2B* Mutations

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Homozygous nonsense mutations in *WNT2B* were identified in three individuals from two unrelated families with severe, neonatal-onset osmotic diarrhea after whole-exome sequencing was performed on trios from the two families. Intestinal biopsy samples from affected individuals were used for histology and immunofluorescence and to generate enteroids *ex vivo*. Histopathologic evaluation demonstrated chronic inflammatory changes in the stomach, duodenum, and colon. Immunofluorescence demonstrated diminished staining for OLFM4, a marker for intestinal stem cells (ISCs). The enteroids generated from WNT2B-deficient intestinal epithelium could not be expanded and did not survive passage. Addition of CHIR-99021 (a GSK3A and GSK3B inhibitor and activator of canonical WNT/β-CATENIN signaling) could not rescue WNT2B-deficient enteroids. Addition of supplemental recombinant murine WNT2B was able to perpetuate small enteroids for multiple passages but failed to expand their number. Enteroids showed a 10-fold increase in the expression of *LEF1* mRNA and a 100-fold reduction in *TLR4* expression, compared with controls by quantitative RT-PCR, indicating alterations in canonical WNT and microbial pattern-recognition signaling. In summary, individuals with homozygous nonsense mutations in *WNT2B* demonstrate severe intestinal dysregulation associated with decreased ISC number and function, likely explaining their diarrheal phenotype. WNT2B deficiency should be considered for individuals with neonatal-onset diarrhea.

Congenital diarrhea and enteropathies (CoDEs) are a heterogeneous group of inherited disorders that present with severe chronic diarrhea in the first few months of life, often requiring significant fluid and nutritional support. These monogenic disorders are rare but can be distinguished from more common acquired causes of diarrhea in infancy such as infections and food-protein intolerance. Features of the diarrhea that should prompt further evaluation for CoDE disorders include neonatal onset, diarrhea severity and chronicity, and the need for nutritional support. These disorders can be grouped into several pathologic categories, including epithelial electrolyte transport disorders, epithelial enzyme and metabolism disorders, epithelial trafficking and polarity disorders, enteroendocrine disorders, and autoimmune disorders. The classification and investigative approach for congenital diarrheas has recently been reviewed in detail.[1]

Normal intestinal homeostasis is thought to require crypt-based columnar (CBC) intestinal stem cells (ISCs) located at the base of the crypt that divide rapidly to give rise to Paneth cells (PCs) and transit-amplifying (TA) progenitor cells.[2–5] CBC-ISCs are marked by production of LGR5, ASCL2, TROY (TNFRSF19), and OLFM4.[3,6–8] In the intestine, canonical WNT signaling has been demonstrated to play a key role in maintenance of the CBC-ISC compartment.[9] Inducible whole complete knock out of *WNTLESS* (transmembrane protein required for secretion of WNTs from the endoplasmic reticulum) in adult mice leads to death and is marked by severe intestinal dysregulation and loss of CBC-ISCs.[10] However, conditional deletion of WNT signaling molecules, specifically from the intestinal epithelium or from the subepithelium, either by deletion of *WNTLESS* or *PORCN* (required for post-translational palmitoylation), led to no appreciable intestinal phenotype in mouse models.[11,12] Based on these findings, it has been hypothesized that several intestinal WNTs from the subepithelium and epithelium act in a redundant manner to maintain the CBC-ISC pool.[11,12] For example, WNT3A and WNT2B are important for CBC-ISC development but have been considered to have redundant function.[10,13]

