Full Title:
Dysregulated phosphatidylinositol signaling promotes endoplasmic reticulum stress-mediated intestinal mucosal injury and inflammation in zebrafish

Running Title:
PI metabolism, ER stress and inflammation

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Abbreviations Used in Manuscript:
Cdipt: CDP-diacylglycerol--inositol 3-phosphatidylinosyltransferase
dpf: days post-fertilization
ER: endoplasmic reticulum
GC: goblet cell
GFP: green fluorescent protein
eGFP: enhanced green fluorescent protein
GI: gastrointestinal
IBD: inflammatory bowel disease
PI: phosphatidylinositol
PI3-K: phosphoinositide-3-kinase
PIS: phosphatidylinositol synthase
SUMMARY

Dysregulated phosphoinositide (PI) signaling has been implicated in human gastrointestinal (GI) malignancies and inflammatory states, underlining the need to study pathophysiological roles of PI in an in vivo genetic model. Here, we study the significance of PI in GI pathophysiology using the zebrafish mutant cdip\textsuperscript{hi559}, which lacks PI synthesis, and unravel a critical role of PI in intestinal mucosal integrity and inflammation.

The cdip\textsuperscript{hi559} mutants exhibit abnormal villous architecture and disorganized proliferation of intestinal epithelial cells (IECs), with pathologies reminiscent of inflammatory bowel disease (IBD), including apoptosis of goblet cells, abnormal mucosecretion, bacterial overgrowth, and leukocyte infiltration. The mutant IECs exhibit vacuolation, microvillus atrophy and impaired proliferation. The cdip\textsuperscript{hi559} gene expression profile shows enrichment of acute phase response signaling and the endoplasmic reticulum (ER) stress factors, hspa5 and xbp1, are robustly activated in the mutant GI tissue. Temporal electron micrographic analyses reveal that PI-deficient IECs undergo sequential ER-Golgi disruption, mitochondrial depletion, macroautophagy, and cell death, consistent with chronic ER stress-mediated cytopathology. Furthermore, pharmacological induction of ER stress by inhibiting protein glycosylation or PI synthase inhibition in leukocyte-specific reporter lines replicates the cdip\textsuperscript{hi559} inflammatory phenotype, suggesting a fundamental role of PI metabolism and ER stress in mucosal inflammation.

Antibiotics and anti-inflammatory drugs resolved the inflammation, but not the autophagic necroapoptosis of IECs, suggesting that bacterial overgrowth may exacerbate
ER stress pathology, whereas persistent ER stress is sufficient to trigger inflammation. Interestingly, the intestinal phenotype was partially alleviated by chemical chaperones, suggesting their therapeutic potential. Using zebrafish genetic and pharmacological models, this study demonstrates a novel link between intracellular PI signaling and ER stress-mediated mucosal inflammation. The zebrafish Cdipt mutants provide a powerful tool to dissect the fundamental mechanisms of ER stress-mediated human GI diseases and a platform to develop molecularly targeted therapies.

INTRODUCTION

Intestinal epithelial cells (IECs) play a major role in mucosal homeostasis, barrier function, and immunity in addition to their digestive functions. Physiological stress to the IECs affects intestinal mucosal integrity, making the host susceptible to various gastrointestinal (GI) diseases. Epithelial disruption is a hallmark pathological feature of GI inflammatory disorders, particularly inflammatory bowel diseases (IBD) and necrotizing enterocolitis (NEC) (Xavier and Podolsky, 2007; Abraham and Cho, 2009; Henry and Moss, 2009).

Endoplasmic reticulum (ER) stress leading to epithelial dysfunction is believed to contribute to GI inflammation (Kaser and Blumberg, 2009; Hotamisligil, 2010; Kaser and Blumberg, 2010). ER stress results from perturbation of ER homeostasis through a multitude of factors and triggers a conserved adaptive response, termed the unfolded protein response (UPR) or ER stress response (ERSR) (Ron and Walter, 2007). The UPR helps in protein folding capacity or ER-associated degradation (ERAD) of misfolded proteins to resolve the ER stress. However, chronic or unresolved ER stress, causing prolonged activation of UPR can exacerbate the pathology of various human diseases.
(Lin et al., 2008). Recent studies on animal models of IBD have provided links between ERSR factors and GI inflammation. Mice lacking a critical ERSR mediator, XBP-1, show cell-specific ER stress in the epithelium and develop spontaneous enteritis (Kaser et al., 2008). In humans, polymorphisms within the \textit{XBP1} locus confer an increased risk for both Crohn’s disease (CD) and ulcerative colitis (UC) (Kaser et al., 2008). In murine models, ER stress-mediated goblet cell (GC) depletion is implicated in the pathogenesis of UC (Heazlewood et al., 2008). Differential expression of the proximal ER stress sensor \textit{HSPA5} is reported in human IBD tissues (Bogaert et al., 2011), and ER stress is hypothesized to activate pro-inflammatory signals through multiple mechanisms (Deng et al., 2004; Hu et al., 2006; Yamazaki et al., 2009). However, the precise molecular pathways leading to ER stress and the pathophysiological roles of various ERSR components in mucosal inflammation are largely unknown, necessitating the development of novel animal models to unravel these mechanisms. Furthermore, only a limited percentage of the estimated genetic heritability of IBD is explained by known genetic determinants identified by genome-wide association studies (Franke et al., 2010; Anderson et al., 2011), indicating the importance of finding new animal models to delineate specific genes and pathways that may contribute to IBD pathogenesis.

The zebrafish, \textit{Danio rerio}, has been an effective tool to decipher mechanisms of human GI diseases, due to the similarity in basic GI tissue structure, function, and gene expression profiles (Stuckenholz et al., 2005; Stuckenholz et al., 2009). The zebrafish intestine is fully developed and becomes functional by 5-dpf, displaying a villous architecture with easily identifiable enterocytes, enteroendocrine cells, and the mucin-secreting GCs (Wallace and Pack, 2003; Ng et al., 2005; Stuckenholz et al., 2005).
Zebrafish IECs secrete defensins and other antimicrobial peptides, and the IBD susceptibility genes *nod1* and *nod2* have been shown to maintain conserved antimicrobial roles in the zebrafish intestine (Oehlers et al., 2011a; Oehlers et al., 2011b). Chemical enterocolitis models in the zebrafish have shown that features of IBD seen in murine models can be rapidly recapitulated in larval zebrafish, emphasizing its utility to study IBD pathogenesis (Fleming et al., 2010). In addition, larval zebrafish models are being utilized to analyze interactions between the commensal microbiota and host innate immunity, providing insights into role of bacteria and inflammation in human IBD (Kanther et al., 2011; Roeselers et al., 2011).

