Intestinal dysmotility in a zebrafish (Danio rerio) shank3a;shank3b mutant model of autism

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Abstract

Background and aims: Autism spectrum disorder (ASD) is currently estimated to affect more than 1% of the world population. For people with ASD, gastrointestinal (GI) distress is a commonly reported but a poorly understood co-occurring symptom. Here, we investigate the physiological basis for GI distress in ASD by studying gut function in a zebrafish model of Phelan-McDermid syndrome (PMS), a condition caused by mutations in the SHANK3 gene.

Methods: To generate a zebrafish model of PMS, we used CRISPR/Cas9 to introduce clinically related C-terminal frameshift mutations in shank3a and shank3b zebrafish paralogues (shank3abΔC). Because PMS is caused by SHANK3 haploinsufficiency, we assessed the digestive tract (DT) structure and function in zebrafish shank3abΔC+/− heterozygotes. Human SHANK3 mRNA was then used to rescue DT phenotypes in larval zebrafish.

Results: Significantly slower rates of DT peristaltic contractions (p < 0.001) with correspondingly prolonged passage time (p < 0.004) occurred in shank3abΔC+/− mutants. Rescue injections of mRNA encoding the longest human SHANK3 isoform into shank3abΔC+/− mutants produced larvae with intestinal bulb emptying similar to wild type (WT), but still deficits in posterior intestinal motility. Serotonin-positive enteroendocrine cells (EECs) were significantly reduced in both shank3abΔC+/− and shank3abΔC−/− mutants (p < 0.05) while enteric neuron counts and overall structure of the DT epithelium, including goblet cell number, were unaffected in shank3abΔC+/− larvae.

Conclusions: Our data and rescue experiments support mutations in SHANK3 as causal for GI transit and motility abnormalities. Reductions in serotonin-positive EECs and serotonin-filled ENS boutons suggest an endocrine/neural component to this dysmotility. This is the first study to date demonstrating DT dysmotility in a zebrafish single gene mutant model of ASD.

Keywords: Digestive transit, Peristaltic rate, Enteroendocrine, Phelan-McDermid syndrome

Background

Autism spectrum disorder (ASD) is estimated to impact more than 1% of the population and is etiologically and clinically heterogeneous [1, 2]. While ASD diagnoses are based upon deficits in social communication and the presence of repetitive behaviors and/or restrictive interests, co-occurring symptoms (comorbidities) are also common. Here, we focus on gastrointestinal (GI) distress, one of the more frequent comorbidities experienced by individuals with ASD [3, 4]. Despite the negative impacts of GI distress on daily life, ASD-associated GI symptoms are poorly understood [3, 5]. Consistent with prospective findings, clinical reports from monogenic causes of ASD regularly document GI distress [3, 6–8]. Our work focuses on a monogenic form of ASD, Phelan-McDermid syndrome, that is caused by mutations that disrupt one copy of the SHANK3 gene resulting in SHANK3 haploinsufficiency [9, 10]. In individuals with PMS, GI distress is characterized by reflux, cyclical vomiting, diarrhea, and/or constipation [11, 12]. To investigate the biological mechanisms underlying GI distress in PMS and ASD, we have generated a zebrafish shank3 mutant model.
The majority of SHANK3 loss-of-function animal models are mammalian and have provided great insight into neural mechanisms related to social and motor behaviors characteristic of ASD [13]. SHANK3 is known to act as a synaptic scaffolding protein in the central nervous system (CNS) where it helps to regulate synaptic development, glutamatergic receptor signaling, actin polymerization, and dendritic spine formation [14–19]. In addition, SHANK3 is also expressed at early developmental stages prior to synapse formation [20, 21], as well as in enterocytes and nitrergic neurons of the enteric nervous system (ENS) [22–24], and has been shown to have important interactions with the Wnt signaling pathway [25]. Studies suggest that SHANK3 may also play important GI-related roles in host/symbiotic interactions and Zn metabolism [22, 26–28] and intestinal barrier function [29]. Studies to explore roles for SHANK3 in relation to GI dysfunction, however, are limited.

To understand the etiology of ASD symptoms, zebrafish is a powerful model system [30–32]. Genetically and physiologically similar to humans and mammalian models, zebrafish provide a complementary model system with accessible developmental stages that are transparent, allowing physiological assessment in vivo [31, 33–35]. Additionally, zebrafish and human digestive tracts are largely conserved, with similar hormonal regulation, morphology, cell types, and physiology, albeit simplified in zebrafish [8, 36–40]. For example, in both zebrafish and mammals, digestion rate adapts to the size of the meal [39]; also in both, serotonin, acetylcholine, motilin, and ghrelin increase DT motility [39, 41, 42] while vasoactive intestinal peptide, pituitary adenylyl cyclase-activating peptide, and nitric oxide decrease DT motility [41, 43]. Like mammals, the zebrafish DT tract can be divided into sections distinguished by differences in cell type and function: digestive secretions are enriched anteriorly where both nutrient absorption and tissue folding are the greatest, while posterior regions are largely devoid of folding and cell types are largely specialized for water absorption [44, 45]. Additionally, the ENS and key DT regulatory brain regions such as the hypothalamus, secondary gustatory nuclei, vagal motor nucleus, sensory nodose ganglia, and spinal dorsal root ganglia are conserved in zebrafish [32, 40, 46, 47]. Experiments that have used zebrafish to investigate GI dysfunction in Hirschsprung’s and chronic intestinal pseudo-obstruction diseases have found that disrupting conserved zebrafish genes linked to these diseases disrupts both GI motility and ENS development [48, 49], mirroring human symptoms and supporting translatability of the zebrafish model system.

Here, we generate a zebrafish shank3 mutant model to investigate a causal link between shank3 loss-of-function mutations and DT dysfunction. As a result of a genome duplication approximately 300 mya [50], the shank3 gene, along with many other synaptic genes, is duplicated in teleost fishes [51, 52], yielding shank3a and shank3b gene paralogues. Therefore, to model human SHANK3 mutations linked to PMS [53], we used CRISPR/Cas9 to generate frame-shift mutations in the C terminal, proline-rich domains of both zebrafish shank3a (chromosome 18) and shank3b (chromosome 4) (referred to as shank3abΔC).