A male individual (I-1) of Kuwaiti origin with neonatal-onset, chronic, parenteral nutrition (PN)-dependent diarrhea was referred to our institution (Boston Children's Hospital, BCH) at 16 months of age due to failure to thrive (Figure 1A; see Supplemental Note). The family was enrolled in an IRB-approved protocol and WES was performed on the trio (proband and both parents). The filtered data (as described in Supplemental Note) were screened for known genes associated with the phenotype and no candidate variants were identified. The data were further evaluated, revealing a homozygous nonsense
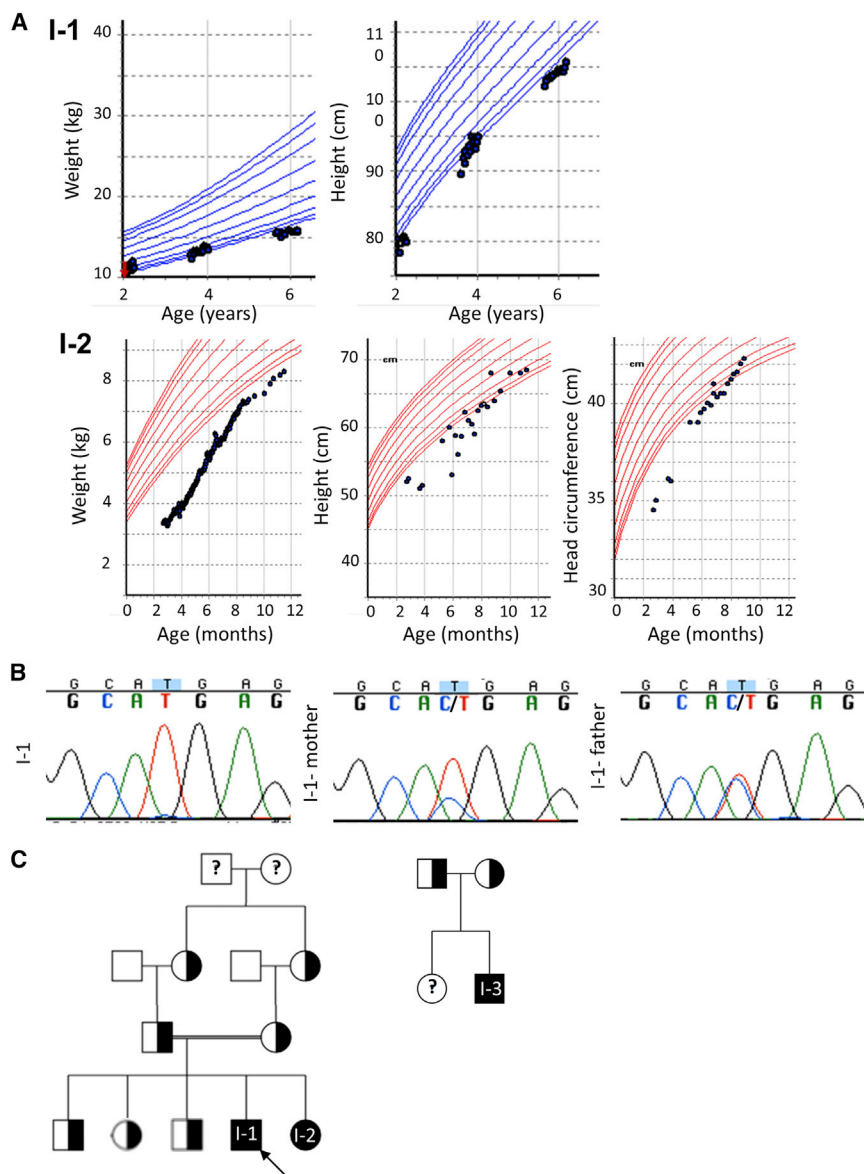
Figure 1. Individual Growth Charts and Sequencing
(A) I-1 growth charts (upper panel, blue lines) and I-2 growth charts (lower, red lines).
(B) Sequencing chromatograms for subject S1 along with maternal and paternal sequencing.
(C) Pedigrees for I-1 and I-2 (left) and I-3 (right). ? = not known, sequencing not done.