Phosphatidylinositol (PI) signaling has been linked to a variety of human diseases and cancer. PI is a critical phospholipid synthesized in the ER and in highly dynamic ER-derived compartments. PI is rapidly metabolized and its levels are tightly controlled in the cell to exert its spatiotemporal intracellular signaling functions (Balla et al., 2009; Kim et al., 2011). Phosphorylated PIs (PIPs) are believed to be the regulators of vesicular transport and secretory pathways. We have previously shown by transcriptome profiling that inositol metabolism and PI3-K pathways are enriched during zebrafish GI development and inhibition of PI3-K signaling results in GI developmental defects (Stuckenholz et al., 2009). To further define the pathophysiological significance of intracellular PI, we identified and characterized the zebrafish insertional mutant *cdipthi559* (*hi559*), which is defective in PI synthesis. Cdipt is a highly conserved enzyme with its active site on the cytoplasmic face of the ER and is responsible for synthesis of intracellular PI from myo-inositol and CDP-diacylglycerol. PI synthesis has been suggested to occur in a dynamic domain of the ER positioned at the leading edge of the
ER tubules (English and Voeltz, 2013). Our earlier studies characterizing the hi559 mutation showed that the lack of de novo PI synthesis leads to ER stress and hepatic steatosis (Thakur et al., 2011). Despite the ubiquitous need for PI, the hi559 mutation does not cause a broad, general developmental defect. The enrichment of Cdip expression in the larval GI tissues accounts for the strong GI defects in hi559 larvae due to functional loss of Cdip and consequent deficiency of de novo PI synthesis, in spite of maternally deposited PI in the yolk. Given the transient roles and dynamics of PI metabolism, it is conceivable that Cdip-controlled de novo PI synthesis is crucial for intracellular availability of PIPs, such as PI(4)P and PI(4,5)P2 to exert their secretory functions.

Cdip mutant zebrafish develop consistent GI defects during late larval stages after tissue differentiation, exhibiting a complex pathology in the intestine: abnormal IEC proliferation and apoptosis, villous atrophy, GC depletion, bacterial overgrowth and inflammation, all of which are hallmarks of human IBD. The hi559 IECs show disruption of ER architecture followed by mitochondrial defects and increased autophagy and cell death, consistent with ER stress-induced cytopathology. Pharmacological induction of ER stress in wild-type larvae results in similar inflammatory pathologies, suggesting a contributory role of aberrant PI synthesis in ER stress-mediated GI inflammation. In addition, akin to IBD treatment strategies, the mutant phenotype is partially ameliorated by antibiotic and anti-inflammatory drugs. This highlights the utility of this system as a tool to study the pathogenesis of ER stress and mucosal inflammation. These studies facilitate novel insights into the mechanistic relationships between intracellular PI signaling, ER stress, and GI pathophysiology in a whole-organism in vivo setting.
RESULTS

Loss of PI synthase causes defects in intestinal architecture

The \textit{hi559} homozygous mutant lacks Cdipt expression due to a retroviral insertion within the \textit{cdipt} gene. Larvae develop normally until 5-dpf, when they begin to exhibit hepatic defects, including hepatomegaly and steatosis (Thakur et al., 2011). Another striking feature of the mutant is a smaller intestine by 5-dpf (Fig. 1A). Analyses of \textit{hi559} larvae expressing GFP in the gut [\textit{hi559Tg(gut:gfp)}] confirmed that the \textit{hi559} mutation is fully penetrant, consistently presenting with a significantly smaller intestine at 5-dpf ($P<0.001$, Fig. 1E). Homozygous mutant larvae die at larval stages between 6.5- and 7-dpf. To prove that loss of Cdipt and its PI synthesis function underlies the mutant GI phenotype, we showed that knockdown of Cdipt by morpholino injection into wild-type embryos or chemical inhibition of PI synthesis in wild-type larvae by $\delta$-HCH replicates the \textit{hi559} phenotype (Supplementary Fig. S1E-G) (Thakur et al., 2011). In addition, larvae with a weaker Cdipt mutant allele, \textit{cdipt}\textsuperscript{lop} (\textit{lop}) (Murphy et al., 2011), which carries a point mutation in the PI synthase (PIS) domain, also replicate the \textit{hi559} GI phenotype (Supplementary Fig. S1C), but they exhibit a milder, delayed phenotype. They develop normally until 7-dpf, subsequently exhibiting similar gross and histological intestinal abnormalities as seen in \textit{hi559} larvae and dying at about 10-dpf. Both \textit{hi559} and \textit{lop} fail to rescue each other in a complementation assay (Supplementary Fig. S1D), supporting the conclusion that \textit{lop} is a hypomorphic allele of \textit{hi559}. In homozygous \textit{hi559} and \textit{lop} mutants, Cdipt function is eliminated, resulting in abrogation of de novo PI synthesis (Murphy et al., 2011; Thakur et al., 2011). The similarity of the intestinal abnormalities in both \textit{lop} and \textit{hi559} larvae suggest that the mutant phenotype is not primarily an early
developmental defect, but reflects a requirement for de novo PI in intestinal function at later larval stages. Since *hi559* mutants offer the advantage of an earlier and consistently stronger GI phenotype, we used them to elucidate the role of PI in GI tissues.