Methods

Additional materials and methods on histology, statistics, and the Igor Pro code can be found in Additional file 1.

Fish maintenance and husbandry

Zebrafish were housed in the University of Miami zebrafish core facility. Both adult and larval zebrafish were maintained at 28 °C in system water and exposed to a 14:10 h circadian light:dark cycle. Zebrafish lines used in this study include AB wild type (WT) and transgenic lines la118Tg:tg(aldoca:gap43-Venus) [54] and Tg(vglut2a:dsRed) [55]. To generate shank3abΔC+/- animals, WT females were crossed with shank3abΔC +/- males. ZFIN zebrafish gene and protein nomenclature conventions were followed.

Single-stranded guide RNA (sgRNAs) design

Site-specific CRISPR-Cas9 sgRNAs were generated using the online software CHOPCHOP [56]. CHOPCHOP-generated guides were chosen based on location within the gene and minimal predicted off-target sites. Guides were designed to target the C-terminal proline-rich domain of shank3a and shank3b (Additional file 1: Table S1). To produce sgRNA templates for in vitro transcription, phage T7 promoter-adapted sgRNA oligonucleotides were annealed to a universal tracrRNA oligonucleotide with complementary overhangs [57]. Each sgRNA template reaction contained: 1× transcription buffer, 1 μM site-specific oligo, 1 μM tracrRNA oligo, 500 nM dNTPs, 0.5 U Phusion high-fidelity DNA polymerase (New England Biolabs, NEB; Ipswich, MA), and nuclease-free H2O to 10 μL. Reaction mixtures were heated to 95 °C for 1 min, cooled to 52 °C (0.1 °C/sec), and extended at 72 °C for 10 min. Templates were then used to transcribe single-stranded sgRNAs with a T7 MEGAscript in vitro transcription kit (Ambion; Foster City, CA) following the manufacturer’s protocol. Transcribed sgRNAs were then cleaned using ammonium acetate/ethanol precipitation and quantified by comparison to an RNA sample of known concentration on an agarose gel.
**CRISPR-Cas 9 mutagenesis and allele screening**

In vitro transcribed sgRNA and Cas 9 protein (PNA Bio, Newbury Park, CA) were mixed and incubated at 37 °C for 5 min. A micro-injector was then used to inject 400:100 pg of sgRNA:Cas 9 into the cell of one-cell stage zebrafish embryos. Injected embryos were allowed to develop for 24 h, and genomic DNA (gDNA) was extracted to screen for mutations. Genes were targeted singly for either shank3aΔC or shank3bΔC then F1 mutants were out-crossed to obtain F2 shank3abΔC heterozygotes. These F2 mutants were then outcrossed to la118Tg:T-aldoca:gap43-Venus and Tg(vglut2a:DsRed) and finally backcrossed to generate F4 and F5 shank3abΔC homozygous mutants. To assay mutations, polymerase chain reaction (PCR) primers were designed to flank the sgRNA target site (Additional file 1: Table S2). Each PCR reaction mixture contained: 1× GO Taq (Promega, Madison, WI), 50 nM forward and reverse primer, 1 μL gDNA, and nuclease-free H₂O to 10 μL. PCR products were then sequenced on an AB3130 Sanger sequencer with each sequence PCR mixture containing: 1× BigDye Buffer, 50 nM forward primer, 0.3× 3.1 BigDye mixture, and 1 μL purified PCR product. Identified mutations were then analyzed for unique restriction digest sites that could be used for genotyping (Additional file 1: Figure S1). Post-experimental analysis genotyping using PCR and restriction enzyme digests (Additional file 1: Figure S1) allowed us to conduct experiments blind to genotype.

**Western**

One hundred milligrams of whole fish brains dissected and pooled from adult shank3ab +/+ (n = 8) and shank3abΔC Δ−/− mutants (n = 8) were used to prepare the postsynaptic density (PSD) fractions following the previously published method [16]. Mouse cortices from Shank3+/+ or Shank3−/−, lacking all Shank3 isoforms, (unpublished) were processed in parallel. Briefly, brains were homogenized in HEPES buffer containing 0.32 M sucrose and protease inhibitors followed by two rounds of low-speed centrifugation (700 g) to remove nuclei. Supernatants were centrifuged at 18,000 g to precipitate crude synaptosomal fraction, which were further homogenized in hypotonic HEPES buffer to eliminate synaptic vesicles. Finally, PSD fractions were obtained by four rounds of washing with HEPES buffer containing 0.5% Triton X-100 and high-speed centrifugation (180,000g) and dissolved in HEPES buffer containing 1.8% SDS and 0.85 M urea. PSD lysates were run on SDS-PAGE gel and transferred to PVDF membrane for immunoblot analysis using antibodies against Shank3 (1:100; sc-30,193, Santa Cruz Biotechnology, CA), raised against amino acids 1431–1590 mapping near the C-terminus of isoform 2 of human SHANK3), Tubulin (1:500, ab18207, Abcam), and Actin (1:500, A2066, Sigma).

**Cryosectioning and immunohistochemistry**

Cryosectioning and antibody staining were performed using previously published methods [58]. Anti-PSD-95 (1:500; Abcam; Cambridge, UK; ab-18,258) and anti-SHANK3 (1:200; sc-30193, Santa Cruz Biotechnology, CA) were used as primary antibodies with secondary antibodies conjugated to Alexa Fluor 568 (Abcam, ab175472) and Alexa Fluor 488 (Thermo Fisher Scientific, R37118), respectively. Images were collected on a SP5 confocal microscope (Leica; Wetzlar, DE) using 40× and 63× oil immersion lenses.