individuals with *WNT2B* mutations. The database identified a 7-year-old male individual (I-3) of Vietnamese origin who had intractable, PN-dependent diarrhea since birth with related growth failure. There was no family history of consanguinity and his parents and older sister were healthy (Figure 1C). He was identified by trio WES to have a different homozygous nonsense mutation, (hg19) chr 1:113057626 (GenBank: NM_024494; c.313C>T [p.Arg105*]). This variant was not previously reported in the ExAC or gnomAD databases. Both mutations, from the two families, were predicted to result in premature stop codons with nonsense-mediated decay and/or protein truncation. To evaluate this, RNA analysis was performed on I-1's cultured skin fibroblasts. In humans, there are two known isoforms of *WNT2B* with 391 and 372 amino acids, respectively, and the identified mutations would affect both isoforms (p.Arg69* and p.Arg50*). Interest-

mutation in *WNT2B* (MIM: 601968), a gene not previously linked to a human disease. The variant, (hg19) chr 1:113057518 (GenBank: NM_024494; c.205C>T [p.Arg69*]) (Figure 1B), has been seen three times in the gnomAD database and once in the ExAC database as a heterozygous variant. Each parent was heterozygous for the same mutation and neither had clinical symptoms of intestinal disease. The parents also had three other children, an older sister and two older brothers of the proband, who were not affected and were heterozygous for the mutation (Figure 1C).

Three years after I-1 was referred to our facility, his parents had a female child (I-2) who also developed intractable diarrhea shortly after birth. Sequencing demonstrated the same homozygous mutation in *WNT2B* (c.205C>T [p.Arg69*]).

After identifying the homozygous *WNT2B* mutation in I-1, we queried GeneMatcher for additional reports of

ingly, the mutation was present in the cDNA by Sanger sequencing, which suggests a lack of nonsense-mediated decay for both transcripts. This was further confirmed by qRT-PCR when no significant difference was noted in the transcript in the proband compared to age-matched control subjects using primers flanking the mutation as well as 5′ and 3′ to the mutation (Figure S1). Further, we were unable to perform immunoblotting because WNT2B is secreted and palmitoylated, with no well-validated antibodies capable of detecting it (see Web Resources, Nusse lab website).

We next sought to understand the molecular pathogenesis of the phenotype. The histological analysis revealed a paucity of crypts and abnormal crypt architecture (Table 1; Figure 2), which raised the possibility of an ISC defect in individuals with WNT2B deficiency. To assess this, we next performed immunofluorescence analysis for OLFM4 (olfactomedin 4) on intestinal samples from I-1 and from

Table 1. Gastrointestinal Pathology Findings

|	Age[a]	Esophagus	Stomach	Duodenum	Left Colon
I-1	3 years	normal	chronic inactive gastritis with oxyntic atrophy	chronic inactive duodenitis with partial villous atrophy	paneth cell metaplasia and mild crypt irregularity
I-2	6 mo.	ND	chronic inactive gastritis with oxyntic atrophy	partial villous atrophy	crypt architecture alteration, crypt dropout, Paneth cell metaplasia
I-3	2 years	normal	active inflammation and lymphoid aggregates	prominent lymphoid aggregates, focal villous blunting; crypt hyperplasia, reactive epithelial changes	ND
	7 years	normal	chronic inactive gastritis, glandular atrophy	normal	ND

ND indicates no data.
[a]Age at endoscopy

a healthy control subject. Duodenal crypts from I-1 demonstrated diminished OLFM4 protein localization compared to the control subject (Figure 3A). Analysis of mean fluorescence intensity of the crypt bases for I-1 demonstrated that WNT2B-deficient samples had lower protein levels of OLFM4 when compared to controls (Figure 3B). While control crypts universally demonstrated the presence of OLFM4, roughly 50% of crypts from I-1 had no expression, and the remaining crypts showed reduced OLFM4 protein localization compared to controls. These data suggest that WNT2B is required to maintain normal ISC numbers and/or activity in the duodenum.

Given the potential for an ISC defect in individuals with WNT2B deficiency, we next performed a functional assay for ISC activity, using intestinal enteroid cultures derived from WNT2B-deficient and healthy control intestinal epithelial biopsies. In contrast to enteroids from control subjects, enteroids derived from I-2 initially grew but then died shortly after passaging (Figures 4A–4C). All enteroids were expanded using standard enteroid culture conditions, including addition of WNT3A and R-SPONDIN1.