The hypomorphic nature of *hi559* intestine is evident by whole-mount staining and histology (Fig. 1A-G, Supplementary Fig. S1). Incorporation of Nile-Red is diminished in the mutants, showing decreased luminal volume (Fig. 1C, Supplementary Fig. S1). The intestinal epithelial structure is reduced in size, as demonstrated by Cy3-SA immunostaining (Fig. 1D), suggesting that the hypomorphic intestinal manifests itself in both smaller lumen and thinner epithelium. To analyze developmental abnormalities in the intestine, we characterized the *hi559* larvae by whole-mount ISH using RNA probes against the intestine-specific markers *fabp2*, *vill* and *anxa2b* (Fig. 1B, Supplementary Fig. S1A). The observed decrease of marker gene expression suggests loss of structural and functional components of the *hi559* intestine by 5-dpf. There is no difference in expression of intestinal markers or Nile-Red staining until 4-dpf in *hi559* compared to wild-type (data not shown), suggesting no gross physical defects during early intestinal development. Intestinal expression of *cdipt* in wild-type lavae (Thakur et al., 2011) and the intestinal defects of *hi559* and *lop* larvae (Fig. 1 & Supplementary Fig. S1) implicate an important role of PI synthase in intestinal integrity and function.

**The hi559 intestine exhibits abnormal villous architecture and mucosal cells**

Histologically, the villous and luminal architecture is disrupted in the *hi559* intestine (Fig. 1F-G). In wild-type larvae, columnar IECs are well polarized, forming a continuous epithelial monolayer with villi. In contrast, *hi559* IECs are disorganized, less columnar with incomplete cytoplasmic maturity, and sporadically detach from the epithelium into
the lumen (Fig. 1F-G and Fig. 2A). The intermittently detached IECs have nuclear condensation, suggesting apoptosis (Supplementary Fig. S2A). TEM analysis of the 5.5-dpf intestinal mucosa demonstrates that the wild-type IECs exhibit a highly elaborate apical brush border with microvilli projecting into the lumen, whereas the hi559 IECs have enlarged cytoplasmic vesicles, abnormal brush border, reduced terminal web and microvillus atrophy (Fig. 2B). The hi559 intestinal lumen is consistently filled with basophilic plaques (Fig. 2A), which TEM and colony formation assays confirm to be largely due to increased bacterial colonization ($P=0.0027$, Fig. 2C).

Mucin-secreting GCs in the esophageal and mid-intestinal regions are typically evident in zebrafish larvae by 5-dpf. In hi559, these cells appear abnormal with pyknotic or fragmented nuclei, suggesting apoptosis (Fig. 2A). We used PAS staining and TEM to analyze these GCs. In wild-type intestine, a thick secreted mucinous layer is consistently seen covering the apical border of the epithelium, which is diminished in the hi559 GI tract, suggesting alteration of GC physiology and their secretory function (Fig. 2D-E). Ultrastructurally, the 5-dpf wild-type GCs show mature theca containing large mucinous vacuoles. In hi559, these appear immature and degenerated (Supplementary Fig. S2B). Interestingly, there is no difference in numbers of GCs at 5-dpf ($P=0.667$), supporting normal IEC differentiation. However, the population of esophageal GCs declined by 6-dpf ($P=0.0456$, Fig. 2D-F), due to apoptosis and detachment. Mid-intestinal GCs and mucus secretion are similarly depleted in hi559 ($P=0.0489$, Supplementary Fig. S2C-E). Concomitant with GC depletion, nearly all hi559 larvae (>90%) showed histological features of bacterial overgrowth in the intestine by 6-dpf ($P=0.002$, Fig. 2F).
**Abnormal proliferation and apoptosis of the hi559 IECs**

We studied the fate of the IECs by analyzing their cell-cycle status by BrdU-incorporation and TUNEL assays. In the wild-type intestine, BrdU-positive cells occur frequently, typically at the base of the epithelial villi; TUNEL-positive cells are rare (Fig. 3A-B). Although BrdU-labeling and TUNEL assays did not reveal abnormal cell proliferation or apoptosis in the hi559 GI tract prior to 5-dpf, IEC proliferation is significantly reduced and disorganized at 5-dpf (P=0.006, Fig. 3A & C) and the frequency of apoptotic cells increases as the hi559 intestinal pathology worsens by 6-dpf (P=0.0003, Fig. 3B-C), resulting in focal ulceration of the intestinal epithelium. TUNEL assays confirm that the GCs with pyknotic nuclei observed in the hi559 GI tract are predominantly undergoing apoptosis, which may account for their depletion (Fig. 3B-C). In the disorganized epithelial region, IECs are often largely vacuolated (Supplementary Fig. S3A). These vacuoles do not show PAS or Oil-Red-O staining, suggesting that they are neither mucinous nor steatotic (data not shown). Taken together, we conclude that PI deficiency impedes proliferation and induces apoptosis of IECs, causing villous atrophy and intestinal hypoplasia.

**PI deficiency causes mucosal pathology and inflammation with IBD-like features**

During larval development, the hi559 intestine exhibits increasing bacterial overgrowth coinciding with depletion of the GCs (Fig. 2F). Since loss of mucinous secretions and aberrant bacterial growth in the gut can cause spontaneous inflammation, as seen in IBD, we assayed intestinal inflammation in hi559 larvae. Onset of an inflammatory response is evident at 5.5- to 6-dpf, as ISH demonstrates infiltration of the epithelium with mpo-positive neutrophils and spi1-positive macrophages (Fig. 3D). Necro-inflammatory injury
is evident histologically by 6-dpf (Supplementary Fig. S3A). To quantify leukocyte infiltration, we utilized the leukocyte-specific reporter line $Tg(lyzc:egfp)$. Analysis of $hi559 Tg(lyzc:egfp)$ mutant larvae reveals significantly higher leukocyte aggregation in the 6-dpf intestine compared to wild-type $Tg(lyzc:egfp)$ ($P=0.008$, Fig. 3E). Interestingly, pharmacological inhibition of PI synthesis by the chemical inhibitor $\delta$-HCH in wild-type larvae replicates the increased intestinal leukocyte aggregation similar to $hi559$ ($P=0.031$, Supplementary Fig. S3B), further substantiating that deficient PI synthesis leads to mucosal inflammation.

Pathway analysis of $hi559$ gene expression identified acute phase response (APR) signaling as the most significantly upregulated canonical pathway, suggesting activated transcription of pro-inflammatory factors ($P=0.002$, Fig. 3F). In addition to the complement cascade pro-inflammatory factors, interleukin (IL-6, IL-8 and IL-17) signaling and NF-$\kappa$B signaling were among the most significantly dysregulated gene sets, suggesting that pro-inflammatory activity may be mediated via these pathways ($P\leq 0.01$, Fig. 3F and Supplementary Fig. S4A-B).