**Peristalsis videography and analysis**

To quantify DT motility, we captured videos of gut peristalsis in intact transparent 7-day-old zebrafish larvae after feeding with a chicken egg yolk emulsion. The yolk emulsion promoted more uniform feeding across the population, making screening easier. To create the emulsion, 1 ml of chicken egg yolk was rapidly and repeatedly pipetted into 1 ml of system water; several drops of the emulsion were then added to a 10 cm petri dish housing 50–100 larva. Both WT controls and shank3abΔC mutant larvae were exposed to the yolk mixture for the same time period (60–90 min). To avoid requiring anesthetic, videos were acquired by embedding single larvae oriented on the sagittal plane in low melt point agarose (LMA; Thermo Fisher, BP165-25) on a glass-bottom 35 mm petri dish. Videos were recorded at 1 frame/second for a total of 10 min on a Canon EOS 5D Mark III; larvae were then processed for genotyping. Videos were compiled blind to genotype, and run through a custom script in Igor Pro. This script allows a user to demarcate a specific area of analysis, or AOA (Fig. 3a, red lines), and determine the number of regions to evaluate along the area of analysis (Fig. 3a, cyan boxes) and the amount of raster averaging for each AOA. Post-analysis, the program produces individual peristaltic periodicity per point of evaluation and overall averages of peristaltic periodicity (see Additional file 1). These outputs were manually annotated to check for artifacts due to muscle twitches that were easy to distinguish from DT peristalsis due to the much faster timescale.

**DT transit**

Seven dpf shank3abΔC Δ−/− mutant and WT larvae were fed a mixture of 6 μm fluorescent beads (Polysciences 17156, fluoresbrite beads packaged as 2.5% aqueous suspension) and chicken egg yolk emulsion for 1.5 h. Approximately 10 μl of fluorescent bead solution in 2 ml of egg yolk emulsion was added to the 10 cm petri dish.
containing the larvae in approximately 20 ml system water. After screening for individuals who had filled their intestinal bulb with the yolk-bead suspension, larvae were embedded in LMA on a 35-mm, glass-bottom petri dish, and a series of pictures of each larva were taken with a Zeiss Axiocam on a stereoscope V20 at 3, 6, 12, and 24 h. Regions of analysis were the pharynx, intestinal bulb, upper intestinal tract, lower intestinal tract, and expelled. This procedure was adapted from [59]. Calculations were made blind to genotype. Using FIJI, the total area encompassed by the fluorescent beads was determined, and percentages of this total were calculated from the total area encompassed by the fluorescent beads was determined, and percentages of this total were calculated per DT region. Averages for 3, 6, 12, and 24 h time periods were compiled for both WT and shank3ab larvae. Supplementary movie figures (shank3abΔC +/-, Additional file 2: Movie S1; and WT, Additional file 3: Movie S2) were processed as described above. Individuals were screened for high quantities of fluorescent intestinal content and were embedded as described. Images were acquired using a Zeiss Axio Imager.Z2 paired with a Zeiss AxioCam MRRm Rev3 camera running Zeiss Zen Blue software. Images were acquired every 10 s for 6 h. Image acquisition began late morning and was concluded before 5 pm; as such, the room lights were left on during the duration of the recording process to prevent any confounding effects with shifting circadian rhythm.

**Rescue experiments**

Fertilized shank3abΔC +/- embryos were injected with a full-length (designated 5t) human SHANK3 mRNA, or a short isoform (designated 32t, Additional file 1: Table S9) [20]. These fish were allowed to grow to 7 dpf before being analyzed with the microsphere transist assay listed above. Additionally, control injections of loading dye and non-injected WT and shank3abΔC +/- fish were analyzed.

**Quantification of gut ENS and enteroneuroendocrine cells**

Seven dpf WT and mutant larvae were incubated on ice for 30 min before being fixed in 4% Formaldehyde (Pierce™, Thermo Fisher Scientific, 28906)-1x phosphate buffer solution/0.25% Triton X-100 (1x PBSTx) for 1 h at room temperature. Whole-mount antibody staining and gut dissections were carried out according to previously published methods [60]. Anti-HuC/HuD (1:1000; Invitrogen; A21271) and anti-5-HT (1:1000; serotonin; Immunostar, New Richmond, WI; 20,080) were used as primary antibodies with secondary antibodies conjugated to Alexa Fluor 568 (1:1000; Abcam, ab175472) and Alexa Fluor 633 (1:1000; Thermo Fisher Scientific, A-31575). To sample the anterior and posterior intestinal tract, a 200 x 200-μm field was captured 200 μm from the intestinal bulb (anterior) and 200 μm from the anus (posterior) as in [61]. Images were captured using a 20x dry objective and analyzed using the cell counter application in Fiji.

**Results**

Generating zebrafish shank3abΔC loss-of-function mutant models of Phelan-McDermid syndrome

Zebrafish shank3a on chromosome 18 shares human SHANK3 synteny, surrounded by six of the seven genes that neighbor human SHANK3, while shank3b has reduced synteny, flanked by three of the genes that are more distant from human SHANK3 (Fig. 1a). Moreover, while zebrafish Shank3a retains all known protein binding domains, Shank3b has an additional ankyrin repeat and lacks cortactin and Abp1 interaction domains (Fig. 1b). CRISPR-Cas9/sgRNA injections produced frameshift mutations in shank3a and shank3b. Both sgRNAs targeted the largest exon in shank3 that encodes a large C-terminal region containing several protein interaction domains. Similar human frameshift mutations in the SHANK3 C-terminal domain have been associated with ASD [53, 62, 63]. Frameshift mutations were induced in both shank3a and shank3b genes, and alleles were selected to minimize the number of amino acids between frameshift and stop codon (Fig. 1c). In shank3a, a four-base pair insertion converted an isoleucine into a histidine and introduced thirteen amino acids before the stop codon. In shank3b, a single-base pair insertion converted an isoleucine into a histidine and introduced thirty-one amino acids before the stop codon.