Biopsies from I-1 were subsequently obtained. Given the precipitous death of enteroids derived from I-2, the biopsies from I-1 were cultured in standard media plus CHIR or recombinant murine WNT2B (rmWNT2B) (Figures 4D–4F). The addition of rmWNT2B to the enteroid cultures was sufficient to promote long-term survival of enteroids over multiple (nine) passages. Despite this, however, the addition of rmWNT2B did not support expansion of enteroid numbers (a surrogate for stem cell number), when compared to control enteroids. Enteroid size was also smaller in rmWNT2B-treated samples compared with healthy controls (Figures 5A and 5B). In fact, at the end of nine passages, the overall number of enteroids was roughly similar to the number of enteroids
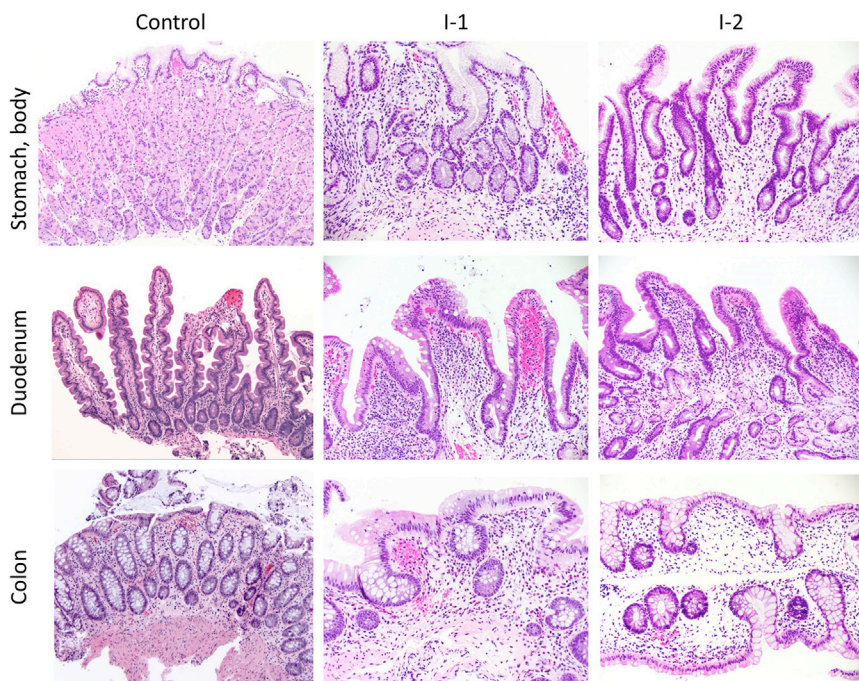
Figure 2. Gastrointestinal Histology
Biopsies from endoscopy or colonoscopy were taken from I-1 and I-2. Results are H&E-stained segments from the body of the stomach, proximal duodenum, and right colon. Note loss of crypt architecture and predominance of inflammatory cells in WNT2B-deficient samples.

A

	DAPI	Autofluorescence	OLFM4 Tx Red	Composite
Control				
I-1 sample 1				
I-1 sample 2				