**Cdipt-deficient zebrafish exhibit unresolved ER stress and macroautophagy in IECs**

Since our microarray-based pathway analyses revealed enrichment of ERSR gene sets in $hi559$ larvae (Thakur et al., 2011), we further investigated the nature of ER stress pathology in the GI tract. Unresolved ER stress is typically marked by persistent UPR induction and subsequent disruption of ER architecture and function. HSPA5 (also known as GRP78) is a heat shock protein that chaperones proteins in the ER lumen and is upregulated in response to ER stress (Marciniak and Ron, 2006), and $XBP1$ mRNA splicing is a key marker of ER stress and UPR activation (Calfon et al., 2002). The
expression of proximal ERSR sensors *hspa5* and *xbp1* is robustly elevated in the *hi559* GI tract as seen by ISH (Supplementary Fig. S5A-B) (Thakur et al., 2011). In addition, splicing of *xbp1* is evident in the micro-dissected GI tissue of *hi559* larvae by RT-PCR (Supplementary Fig. S5C). The elevation of both unspliced and spliced transcripts of *xbp1* indicate that the *hi559* GI tissue is experiencing ER stress. To further clarify the ER stress within tissues, we performed immunohistochemistry to detect active Hspa5 protein. Robust enrichment of Hspa5 protein is seen within *hi559* GI tissues, specifically in the mucin secreting GCs and subsequently in the IECs of the intestinal mucosa (Fig. 4A).

Since, these ER stress UPR factors are associated with molecular pathogenesis of IBD (Kaser et al., 2008; Bogaert et al., 2011), we wanted to further analyze the temporal ultrastructural defects within *hi559* GI tissues to dissect the sequence of ER stress-mediated pathology at cellular level. We performed extensive TEM analysis of the intestinal mucosa at different stages of the phenotype (Fig. 4B-D). At 5-dpf, the most striking defect in *hi559* IECs is a disruption of the ER-Golgi architecture, without any overt changes to other cellular components (Fig. 4B). Large double-membrane macroautophagic bodies (autophagosomes) causing focal cytoplasmic necrosis are evident by 5.5-dpf (Fig. 4B). At 6-dpf, the IECs show extensive mitophagy, depletion of mitochondria, and large multilamellar autophagosomes containing cytoplasmic organelles (Fig. 4C). The lumens of the distended ER-golgi compartments in the *hi559* IECs are often filled with aggregates of variable electron density, suggesting protein accumulation. Significantly increased autophagy, and loss of mitochondria may account for the large cytoplasmic vesicles of 6-dpf IECs (*P*≤0.003, Fig. 4E). ER stress-associated cytopathology and autophagic vesicles are also evident in the secretory enteroendocrine
cells (Fig. 4D). ER expansion and autophagy occur in both the pancreatic endocrine cells and the zymogen-rich acinar cells by 6-dpf, suggesting that the ER stress-induced pathology is subsequently propagated in the majority of the secretory cells of the digestive system (Supplementary Fig. S5D-E). Disrupted ER architecture, grossly expanded ER lumens and vacuolization, consequent mitochondrial damage, and autophagy are consistent with ER stress-induced cytopathology. These results demonstrate that unresolved ER stress in the highly secretory GI cells is the major etiology of the \textit{hi559} phenotype, implying that the lack of \textit{de novo} PI impedes secretory function, leading to pathological ER stress-induced GI defects in \textit{cdipt} mutants.

\textit{ER stress is causal and sufficient to the induction of GI inflammatory pathology}

Our analysis of \textit{hi559} mutants has not yet addressed the sequence of events leading to the overt phenotype: Is the ER stress a direct consequence of functional loss of Cdipt, or induced by another unrecognized process, such as inflammation? To help distinguish between these possibilities, we tested whether tunicamycin, a compound known to induce ER stress by inhibition of N-glycosylation, can cause GI inflammation. Chronic treatment of wild-type zebrafish with 1 µM tunicamycin from 3.5-dpf through 6-dpf results in a smaller intestine ($P=0.002$) and defects in intestinal architecture (Fig. 5A-C and Supplementary Fig. S6A). Interestingly, tunicamycin-treated larvae exhibit increased bacterial growth ($P=0.0006$), GC depletion ($P\leq0.02$), and increased intestinal macrophage and neutrophil infiltration ($P\leq0.006$, Fig. 5C-E and Supplementary Fig. S6B-D). Necro-inflammatory lesions containing large numbers of vacuolated or autophagic IECs in tunicamycin-treated \textit{Tg(lc3-gfp)} larval intestines are clearly evident ($P=0.005$, Fig. 5F and Supplementary Fig. S6C). These results clearly suggest that
chronic ER stress is sufficient to trigger necro-inflammatory injuries in the zebrafish intestine, leading to hi559-like GI pathology.

**Anti-inflammatory drugs and chemical chaperones ameliorate ER stress-induced GI inflammation**

Recruited macrophages and neutrophils are potent sources of cytokines and tissue destructive enzymes, contributing to necro-inflammatory injury by loss of IEC integrity. Anti-inflammatory agents, such as 5-aminosalicylic acid (5-ASA) and prednisolone, are thus widely used therapies for alleviating human inflammatory disorders, and remain an important option for treating patients presenting with moderate to severe IBD. In recent years, co-administration of antibiotics or probiotics with anti-inflammatory drugs is also being prescribed as an effective regimen for IBD treatment (Perencevich and Burakoff, 2006). To illustrate the similarity of cdip mutants to human inflammatory states and as an in vivo system in which to assay potential suppressors of the inflammatory pathology, we assessed the response of hi559 mutants to antibiotics and anti-inflammatory drugs. Treatment with antibiotics or anti-inflammatory drugs alone fails to rescue the hi559 intestinal phenotype and does not increase gut size ($P=0.72$ and 0.39 respectively), although leukocyte infiltration is effectively reduced ($P \leq 0.01$, Fig. 6E, Supplementary Fig. S7A-C). Co-administration of anti-inflammatory drugs 5-ASA and prednisolone together with antibiotics from 3.5 to 6-dpf results in marginal alleviation of hi559 intestinal size, as seen by ISH with fabp2, showing a minor increase in gut size when compared to the mutant ($P=0.051$, Fig. 6A, E), clearing of luminal bacterial plaques, and reduced intestinal leukocyte infiltration seen by decreased mpo expression and lyzc:egfp
punctates \( (P=0.0006, \text{Fig. } 6B-E) \). The similar response to anti-inflammatory agents in \textit{hi559} larvae and to anti-inflammatory treatment in humans in reducing leukocyte infiltration suggests a conserved mode of action. Intriguingly, the antibiotic and anti-inflammatory treatments do not significantly alleviate GCs, autophagy, and apoptosis of the \textit{hi559} intestinal mucosa \( (P=0.527, 0.59, 0.298 \text{ respectively, Fig. } 6C \& E) \), suggesting that ER stress is a precursor to bacterial overgrowth and inflammation. Subsequently, inflammation may then further exacerbate GI pathology. Hence, we hypothesize that inflammation is a downstream pathological event of ER stress and relieving ER stress in \textit{hi559} larvae would rescue the GI inflammation.