A western blot of protein samples enriched for membrane fractions including post-synaptic densities (PSDs) was then used to determine whether the mouse Shank3 antibody was cross-reactive in zebrafish and to characterize the isoform complexity of zebrafish Shank3ab. Zebrafish protein extracts from adult brain showed multiple isoforms as indicated by the five bands of ranging from 75 to 250 kD in WT. For comparison, PSDs isolated from WT and Shank3/-/- mouse brains showed similar Shank3 isoform complexity (Fig. 2a). By contrast, PSD fractions from zebrafish mutant for both shank3a and shank3b (shank3abΔC -/-) showed no visible bands confirming the specificity of the mouse Shank3 antibody for zebrafish Shank3ab.

To test for localization of Shank3ab protein in 6 days post-fertilization (dpf) zebrafish brain tissue, we stained fresh frozen sections from both shank3abΔC -/- and WT larvae. Sectioned tissue was co-labeled with PSD-95 antibody because Shank3 is known to co-localize with PSD-95 at mammalian glutamatergic post-synapses [64]. As in mammals, we found that PSD-95 and Shank3 expression co-localized in post-synaptic puncta of WT larvae (Fig. 2b). These puncta were found in the cerebellum that most likely label glutamatergic granule cell
synapses. By contrast, Shank3ab expression was absent in \textit{shank3ab}\(\Delta \)C\(\sim\) mutants despite robust PSD-95 staining.

**DT peristalsis frequency is reduced in \textit{shank3ab}\(\Delta \)C larvae**

To test whether \textit{shank3ab}\(\Delta \)C mutations impact DT function, we compared peristaltic rates in WT and different combinations of \textit{shank3ab}\(\Delta \)C mutant alleles in 7 dpf larvae. After feeding, larvae were filmed for a period of 10 min to capture the muscular contractions moving along the DT tract. To determine peristalsis frequency, a custom script in Igor Pro quantifies pixel intensity changes to produce individual plots for regions along the DT (Fig. 3a, cyan boxes). These data show that \textit{shank3ab}\(\Delta \)C\(\sim\) larvae have significantly less frequent peristaltic contractions compared to WT larvae (Fig. 3d, WT \(n = 9\), \textit{shank3ab}\(\Delta \)C\(\sim\) \(n = 29\), \(p < 0.001\)). Peristaltic contractions are shown for the intestinal bulb, as this region is the most responsive to a feeding event. It should be noted, however, that this pattern of significantly reduced motility was also seen in upper and lower intestines (Additional file 1: Table S7). In addition to \textit{shank3ab}\(\Delta \)C\(\sim\) larvae, this dysmotility phenotype was also seen in both CRISPR/Cas9/\textit{shank3b} sgRNA injected embryos (\(F_0\) generation; \(n = 13\), \(p = 0.0001\)) and \textit{shank3ab}\(\Delta \)C\(\sim\) larvae (\(n = 9\), \(p < 0.0001\)) compared to WT
(similar results were found in the CRISPR/Cas9/shank3a
sgRNA injected embryos, but were not recorded with
sufficient n to include in the data). Differences between
heterozygous and homozygous shank3abΔC mutants
were not found to be significant (p = 0.978), suggesting a
maximum level of DT effect in shank3abΔC+/− geno-
types. Our subsequent analyses therefore focus on this
shank3abΔC+/− phenotype that best models human
SHANK3 haploinsufficiency.

DT transit time is prolonged in shank3abΔC+/− larvae

Having established a DT motility phenotype associated
with shank3abΔC+/− mutants, we next tested whether
the reduced peristaltic frequencies translated into pro-
longed DT transit times by feeding larvae fluorescent
6 μm microspheres in an emulsion of egg yolk and timing
microsphere transit through the DT. Both WT and
shank3abΔC+/− larvae were embedded in 1% LMA,
immersed in system water, and photographed at 3, 6, 12,
and 24 h. Transit differed between WT and shank3abΔC
+/− mutants, both in terms of overall rate and the
amount of time spent in the upper DT (Fig. 4;
Additional file 1: Tables S3–S6). To quantify these differ-
ences, we compared the proportions of fluorescent
microspheres in DT regions across time and genotypes.

After permutation (Additional file 1: Table S8), a re-
peated measures ANOVA found significant interactions
in the percentage of microspheres between genotype
(shank3abΔC+/−, n = 15 and WT, n = 13) and time (p <
0.004). When analyzing the rate of bead expulsion spec-
cifically (analogous to completed DT transit), significant
differences were found between WT and shank3abΔC
+/− larvae at 6 h and 12 h transit times (p < 0.006), but
not the 24 h time point (p = 0.330). This is indicative of a
severely delayed but not entirely obstructed transit. Most
WT larvae had passed a large portion of the consumed
microspheres by 6 h and had passed the remainder be-
tween 12 and 24 h post consumption (Fig. 4b). Com-
paratively, shank3abΔC+/− larvae took longer than 12 h
to begin passing the microspheres and some individuals
had not passed the remainder even after 24 h post con-
sumption (Fig. 4c). Of particular interest is the amount
of “sloshing” that occurred in shank3abΔC+/− larvae,
where the microspheres would repeatedly move ante-
riorly and posteriorly between the intestinal bulb and
upper-intestine. In shank3abΔC+/− larvae, the passage
of particles seemed to get delayed in two key areas: the
intestinal bulb to upper-intestine transition and near the
anus (Additional file 2, shank3ab mutant). This intestinal
bulb to upper-intestine transition marks a key region

Fig. 2 Zebrafish Shank3ab protein shares complex isoform expression with mammals and shows expression in the cerebellum. a Shank3 western
blots of postsynaptic densities (PSD) isolated from mouse (M.m) and zebrafish (D.r.) show similar complex isoform expression. Molecular marker
weight is expressed in kilodaltons. Tubulin and actin expression were used as loading controls for zebrafish and mouse immunoblots, respectively.
Loss of Shank3ab staining in IHC and the Western blot supports the immunoreactive specificity of Shank3 antibody. b Shank3ab protein is expressed
as distinct overlapping puncta with PSD-95 in the cerebellum from larvae 6 days post-fertilization. Transverse cerebellar sections (see diagram) with
enlarged insets show neuropil area dorsal to the rhombencephalic ventricle (RVe). Scale bar represents 10 μm for the first column and 5 μm for the
insets (i–vi)
Fig. 3 (See legend on next page.)
where both anterograde and retrograde peristaltic propulsion transitions to just retrograde propulsion [36]. WT larvae displayed no sloshing and passed the microspheres in a clear anterior to posterior direction; WT similarly lacked the delay in the intestinal bulb to upper-intestine transition that was seen in shank3abΔC+/- larvae.