B
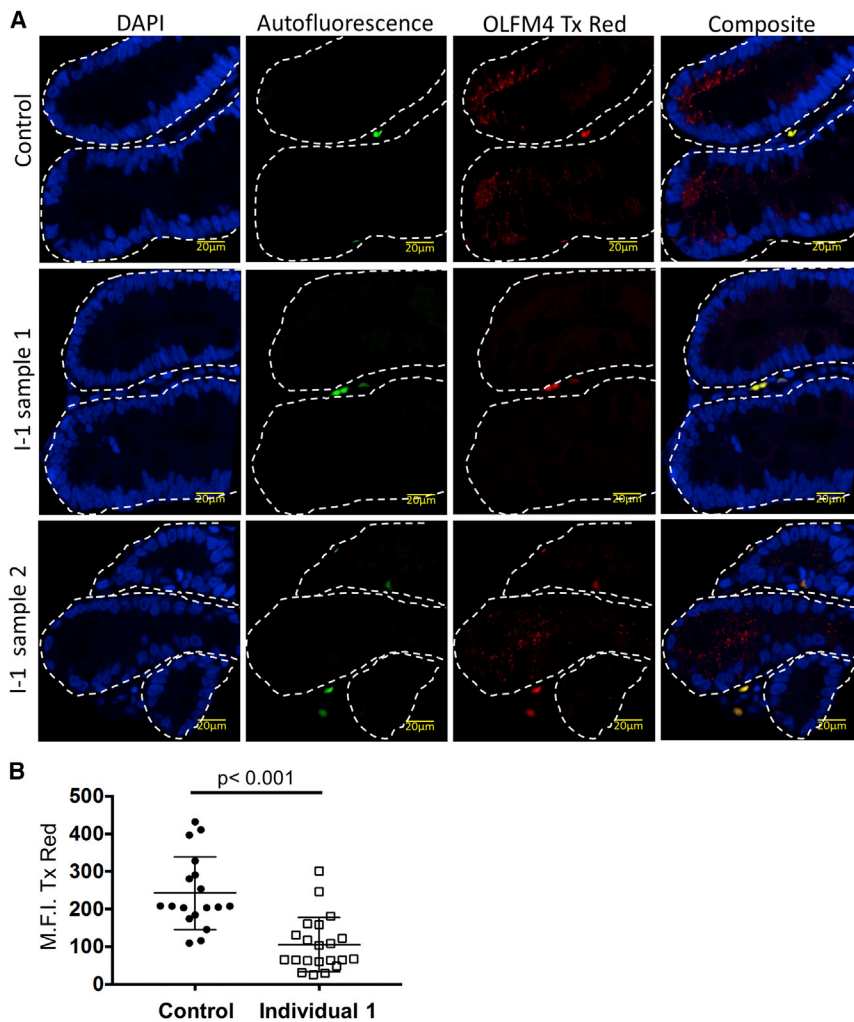
Figure 3. OLFM4 Localization in WNT2B-Mutated Duodenal Crypts

(A) Duodenal sections from I-1 compared with control duodenum. Control samples uniformly demonstrated OLFM4 localization at crypt bases similar to depicted section. Representative WNT2B-deficient samples were selected, with I-1 sample 1 showing no OLFM4 and I-1 sample 2 showing some OLFM4. Blue, DAPI; green, auto-fluorescence; Tx red, OLFM4. 100× mag.

(B) Mean fluorescence intensity of crypt bases. Crypt base was identified using the DAPI channel, then selected base of crypt was analyzed for Texas red fluorescence. Error bars represent 25th and 75th quartiles. p value for unpaired t test with Walsh adjustment.
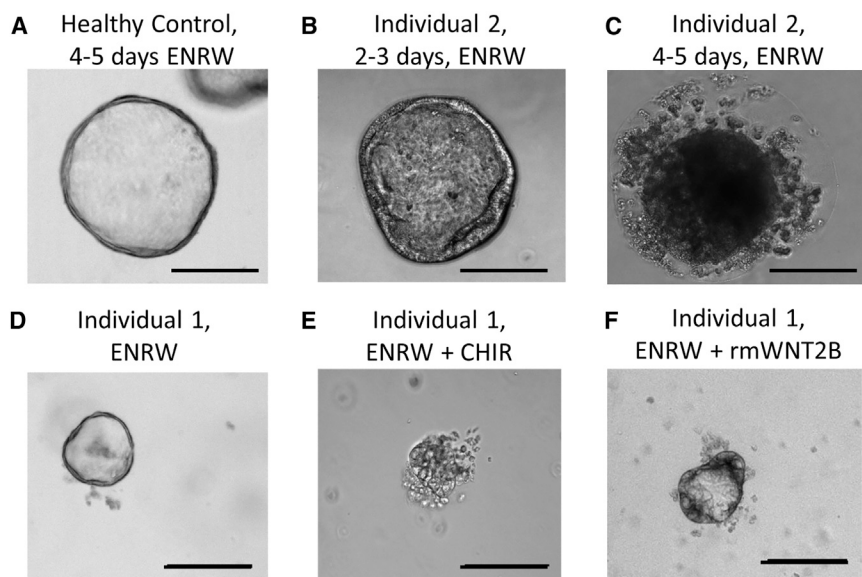
present during the first passage. In contrast, as expected, parallel cultures of control enteroids showed an exponential expansion in the number of enteroids during the same number of passages (Figure 5C). In contrast to enteroids, addition of either exogenous rmWNT2B or CHIR 99021 failed to rescue colonoid or gastroid cultures from WNT2B-deficient samples, which died after four passages (not shown). Taken together, these data imply that duodenal ISCs from WNT2B-deficient samples have an intrinsic defect in their ability to expand and that addition of rmWNT2B is required for ISC survival but is not sufficient for their expansion.