Chemical chaperones enhance the protein folding and adaptive capacity of the ER and thus act as a potent suppressor of ER stress. As a proof of principle, we investigated if the \textit{hi559} larvae are responsive to chemical chaperones that may alleviate ER stress. Phenylbutyric acid (PBA) is a small chemical chaperone and a well-established drug proven to reduce ER stress in both in vivo and in vitro studies (Ozcan et al., 2006). Prolonged exposure of \textit{hi559} larvae to 4-PBA ameliorates the intestinal phenotype showing significant increase in gut size \( (P=0.0006, \text{Fig. } 7A \& D) \), improvement in intestinal villous architecture (Fig. 7B), mitigation of inflammation with a significant reduction of intestinal leukocyte infiltration \( (P=0.0037, \text{Fig. } 7C-D) \), and increased survival of GCs \( (P=0.0031, \text{Fig. } 7D) \). Exposure of 4-PBA in \( \delta \)-HCH-treated \textit{Tg(lc3:gfp)} larvae shows reduction of IEC autophagosomes \( (P=0.0028, \text{Fig. } 7D) \), suggesting that alleviating ER stress may potentially reduce autophagy in PI-deficient larvae. Notably, the alleviation of \textit{hi559} GI phenotype by 4-PBA also results in improved survival of the mutant larvae \( (P=0.0008, \text{Fig. } 7E) \). Taken together, these results strongly support a
feedback model in which unresolved ER stress initially triggers inflammation, which in turn may further worsen ER stress, exacerbating the GI pathology.

**DISCUSSION**

Using zebrafish genetic and pharmacological models, this study demonstrates that intracellular PI synthesis plays a vital role in maintaining physiological homeostasis and integrity of intestinal mucosa. Analysis of hi559 mutants has proven that loss of Cdipt function abrogates de novo PI synthesis, resulting in multiple GI pathologies. The cdip mutant intestine fails to maintain its integrity and villous architecture as IECs exhibit reduced proliferation, reduced columnar shape, and degeneration at later larval stages. PI signaling has been linked to both cytokinesis and specification of apicobasal polarity in epithelial organs (Janetopoulos et al., 2005; Janetopoulos and Devreotes, 2006; Comer and Parent, 2007). As IECs are rapidly proliferating at larval stages (Fig. 3A), they may require increased levels of de novo PI synthesis. Thus, despite maternal deposits of PI within the embryo itself (Thakur et al., 2011), these PI pools may not satisfy the specific needs of IECs in the hi559 larval intestine, leading to aberrant cytological architecture and a decrease in proliferation.

Loss of de novo PI synthesis may impede various cellular functions, however our data suggest a primary role for secretory pathways in the development of hi559 phenotype. Highly secretory cells of the digestive system of Cdipt-deficient larvae exhibit ER stress-associated cytopathology, such as extensive disruption of the ER-Golgi complex and aggregates of electron-dense granules within the ER-Golgi lumen, suggesting protein accumulation consistent with pathological ER stress (Fig. 4). Furthermore, the observed
diminution of mucus secretory functions of GCs and the aberrant ER-Golgi architecture of enteroendocrine and pancreatic cells clearly implicate a secretory defect in the hi559 digestive system. A mutation in sec13, which is primarily involved in protein trafficking from ER to Golgi, causes similar ER disruption, cell-cycle arrest, and cell death phenotypes in zebrafish (Niu et al., 2012), thus substantiating the hypothesis that a secretory defect may be linked to the persistent ER stress pathology. Given the role of PI metabolism and its phosphorylated derivatives (PIPs) in vesicular trafficking at ER exit sites and within the secretory pathway (Hama et al., 1999; Blumental-Perry et al., 2006; Yakir-Tamang and Gerst, 2009), it is likely that the tissue-specific expression of cd ipt reflects a requirement for de novo intracellular PI pools by IECs to support the secretory function of intestinal cells, which may make them particularly vulnerable to ER stress. Therefore, specific intracellular PI signaling components may be intrinsic regulators of ER stress and UPR (Jesch et al., 2006), and we are pursuing their identification.

We observed abnormal cell death in the mutant intestine, predominantly of GCs by apoptosis. Interestingly, loss of GCs has been linked to human IBD pathogenesis (Xavier and Podolsky, 2007). Chronic ER stress and UPR can lead to cell death through different pathways, such as upregulation of apoptotic factors, including casp3 or ddit3/chop, which are found to be upregulated in hi559 larvae (Thakur et al., 2011), or via release of Ca²⁺ from the ER, which perturbs mitochondria and triggers oxidative stress-induced cell death and inflammation (Kim et al., 2008; Lin et al., 2008). Additionally, ER stress is linked to increased autophagy (Yorimitsu et al., 2006), which is clearly evident in hi559 GI cells. These mechanisms could plausibly explain the increased apoptosis,
mitochondrial damage and autophagy in hi559 GI cells, which collectively manifest in GC loss and intestinal dysfunction.