Partial rescue of shank3abΔC +/- transit time with human SHANK3
To test whether providing human SHANK3 mRNA to shank3abΔC +/- zebrafish embryos is sufficient to rescue the DT dysmotility phenotype, we injected mRNA encoding either the longest human SHANK3 isoform that includes all SHANK3 protein domains (5t, n = 19) or a
shorter human \textit{SHANK3} isoform that includes only the C-terminal proline-rich and SAM domains (32t, \( n = 6 \)) into fertilized eggs from \textit{shank3ab}\(\Delta C \) \( +/- \) mutants. After injection, larvae were reared to 7 dpf and were put through the fluorescent microsphere digestive transit assay. While injecting \textit{shank3ab}\(\Delta C \) \( +/- \) larvae with the short 32t \textit{SHANK3} isoform further slowed mutant transit times (Fig. 5cii), injecting the long 5t \textit{SHANK3} isoform partially rescued mutant transit times (Fig. 5ci); microspheres moved in a clear anterior-posterior direction without any sloshing or pause at the intestinal bulb to upper-intestine transition. The rescue did not return the WT phenotype entirely; while the progression of microspheres was more consistent, the transit rates in rescued \textit{shank3ab}\(\Delta C \) \( +/- \) mutants resembled a mid-point between unrescued \textit{shank3ab}\(\Delta C \) \( +/- \) and WT larvae (Fig. 5a). By contrast to the partial rescue in the upper intestine, transit rates in the lower intestine were not rescued (Fig. 5b). Therefore, it is likely that RNA rescue was associated with overexpression phenotypes in addition to the partial functional rescue seen with injection of the longest \textit{SHANK3} isoform.

![Fig. 5](image-url) Full-length human \textit{SHANK3} mRNA partially rescues transit time in \textit{shank3ab}\(\Delta C \) \( +/- \) larvae. a When measuring the total percentage of fluorescent beads within the intestinal bulb (highlighted region of analysis for in magenta), total content of the long isoform (5t) rescue (dark gray) decreases at a rate quicker than the unrescued \textit{shank3ab}\(\Delta C \) \( +/- \) (black) but not as quickly as the WT (light gray). Significant differences of intestinal bulb emptying (total intestinal bulb content) were found between the long isoform rescued and unrescued \textit{shank3ab}\(\Delta C \) \( +/- \) larvae at 12 h \( (p < 0.006) \) and 24 h \( (p < 0.03) \), and between WT and unrescued \textit{shank3ab}\(\Delta C \) \( +/- \) at 6 h \( (p < 0.02) \) and 12 h \( (p < 0.01) \). b Fluorescent microsphere expulsion (analogous to completed digestion; highlighted region of analysis in orange) was not rescued, however, and the long isoform rescued larvae showed expulsion rates closer to \textit{shank3ab}\(\Delta C \) \( +/- \) than that of WT. Significant differences were found at 6 h between WT, the long isoform rescue, and unrescued \textit{shank3ab}\(\Delta C \) \( +/- \) \( (p < 0.006) \), at 12 h between WT and unrescued \textit{shank3ab}\(\Delta C \) \( +/- \) \( (p < 0.001) \), WT and the long isoform rescue \( (p < 0.001) \), and at 24 h between unrescued \textit{shank3ab}\(\Delta C \) \( +/- \) and the long isoform rescue larvae \( (p < 0.01) \), and WT and the long isoform rescue larvae \( (p < 0.005) \). c Graph of bead distribution per time period for the long isoform (5t) rescue larvae \( (n = 19) \) and cii short isoform (32t) rescue larvae \( (n = 6) \). Each point represents the average +/- standard error percentage of total beads held by each region.
Larval DT morphology

In order to assess the integrity of both WT and shank3abΔC+/− mutant gut tissues, we processed 7 dpf larvae for paraffin sectioning. Larvae were sectioned along the sagittal plane at a thickness of 5 μm and stained with alcian blue and Eosin B as a counterstain, with a left-right anterior to posterior orientation (Fig. 6). Typical features of gut morphology at this stage of development are captured in WT sections (Fig. 6a, b). The intestine is comprised of polarized epithelium and is well-defined with dense cytoplasm. Folding of the epithelium (plicae) can be seen readily in the intestinal bulb extending into the upper-intestine (arrows), but these plicae do not extend any significant distance into the luminal space. Goblet cells and mucin production can be seen throughout the upper-intestine (black arrowheads). In shank3abΔC+/− larval sections (Fig. 6c, d), like that seen in WT, folding can be seen in the intestinal bulb, and extending into the upper-intestine; goblet cells and heavy mucin production can be seen throughout the upper-intestine (black arrowheads); and enterocytes have similar large supranuclear vesicles (white arrowheads) [65]. Statistically, shank3abΔC+/− larvae did not show a significant increase in goblet cells (p = 0.750, n = 25 for shank3abΔC+/− fish, n = 15 for WT).