To gain additional insight into the potential mechanisms associated with WNT2B deficiency in the duodenum, we performed gene expression analysis on WNT2B-deficient and control enteroids. We hypothesized that dysregulated pathways might include canonical WNT signaling, non-canonical WNT signaling, senescence, and/or inflammation. Because limited enteroid material was available, we selected key target genes from each of these pathways. mRNA derived from I-2 duodenal enteroids maintained in rmWNT2B was reverse transcribed into cDNA and then pre-amplified for target genes. The pre-amplified gene regions were then quantified against cDNA from control enteroids using quantitative PCR. Because the organoids had been cultured with exogenous rmWNT2B, we assessed gene expression for control enteroids treated with rmWNT2B as well. No differences in expression were detected between these control samples for any of the genes analyzed (data not shown). Notably, expression of several canonical WNT pathway members was altered in the WNT2B-deficient enteroids (Figure 6). *TCF4* expression was decreased, while *LEF1* expression was slightly increased. *WNT5A* expression was also increased, as was the senescence pathway gene *CDKN1A*. Interestingly, the biggest change in expression of the genes surveyed was in *TLR4*, which was decreased nearly 100-fold in WNT2B-deficient enteroids. Despite this change, expression of *NFKB1*, a downstream target of TLR4 signaling, was not changed. This suggests that loss of WNT2B leads to impaired expression of downstream canonical WNT targets, increased expression of WNT5a, and alterations in microbial pattern recognition receptors. The variety of target gene pathways altered may indicate that there are overlapping consequences of WNT2B deficiency that, in concert, lead to the phenotype.

Homozygous nonsense mutations in *WNT2B* represent a distinct genetic etiology for CoDE.[1] The finding of disordered ISC number and/or function in individuals lacking WNT2B underscores an essential role for WNT2B in human intestinal homeostasis. In addition, the finding that WNT2B is of singular importance in ISC health, rather than being completely redundant with WNT3A, highlights the need for more investigation in this area, as there may be implications for the role of WNT2B in other intestinal disorders. Indeed, *WNT2B* expression has been

A Healthy Control, 4-5 days ENRW

B Individual 2, 2-3 days, ENRW

C Individual 2, 4-5 days, ENRW

D Individual 1, ENRW

E Individual 1, ENRW + CHIR

F Individual 1, ENRW + rmWNT2B

Figure 4. Intestinal Enteroids Derived from Biopsy Samples and Grown in Matrigel with Standard Enteroid Media
(A–C) Representative images of enteroids from (A) a healthy control individual and from (B) WNT2B-deficient individual (I-2) after 2–3 days and (C) after 4–5 days, when enteroids have collapsed.
(D–F) Representative enteroids from WNT2B-deficient individual (I-1) (D) treated with standard media, (E) treated with CHIR-99021, or (F) treated with recombinant murine WNT2B (rmWNT2B). Scale bars are shown at 200 μm.