Increased inflammation in the hi559 intestine may be the result of at least two complementary mechanisms. Dysfunction and apoptosis of GCs and other IECs may result in reduced secretion of antimicrobial mucus and peptides. This loss would facilitate bacterial overgrowth resulting in an inflammatory response to the increased intraluminal microbial load. Separately, in hi559 larvae, we observe the reported ER-UPR dependent dysregulation of NF-κB and pro-inflammatory interleukin signaling (Fig. 3 & Supplementary Fig. S4), likely contributing to inflammation (Yamazaki et al., 2009; Kaser and Blumberg, 2010). This may set up a positive feedback loop, in which bacterial overgrowth causes upregulation in the synthesis of secreted antimicrobial peptides and mucus, thus adding to the stress of the ER-Golgi secretory complex, further straining the intestinal mucosa. Additional studies using a recently developed NF-κB reporter line (Kanther et al., 2011), reared in a gnotobiotic environment could serve as a tool to dissect the complex interplay of gut microbiota, NF-κB signaling, and ER stress in intestinal inflammation.

While inflammation itself may contribute to ER stress, multiple lines of evidence in our study support a model in which ER stress initially triggers the development of necro-inflammatory injury. Temporal analysis of the hi559 ultrastructural pathology showed that hallmarks of ER stress occur prior to the onset of gross intestinal inflammatory pathology. Furthermore, pharmacological induction of ER stress in wild-type larvae using tunicamycin resulted in apoptosis of intestinal GCs, bacterial overgrowth, and increased inflammation similar to hi559. Lastly, co-administration of antibiotics and anti-
inflammatory drugs suppressed bacterial overgrowth and mucosal inflammation, but
failed to alleviate the ER stress-associated autophagy and necro-apoptosis (Fig. 6).
Interestingly, treatment with the chemical chaperone 4-PBA, a known alleviator of ER
stress, shows amelioration of the mucosal inflammation and increased survival of hi559
larvae (Fig. 7). Collectively, these results strongly suggest that bacterial overgrowth and
inflammation do not directly cause ER stress, but result from it and exacerbate the ER
stress-induced pathology in our model.

This finding has important implications for the treatment of multiple human GI
diseases. It suggests that pharmacologic manipulation of the ER stress pathway may be a
novel treatment paradigm for particular GI diseases, such as IBD, and other disparate
diseases, including cancer, that have been linked with chronic inflammation.
Phospholipids are believed to have potential anti-inflammatory roles and can suppress
activation of pro-inflammatory cells in vivo. Phosphatidylserine has been shown to
inhibit macrophage activation (Gilbreath et al., 1985), and administration of
phosphatidylcholine prevented stricture formation in a rat model of colitis (Mourele et
al., 1996). Interestingly, the PI3-K subunit p110δ was shown to play a vital role in
maintaining mucosal homeostasis. Its inactivation caused defects in B-and T-cell
signaling and in bactericidal activity, resulting in chronic colitis in mouse (Uno et al.,
2010). Recently, PI itself has been shown to inhibit T-cell proliferation and function,
implicating it as a novel physiological immune suppressant (van Dieren et al., 2011). The
μ-opioid receptor ligand DALDA, a compound that may activate PI3-K signaling, has
been shown to protect glafenine-induced intestinal injury in zebrafish by ameliorating ER
stress (Goldsmith et al., 2013). Collectively, we hypothesize that PI may exert its anti-inflammatory function via its ability to alleviate pathological ER stress.

This study provides the first evidence linking PI synthase to ER stress-mediated GI pathologies, including bacterial overgrowth, mucosal apoptosis, and inflammation, that are reminiscent of human IBD. In addition to genes regulating the immune system, mutations in genes affecting epithelial ER stress and function have been associated with IBD risk factors (Khor et al., 2011). Since ER homeostasis and inflammatory pathways appear to be conserved between zebrafish and human, investigating mechanisms of ER stress in the zebrafish might reveal novel markers for IBD treatment. Currently, metabolically stabilized PI-derivative analogs and ER stress-modulating compounds are being tested for their physiological relevance (Kim et al., 2008; He et al., 2011). Within this context, the cdip mutants may provide an excellent platform for preclinical in vivo whole-organism studies evaluating the therapeutic potential of such compounds in ameliorating epithelial injury and inflammation.

MATERIALS AND METHODS

Zebrasfish lines, Embryo collection and Genotyping

The zebrafish line cdip hi559 was isolated from a large-scale insertional mutagenesis screen (Amsterdam et al., 2004). Heterozygous and homozygous mutants were sorted by genotyping using PCR (Thakur et al., 2011). The cdip lop mutant was isolated from a chemical mutagenesis screen (Murphy et al., 2011). The cdip hi559/lop trans-heterozygotes were generated by crossing hi559 heterozygotes with cdip lop. Fishes were maintained in accordance with the institutional animal care and use committee protocols.
Development of Tg(fabp2:egfp) transgenic zebrafish

We used the regulatory region of the zebrafish fatty acid binding protein 2, intestinal (fabp2) to generate a transgenic zebrafish line. A 1.2 kb upstream fragment of the fabp2 promoter was cloned into the plasmid vector pEGFP. The plasmids pEGFP-fabp2 and pTOL2 (pT2KxIG Vin) were double digested with BamHI and XhoI. The linearized ~3.5 kb fragment from pEGFP-fabp2 and the 6.8 kb fragment from pTOL2 were ligated using the T4 DNA ligase. The linearized construct pTOL2-EGFP-fabp2 were microinjected into 1-cell zebrafish embryos to obtain a germ-line transgene integration of fabp2-TOL2-EGFP. The founder fish were screened for the stable integration of the transgene and subsequent transgenic fish generations were maintained.

Live imaging of transgenic zebrafish

Double transgenic fishes used in this study were generated by crossing hi559 heterozygotes with Tg(gut:gfp) and Tg(lyzc:egfp) lines. The Tg(gut:gfp) transgenic zebrafish line expresses GFP throughout the digestive system and are used as a tool to analyze development of GI tract and digestive organs (Field et al., 2003). The Tg(lyzc:egfp) transgenic line expresses EGFP under the regulatory regions of the zebrafish lysozyme-C (lyzc) gene, and are used to study infiltration of myeloid-derived inflammatory cells, representing a subset of macrophages and granulocytes (Hall et al., 2007; Hall et al., 2009). The Tg(mpx:gfp) transgenic line expresses GFP under the neutrophil-specific myeloperoxidase (mpx, also known as mpo) promoter and are used effectively to analyze intravital inflammatory response in vivo in zebrafish larvae (Renshaw et al., 2006). The Tg(lc3:gfp) transgenic line expresses GFP-fused Lc3 (GFP-Lc3) that can be visualized in vivo to monitor autophagy, as Lc3 specifically label the
growing phagophores and completed autophagosomes (Kabeya et al., 2000; He et al., 2009; He and Klionsky, 2010).