We also quantified goblet cells in adult DTs from adult (>1 year old) WT, shank3abΔ+/−, and shank3abΔC−/− animals that had been sacrificed to acquire adequate CNS tissue for western analysis. Paraffin sections were collected near the IB and UI transition zone and stained similar to 7 dpf larvae. In contrast to larvae, goblet cell counts from adult gut tissue were significantly increased in shank3abΔC+/− and shank3abΔC−/− mutant as compared to...
Larval Shank3abΔC<sup>+/−</sup> and Shank3abΔC<sup>−/−</sup> DTs contain fewer serotonin (5-HT+) cells

To determine whether impaired gut motility was due to deficits in the ENS or enteroendocrine cells (EECs), Shank3abΔC mutants and WT larvae were immunostained for the pan-neuronal marker HuC and 5-HT (Fig. 7, WT, n = 15; Shank3abΔC<sup>−/−</sup>, n = 19; Shank3abΔC<sup>+/−</sup>, n = 9). Enteric neurons (HuC+), serotonin enteric neurons (HuC+/5-HT+), and enteroendocrine (5-HT+) cell bodies from representative anterior and posterior regions (Fig. 7a, (i and ii)) were counted using ImageJ and confocal Z-stacks. There was no significant decrease in enteric neurons along the intestinal tract of Shank3abΔC mutants compared to WT larvae. However, there was a significant decrease in serotonin-positive EECs in the intestinal tract of Shank3abΔC<sup>+/−</sup> (anterior, p = 0.0128; posterior, p = 0.0003) and Shank3abΔC<sup>−/−</sup> (anterior, p = 0.0013; posterior p < 0.0001) larvae compared to WT. EECs are mechanosensitive cells that release serotonin in response to mechanical and/or chemical stimulation, which in turn causes the intestine to increase mucus secretion and peristalsis, both of which facilitate transit [66].

**Discussion**

**Zebrafish as a model system for GI distress in ASD**

Our studies are the first to establish DT dysmotility as a robust phenotype in any SHANK3 mutant animal model of ASD. The developmental accessibility and transparency of the zebrafish model system allows highly quantitative assays to be carried out in an intact animal [40, 46]. This type of systems-level analysis is important because GI motility is coordinately regulated by diverse neuronal tissues [67]. For example, the central nervous

![Figure 7](image-url)
system (CNS) and parasympathetic ganglia increase GI motility in anticipation of the arrival of food; the vagus nerve releases acetylcholine to promote stomach emptying [53–56]; and coordinated regulation by the enteric nervous system [36, 68–70], enterochromaffin cells [71, 72], interstitial cells of Cajal [73], and microbiome [74–76] all help to promote motility and digestion. In the zebrafish larva, it is possible to quantify this coordinated regulation as food passes through the DT tract. Our analyses of DT function in WT and shank3abΔC zebrafish reveal hypomotility as a phenotype with likely parallels to GI distress described in individuals with PMS. Based on our findings, our working hypothesis is that mutations in shank3ab disrupt EEC regulation of DT motility.

**Zebrafish Shank3ab proteins show similar isoform complexity and post-synaptic density enrichment to that seen in mammals**

Zebrafish and mouse both express multiple Shank3 isoforms in the brain. Previous studies of the mouse Shank3 gene have identified ten to twelve Shank3 isoforms arising from five transcriptional start sites as well as alternative splicing [62, 77]. While zebrafish shank3ab genes have also been shown to encode at least six isoforms [21], these likely represent an underestimate based on our western data. Despite using an antibody targeting the C-terminus of Shank3 that would only recognize three of the six previously characterized splice-variants, our western identifies five abundant and additional, less abundant Shank3 isoforms that range in size from 75 to 250 kD. These bands are absent in brain-specific PSD extracts from shank3abΔC +/- animals, showing that these bands represent specific Shank3ab protein products. This antibody recognizes residues downstream of the induced mutations; therefore, it is possible that there is residual Shank3 protein in our zebrafish shank3abΔC +/- mutants. Future experiments are needed to assess the full complement of protein products produced by shank3ab genes as they relate to different phenotypes associated with the shank3abΔC mutant genotype.

Immunohistochemistry of larval cerebellar tissue shows that zebrafish Shank3ab proteins co-localize with PSD-95 at excitatory post-synaptic densities, as has been reported previously in mammalian cultured hippocampal neurons [78]. The lack of Shank3ab puncta in shank3abΔC mutants shows that the mammalian Shank3 antibody also specifically recognizes zebrafish Shank3ab in tissue. Taken together, these findings support that there is functional conservation between zebrafish and mammalian Shank3 proteins.

**Mutations in shank3ab result in DT hypomotility**

Functional comparisons of WT and shank3abΔC +/- mutant larvae show significantly reduced DT peristaltic frequencies with correspondingly prolonged DT transit times. It is important to note that our assay did not test appetite or potential differences in jaw shape that might impact their ability to forage. Comparisons of overall size between WT and shank3abΔC mutant larvae found no significant differences (data not shown), but further study of aspects such as jaw size/shape and potential differences in appetite should be investigated in future research. Although we saw no signs of regurgitation in larval shank3abΔC mutants, fluorescent microspheres remained in the intestinal bulb for prolonged periods of time, and in a small number of cases, fluorescent microspheres moved back into the pharynx in shank3abΔC +/- individuals, a behavior not seen in the WT larvae. Such prolonged transit times and longer dwell times in anterior DT in shank3abΔC +/- larvae are consistent with reflux and vomiting in individuals with PMS [11, 12] and phenotypes reported in a zebrafish chd7 mutant model of CHARGE syndrome [79].

**Hypomotility in shank3abΔC +/- larvae can be partially rescued by human SHANK3 mRNA**

Prolonged dwell times in the intestinal bulb of 7-day-old shank3abΔC +/- larvae could be partially rescued by injecting human SHANK3 mRNA at the one cell stage. Human SHANK3 RNA specifically rescued transit from the intestinal bulb (analogous to the mammalian stomach) to the upper intestine but not overall intestinal motility. Unlike endogenous transcripts, RNAs injected at fertilization are expressed transiently, for approximately 48 h, throughout the embryo [80]. Therefore, such a manipulation would likely rescue early shank3ab-dependent processes like hindbrain morphogenesis [20], but perhaps not later shank3ab-dependent processes like ENS migration, EEC differentiation, growth and function, and general epithelial maintenance.