shown to be upregulated in ulcerative colitis (UC) and *WNT2B* methylation was increased in human intestinal fibroblasts from fibrotic Crohn disease (CD) samples.[14,15]

Phenotypically, WNT2B deficiency presents with an osmotic diarrhea that is not substrate specific (carbohydrate, fat, protein), abnormal crypt and villus architecture, and significant fat malabsorption evidenced by high levels of fecal fat. These features overlap with findings seen in several existing categories of CoDEs including enteroendocrine dysfunction (non-substrate specific osmotic diarrhea), abnormal epithelial structure (e.g., tufting enteropathy, *TTC7A* mutation, and tricho-hepatic-enteric syndrome), and disorders of fat transport (e.g., chylomicron retention disease).[1] Some of these disorders also feature dysregulated immunity, which is of note given the altered *TLR4* expression in WNT2B deficiency. However, the constellation of findings in WNT2B deficiency do not fit easily with previous clinical categorization of CoDEs and WNT2B deficiency may be considered as a separate clinical category of CoDE associated with multi-potent epithelial stem cell dysfunction. The associated mixed phenotype seen in WNT2B deficiency may be due to the loss of ISC function causing a multi-lineage impact on the intestinal barrier epithelium.

The lack of reliable antibodies against WNT2B prevented confirmation of the lack of WNT2B protein in the individuals. In addition, Sanger sequencing and qRT-PCR performed on the cDNA derived from I-1's fibroblasts ruled out an obvious nonsense-mediated decay of the transcripts despite the proximal location of the mutation. This has been previously described for proximal nonsense mutations in other genes that do not result in nonsense-mediated decay but still cause protein deficiency.[16] Furthermore, if the mutant transcript led to the generation of a dominant negative protein, we would expect heterozygotes to variably demonstrate the phenotype. In the described individuals and th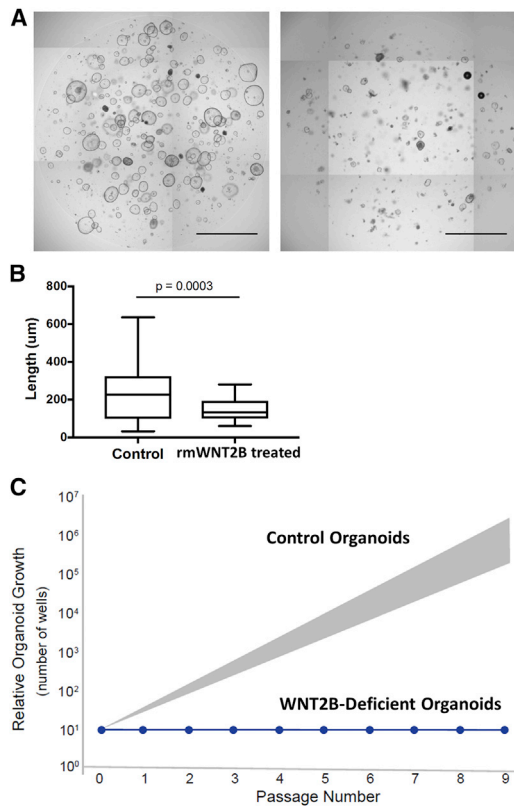eir families, only those with homozygous mutations demonstrated the phenotype. Unfortunately, we did not have samples from I-3 to assess for mRNA transcripts.

The OLFM4 immunofluorescent analysis shows diminished staining in the CBC-ISC compartment of WNT2B-deficient intestinal crypts. I-1 had variable OLFM4 localization, and the intensity of signal was decreased compared with control subjects. It has previously been suggested that WNT2B has a critical role in CBC-ISC development and maintenance; however, such a role has been questioned due to the lack of an intestinal phenotype in *Wnt2b* knock-out mice and evidence suggesting functional redundancy between WNT ligands *in vivo*.[11,12,17] We hypothesize that the phenotypic difference seen between WNT2B-deficient humans and the published mouse model is that mice housed in specific-pathogen-free conditions lack the microbial or inflammatory stimuli required to manifest the phenotype seen in human WNT2B deficiency.