Live imaging of zebrafish larvae were done by bright-field or fluorescent microscopy (Leica or Zeiss Axiovert). Confocal Imaging was performed using a laser scanning confocal microscope (Leica or Zeiss), and the acquired images were analyzed using ImageJ. The GFP intensity and puncta were quantified to assess leukocytes and autophagosomes in the GI tract of the respective transgenic larvae (n≥12).

**Whole-mount Staining**

For Nile-Red staining, larvae were treated with 10 ng/ml Nile Red in E3 medium, starting at 3-dpf. The size and morphology of the gut lumen was assessed at different stages by observing Nile-Red incorporation using fluorescent microscopy (Leica). CY3-streptavidin (CY3-SA) labeling and whole-mount in situ hybridization (ISH) were performed as illustrated previously (Sadler et al., 2005; Stuckenholz et al., 2009). Quantitative analyses of gut size was performed by ImageJ analyses of GFP positive intestinal area using the Tg(gut:gfp) or Tg(fabp2-egfp) transgenic fish.

**Quantification of histological data**

Histological sectioning, Hematoxylin and Eosin (H&E) staining were performed as described previously (Thakur et al., 2011). The IEC morphology, villous architecture and histological evidence of intraluminal bacteria were assessed by microscopic examination (Zeiss Axiovert) of atleast 10 alternate H&E-stained sagittal sections (5 µM) each representing larvae from wild-type and mutants (n≥15), and larvae from DMSO control and drug treatment groups (n≥7). This allowed us to cover the analyses of histological features of the entire GI tract. For GC enumeration, Periodic Acid Schiff (PAS)- and
Hematoxylin-stained sections prepared at various time points during larval growth were imaged, and the total numbers of IECs and PAS-positive cells were determined for at least 12 alternate sections (4 µM) representing at least 8 different larvae each from wild-type, mutants, DMSO and drug treatment groups. Phenotypically mature GCs were assessed based on the intensity of staining, the size of the apical region, the location in the intestinal epithelium and morphological appearance.

**Immunofluorescence assay**

To assess the differential expression of Hspa5, sagittal cryosections (8 µM) through the entire GI tract of wild-type and mutant larvae were used for fluorescent immunohistochemistry using anti-HSPA5 primary antibody (Sigma) and FITC-conjugated anti-rabbit IgG secondary antibody (Sigma) and counter stained with DAPI (Sigma) for nuclear staining. Fluorescent image acquisition was performed using confocal microscope (Zeiss) followed by analyses using ImageJ.

**TEM data analyses**

TEM was performed in the EM facility at Center for Biological Imaging, University of Pittsburgh. For semi-thin sections, the epoxy resin embedded larvae were transverse sectioned (350 nm) and stained with Toluedene-blue, and at least 10 ultra-thin sections (70 nm) were collected corresponding to the esophageal and intestinal region for TEM staining and analyses. Sectioning depth from the beginning of the tissue as reference point and visualization of Tolueden-blue stained sections at regular intervals allowed us to select TEM sections from the same area of the tissue for wild-type and mutants. Number of mitochondria, autophagosomes and lysosomes were counted from a set field of specific magnifications facilitating observation of GI cells and represented as
numbers/field of observation. GI cells containing double or multi-membrane autophagosomes were considered positive and counted manually and data were presented as percentage of IECs with autophagy in each field of observation. Mitochondrial counts were presented as total number of mitochondria per IECs.

**Reverse transcriptase PCR**

Total RNA from the micro-dissected GI tissue (n=5) was isolated using RNA purification kit (Stratagene), and cDNA was prepared by reverse transcription using Superscript II (Invitrogen). RT-PCR to detect xbp1 splicing was performed as described previously (Cinaroglu et al., 2011).

**Analyses of intestinal bacteria**

Quantification of intestinal bacterial density was adapted from previously described methods with applicable modifications (Oehlers et al., 2011b). Zebrafish larvae (6-dpf) were euthanized with tricaine (MS-222, Sigma), and were washed three times with sterile PBS-0.1% Tween to remove non-adherent or loosely attached surface bacteria. Individual guts from each larvae were micro-dissected using disposable sterile needles (n=3 for each genotype or treatment groups) and the isolated gut tissues were washed three times with sterile PBS followed by homogenization with 500 µL PBS in sterile microfuge tubes with disposable microfuge pestles. Serial log10 dilutions of the homogenates were plated on LB agar plates and incubated overnight at 28.5° C. Intestinal bacterial density was enumerated based on total colony forming units (CFU) per individual gut.

**Cell Proliferation and Apoptosis**

Cell proliferation was estimated by *in vivo* labeling with 5-bromo-2’-deoxy-uridine (BrdU, Roche) and apoptosis was quantified by TUNEL assay on histological sections
using the ApopTag peroxidase kit (Chemicon). Larvae (4, 5 and 6-dpf) were incubated in E3 with 10 mM BrdU for 6 h at 28.5°C and fixed in 4% PFA overnight. Incorporated BrdU was detected with an anti-BrdU antibody (Amersham) and visualized with Peroxidase substrate kit (Vector). Quantification of proliferating and apoptotic cells was represented in percentages of relative proportion of BrdU-positive and TUNEL-positive IECs to the total number of IECs in at least 10 alternate sagittal sections (5 μM) representing the entire GI tract of at least 8 different larvae.

**Pharmacological Assays**

Tunicamycin is known to prevent N-linked glycosylation of proteins, affecting their proper folding and accumulation in ER, thus inducing ER stress in eukaryotic cells (Yoshida, 2007). Tunicamycin (Calbiochem, EMD Biosciences) treatment assay in larval zebrafish was optimized using the methods described in our previous study (Thakur et al., 2011).