**Structurally intact DT tissue in shank3ab mutant larvae contains fewer serotonin-expressing enteroendocrine cells**

Histological analysis of shank3abΔC +/- and WT larval DTs reveals normal tissue structure in mutants. Despite the fact that inflammation and leaky gut have been implicated in ASD-linked GI distress [81, 82], at least at larval stages, shank3abΔC +/- zebrafish exhibited an intact columnar epithelium and similar numbers of goblet cells to their WT counterparts and to the literature. Likewise, analysis of the larval ENS showed similar numbers of enteric neurons in WT and shank3abΔC mutants.

By staining for serotonin in the DT, we showed that EECs are significantly reduced in both shank3abΔC +/- and shank3abΔC +/- larvae. EECs in the gut synthesize 95% of the body’s serotonin and, during digestion,
serotonin release helps to coordinate secretion and motility as well as satiety and pain sensation [83, 84]. RNA-seq analysis from cells dissociated and sorted from 6 dpf WT larvae show shank3a and shank3b are detectable in EECs as well other intestinal epithelial cells, with shank3a expressed at higher levels than shank3b (personal communication from Lihua Ye, Roger A. Liddle, and John F. Rawls, Duke University). Therefore, Shank3 dosage-dependent reductions in DT EECs could help to explain dysmotility phenotypes. Future experiments are required to establish a causal relationship between EEC reductions and DT dysmotility phenotypes. Additionally, future studies will need to explore other cell types, such as interstitial cells of Cajal and smooth muscle cells, as defects in these may also explain the observed motility phenotype.

Unlike larval shank3abΔC +/-, DTs from both shank3abΔC +/- and shank3abΔC +/− adults show significantly more goblet cells. Such a goblet cell expansion could result from disrupting Delta-Notch signaling and/or inflammation. In mammals, zebrafish goblet cell differentiation (and DT epithelial maintenance in general) is reliant on Delta-Notch signaling [85, 86]; therefore, it is possible that Shank3ab normally interacts with Delta-Notch signaling in a way that is pivotal to GI differentiation and maintenance. Recent research reports such interactions between ascl1a (a transcription factor important for neuronal commitment and differentiation) and Delta-Notch have drastic impacts on both motility and secretory cell expression [87]. An alternate possibility is that reduced motility feeds back on microbial communities to cause inflammation [88]. Zebrafish serve as an excellent model system for studying inflammation, and it has been observed that such inflammation can cause a dramatic expansion of goblet cells [89]. Importantly, inflammation is reported to occur in ASD, and the potential impact it might have on patients is not yet known [90]. Distinguishing between these possibilities and expanding on their potential impact is an area of interest for future studies.

Conclusions
The shank3abΔC mutant zebrafish generated herein mimics clinically relevant mutations shown previously to cause Phelan-McDermid syndrome. Westerns and immunohistochemistry show zebrafish Shank3 to be functionally conserved with mammalian Shank3 in terms of isoform complexity and enrichment at post-synaptic sites in the cerebellum, validating the zebrafish model system. Shank3abΔC mutant zebrafish show reduced DT peristalsis, increased digestive transit time, and reductions in EECs consistent with reduced GI functionality and symptoms of GI distress reported in people with Phelan-McDermid syndrome. Prolonged passage time in shank3abΔC mutants can be rescued by human Shank3 mRNA establishing reduced Shank3 in the zebrafish model system as causal for GI dysmotility. This zebrafish model system provides a basis for studying the functional relationships between the brain, gut, and microbiome as these relate to GI distress common in Phelan-McDermid syndrome.

Additional files

**Additional file 1: Figure S1.** Insertion mutations in zebrafish shank3 orthologues produce unique restriction maps for genotyping. **Figure S2.** A Transverse 5 μm section of adult WT upper intestinal tissue stained with alcian blue and Eosin B (n = 6 for WT, n = 6 for shank3abΔC +/- and n = 6 for shank3abΔC −/−). b. 40x magnification shows dense plicae that extend to the point of nearly occluding the luminal space. c. d In shank3abΔC upper intestinal tissue, increased counts of goblet cells (black arrowheads) suggest inflammation. e. −/−/−/− adults also show increased goblet cell count. **Table S1.** Oligonucleotides used for sgRNA synthesis. **Table S2.** Primers used for PCR andanger sequencing. **Table S3.** % bead occupancy in WT and shank3abΔC +/- larvae at 3 h post-feed. **Table S4.** % bead occupancy in WT and shank3abΔC +/- larvae at 6 h post-feed. **Table S5.** % bead occupancy in WT and shank3abΔC +/- larvae at 12 h post-feed. **Table S6.** % bead occupancy in WT and shank3abΔC +/- larvae at 24 h post-feed. **Table S7.** Upper and lower GI rate of periodic period (seconds between contractions). **Table S8.** R script for Permutation. **Table S9.** Shank3abΔC +/- injected with either Human Shank3 mRNA short (32T) or long (5 T) isoform, 3–24 h post feed. (DOCX 4255 kb)

**Additional file 2: Movie S1.** Playback at 5 frames per second, each frame represents 1 z-stack capture per minute. This movie shows the abnormal mutant peristaltic process in a 7 dpf shank3abΔC −/− zebrafish. The fluorescent microspheres can be seen near the intestinal bulb-upper intestine junction; this was a common catch point and the mutant model seems to have the greatest difficulty moving food particles out of the intestinal bulb. Post processing was done to reduce size, change orientation of images, and speed playback up. (MP4 169887 kb)

**Additional file 3: Movie S2.** Playback at 5 frames per second, each frame represents 1 z-stack capture per minute. This movie shows “normal” wild type peristaltic movements of a 7 dpf zebrafish, highlighting the intestinal region starting at the intestinal bulb-upper intestine junction. Fluorescent microspheres can be seen moving posteriorly down the digestive tract. Post processing was done to reduce size, change orientation of images, and speed playback up. (MP4 23290 kb)