Addition of CHIR was unable to rescue WNT2B-deficient enteroid cultures. This was surprising because WNT2B is thought to function via the canonical WNT/β-CATENIN pathway, and we had hypothesized that CHIR would enhance WNT pathway signaling in the absence of WNT2B, bypassing the need for WNT2B. It is possible that WNT2B also has non-canonical functions that have not previously been appreciated that would not be rescued by CHIR supplementation. The ability of exogenous rmWNT2B to propagate WNT2B-deficient enteroids from I-1, while the addition of CHIR alone did not, is consistent with a non-canonical role for WNT2B. However, recombinant rmWNT2B did not rescue the phenotype completely. Whether this is a dose-related effect or due to another variable will require further study.

Microbial pattern recognition changes may also contribute to the intestinal phenotype seen in WNT2B-deficient humans, although the clinical phenotype is different from known autoimmune enteropathies and very early-onset inflammatory bowel diseases. Quantitative RT-PCR showed decreased *TLR4* message in the WNT2B-deficient enteroids. TLR4 stimulation has been shown to increase phosphorylation of β-CATENIN in an

Figure 6. *WNT2B* Mutant Enteroids Demonstrate Altered Expression of βCATENIN-Target and Inflammatory Genes
Duodenal enteroid cultures from I-1 were cultured with recombinant murine WNT2B and gene expression assessed by qRT-PCR. Relative expression is displayed as compared with control enteroids. Expression was normalized to *GAPDH*.

Accession Numbers

The genetic data of the affected probands I-1 and I-2 have been deposited to Clinvar; the accession number is pending and will be available publicly. The genetic data for subject I-3 have also been deposited to ClinVar and the accession number is SCV000297313.2.

Supplemental Data

Supplemental Data include one figure, one table, and a Supplemental Note and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.05.007.

Acknowledgments

Declaration of Interests

The authors declare no competing interests.

Figure 5. Comparison of Enteroid Cultures in Standard Enteroid Media
(A) Stitched low-magnification comparison of enteroid cultures from healthy control individual (left) versus WNT2B-deficient individual (I-1) treated with recombinant murine WNT2B (rmWNT2B, right) at passage 4. Scale bars are shown at 2 mm.
(B) Enteroid size comparison between healthy control samples and WNT2B-deficient enteroids treated with WNT2B. Boxplot is max and min numbers with 75th and 25th quartiles. p value for unpaired t test with Walsh adjustment.
(C) Semiquantitative analysis showing exponential expansion of enteroid numbers from the control individual scored for the number of wells at each passage compared with enteroids from the WNT2B-deficient individual, which survived each passage but failed to expand in number.

intestinal neoplasia model.[18] There may be a negative feedback mechanism that downregulates *TLR4* transcription in the absence of WNT2B. Microbe-derived stimuli are known to be a factor in driving intestinal epithelial proliferation, and TLR4 signaling may be involved in maintenance of ISCs.[19–22] The relationship between WNT2B signaling, host-microbe signaling, and epithelial TLR4 responses requires further study.

In summary, WNT2B deficiency is a cause of neonatal-onset chronic diarrhea resulting in failure to thrive and should be considered in the differential diagnosis of CoDEs. Individuals with homozygous nonsense mutations in *WNT2B* highlight a critical role for WNT2B in intestinal homeostasis. Our data suggest that the impact of WNT2B deficiency on intestinal health is mediated through impaired generation or maintenance of ISC and possible dysregulation of host-microbial pattern recognition signaling.

Web Resources

ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
ExAC Browser, http://exac.broadinstitute.org/
GenBank, https://www.ncbi.nlm.nih.gov/genbank/
GeneMatcher, https://genematcher.org/
gnomAD Browser, http://gnomad.broadinstitute.org/
OMIM, http://www.omim.org/
Roel Nusse lab, https://web.stanford.edu/group/nusselab/cgi-bin/wnt/reagents#antibod

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