**Abbreviations**

- 5-HT: Serotonin
- ASD: Autism spectrum disorder
- CNS: Central nervous system
- DT: Digestive tract
- ENS: Enteric nervous system
- GI: Gastrointestinal
- PMS: Phelan-McDermid syndrome
- WT: Wild type

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37. Chen YC, et al. Zebrafish Ag2 is required for terminal differentiation of intestinal goblet cells. PLoS One. 2012;7(4):e34408.
38. Rich A, et al. Kit signaling is required for development of coordinated motility patterns in zebrafish gastrointestinal tract. Zebrafish. 2013;10(2):154-60.
39. Jordi J, et al. A high-throughput assay for quantifying appetite and digestive dynamics. Am J Phys Regul Integr Comp Physiol. 2015;309(4):R345–57.
40. Zhao X, Pack M. Modeling intestinal disorders using zebrafish. Methods Cell Biol. 2017;138:241–70.
41. Holmberg A, et al. Ontogeny of the gut motility control system in zebrafish Danio rerio embryos and larvae. J Exp Biol. 2004;207(Pt 23):4085–94.
42. Olsson C, Holmberg A, Holmgren S. Development of enteric and vagal innervation of the zebrafish (Danio rerio) gut. J Comp Neurol. 2008;508(5):756–70.
43. Holmberg A, Olsson C, Holmgren S. The effects of endogenous and exogenous nitric oxide on gut motility in zebrafish Danio rerio embryos and larvae. J Exp Biol. 2006;209(Pt 1):2472–9.
44. Wallace KN, et al. Intestinal growth and differentiation in zebrafish. Mech Dev. 2005;122(2):157–73.
45. Wallace KN, Pack M. Unique and conserved aspects of gut development in zebrafish. Dev Biol. 2003;255(1):12–29.
46. Ganz J, Melancon E, Eisen JS. Zebrafish as a model for understanding enteric nervous system interactions in the developing intestinal tract. Methods Cell Biol. 2016;134:139–64.
47. Yanez J, et al. Gustatory and general visceral centers and their connections in the brain of adult zebrafish: a carbocyanine dye tract-tracing study. J Comp Neurol. 2017;525(3):333–62.
48. Jiang Q, et al. Functional loss of semaphorin 3C and/or semaphorin 3D and their epistatic interaction with ret are critical to Hirschsprung disease. Neurobiol Dis. 2013;60:139–57.
49. Wallace P, et al. Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. Neuron. 1999;23(3):583–92.
50. Ng AN, et al. Formation of the digestive system in zebrafish: III. Intestinal epithelium morphogenesis. Dev Biol. 2005;286(1):114–35.
51. Linan-Rico A, et al. Mechanosensory signaling in enterochromaffin cells and S-HT release: potential implications for gut inflammation. Front Neurosci. 2016;10:564.
52. Hottmann G, Talley NJ. The stomach-brain axis. Best Pract Res Clin Gastroenterol. 2014;28(6):967–79.
53. Gronenberg D, Vouwen B, Friebie A. Integrative control of gastrointestinal motility by nitric oxide. Curr Med Chem. 2016;23(24):2715–35.
54. Grundy D, et al. Fundamentals of neurogastroenterology: basic science. Gastroenterology. 2006;130(5):1391–411.
55. Holmberg A, Olsson C, Henning GW. TTX-sensitive and TTX-insensitive control of spontaneous gut motility in the developing zebrafish (Danio rerio) larvae. J Exp Biol. 2007;210(Pt 6):1084–91.
56. Browning KN. Role of central vagal 5-HT3 receptors in gastrointestinal physiology and pathophysiology. Front Neurosci. 2015;9:413.
57. Belloso NW, et al. Enterochromaffin cells are gut chemosensors that couple to sensory neural pathways. Cell. 2017;170(1):185–198.e16.
58. Uyttendaele L, et al. The zebrafish mutant lesser: an experimental model for congenital enteric neuropathies. Neurogastroenterol Motil. 2016;28(3):345–57.
59. Miles TJ, et al. Host gut motility promotes competitive exclusion within a model intestinal microbiota. PLoS Biol. 2016;14(7):e002517.
60. Parracho HM, et al. Differences between the gut microbiota of children with autism spectrum disorders and that of healthy children. J Med Microbiol. 2005;54(Pt 10):987–91.
61. Yano JM, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell. 2015;161(2):264–76.
62. Waga C, et al. Identification of two novel Shank3 transcripts in the developing mouse neocortex. J Neurochem. 2014;128(2):280–93.
63. Qualmann B, et al. Linkage of the actin cytoskeleton to the postsynaptic density via direct interactions of Abp1 with the ProSAP/Shank family. J Neurosci. 2004;24(10):2481–95.
64. Cloney S, et al. Ectopic and functional validation of gastrointestinal motility dysfunction in a zebrafish model of CHARGE syndrome. FEBS J. 2018;285(1):2125–2140.
65. Hyatt TM, Eikker SC. Vectors and techniques for ectopic gene expression in zebrafish. Methods Cell Biol. 1999;59:17–26.
66. Samsom M, Ahangari R, Naser SA. Pathophysiology of autism spectrum disorders: revisiting gastrointestinal involvement and immune imbalance. World J Gastroenterol. 2014;20(29):9942–51.
67. de Magistris L, et al. Alterations of the intestinal barrier in patients with autism spectrum disorders and in their first-degree relatives. J Pediatr Gastroenterol Nutr. 2010;51(4):418–24.
68. Rolig AS, et al. The enteric nervous system promotes intestinal health by constraining microbiota composition. PLoS Biol. 2017;15(2):e2000698.
69. Brugman S. The zebrafish as a model to study intestinal inflammation. Dev Comp Immunol. 2016;64:82–92.
70. Ashwood P, et al. Intestinal lymphocyte populations in children with regressive autism: evidence for extensive mucosal immunopathology. J Clin Immunol. 2003;23(5):504–17.