δ-Hexachlorocyclohexane (δ-HCH), a compound with similar configuration of myo-inositol, inhibits PI synthesis by affecting the incorporation of myo-inositol into PI (Hoffmann et al., 1980; Thakur et al., 2011). Wild-type, Tg(gut:gfp), Tg(lyz:egfp) or Tg(lc3:gfp) larvae were treated with 5 μM δ-HCH (Sigma) from 3.5- to 6-dpf and analyzed at 6-dpf.

For antibiotics treatment, zebrafish larvae were exposed to a cocktail of antibiotics from 3.5- to 6-dpf. Antibiotics consisted of ampicillin (Sigma, 100 μg/ml final concentration), kanamycin (Sigma, 5 μg/ml final concentration), and penicillin-streptomycin pre-mix (Invitrogen, 100 units/ml penicillin and 100 μg/ml streptomycin) in E3 media. Anti-inflammatory drugs consisted of 5-aminosalysilic acid (ASA, Sigma, 50
µg/ml) and 6-alpha-methylprednisolone (Sigma, 25 µg/ml) dissolved in 0.05% DMSO v/v in E3 media.

For chemical chaperone treatment, the sodium 4-PBA (Sigma) was dissolved in E3 water and the fishes were exposed to various dosages of 4-PBA to optimize a non-toxic dosage that did not cause any developmental defects. A final concentration of 50 µM 4-PBA (3.5- to 9-dpf) was used for the rescue experiment in this study. For control groups, larvae were treated with equivalent concentration of DMSO alone. The drug treatments were performed in 12-well plates containing 15 larvae each from 3 different biological clutches.

Statistics

Data were representative of larvae from at least 3 different biological clutches. Statistical significance was calculated by using a two-tailed student $t$-test and a $P$-value of less than 0.05 was considered significant. The results are expressed as means and standard deviations as error bars in the respective bar-charts.

Pathway analyses

Gene expression and pathway analyses were performed as described previously using our microarray data deposited with GEO (GSE17711) (Stuckenholz et al., 2009; Thakur et al., 2011). We used Ingenuity’s pathway analysis tool (IPA; http://www.ingenuity.com) and Gene Set Enrichment Analysis tool (GSEA; http://www.broad.mit.edu/gsea/) (Subramanian et al., 2005) to decipher the dysregulated pathways in the mutants. A $P$-value of less than 0.05 ($n=3$) after adjusting for false discovery rate was considered significant.
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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

Study concept and design: PT, JD, CS, NB
Acquisition of data: PT, JD, NB
Analysis and interpretation of data: PT, JD, CS, NB
Performed experiments: PT, NB, LL
Drafting of the manuscript: PT
Critical revision and editing of the manuscript: PT, CS, JD, NB
Obtained funding: NB
Study supervision: NB

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REFERENCES


FIGURE LEGENDS

Fig. 1: Morphological defects of hi559 GI tract. (A) Intestinal morphology at 5-dpf (bright-field, red outline). (B) ISH with intestinal marker fabp2 at 5-dpf. (C) Nile-Red staining shows hi559 intestinal luminal atrophy (arrow). (D) Cy3-SA staining shows reduced epithelial strucutre in hi559 intestine (white outline). (E) Bar-chart showing reduced gut size in hi559Tg(gut:gfp) mutant larvae show smaller intestine (n=7, **P<0.001). (F-G) H&E-stained sagittal sections of 5-dpf wild-type (F) and hi559 larvae (G). The hi559 intestinal epithelium is thinner, loses villous architecture with cellular aggregates in a smaller lumen. (villi, arrow; cells and debris, arrowhead). Wild-type on top, mutant below in each panel. Es, esophagus; Gb, gas-bladder; Ib, intestinal bulb; P, pancreas; L, liver; Y, yolk; cm, cell membrane. Scale bar: F-G, 20 µM.

Fig. 2: Disrupted epithelial architecture, abnormal IECs, and increased luminal bacteria in hi559 intestine. (A) In hi559 anterior GI tract, the IECs appear less columnar, a few IECs detach from the mucosa (arrowhead), and esophageal GCs (arrow) appear disorganized with nuclear pyknosis. Asterisk indicates luminal bacterial plaques. (B) TEM comparison of intestinal epithelium of 6-dpf wild-type (left) and hi559 (right). Wild-type intestine shows columnar IECs, thick terminal web (tw, red line) and long microvilli (mv, arrows). hi559 IECs have thinner terminal webs, shorter microvilli, and increased cytoplasmic vacuoles (asterisk). (C) TEM showing dense bacterial colonies in hi559 intestinal lumen (red arrow), but not in wild-type. Bar-chart (right panel) showing significant increase in intestinal bacterial density in hi559 (n=5). (D-E) The mucin-rich esophageal GCs (arrow) at 5-dpf (D) and 6-dpf (E) are shown by PAS-staining (pink). The secreted mucinous layer (arrowhead) on the epithelial border seen in wild-type is diminished in hi559 with frequent detachment of GCs. (F, left) Bar-chart showing PAS-positive GC numbers in 5- and 6-dpf wild-type and hi559 esophagus (n=7). (F, right) Bar-chart showing the percentage of larvae with intra-luminal bacterial overgrowth at 5- and 6-dpf (n≥21). Es, esophagus; Ib, intestinal bulb. *P<0.05, **P<0.01. Scale bars: A, D-E, 20 µM; B-C, 500 nm.
**Fig. 3: Abnormal cell proliferation, apoptosis and inflammation in hi559 intestine**

(A) BrdU-staining (red) shows decreased proportion of proliferating cells (*arrow*) in the hi559 intestine. (B) TUNEL-staining (brown) showing several apoptotic cells in hi559 GI tract (*red-dashed esophageal GC region magnified in inset*). (C) Bar-charts showing the proportion of BrdU-positive cells at 5-dpf, and TUNEL-positive cells at 6-dpf (*n*=8). (D) ISH showing increased expression (*arrow*) of neutrophil marker *mpo* (*left*) and macrophage marker *spi1* (*right*) in hi559 intestines at 6-dpf. (E) Confocal projections of 6-dpf *Tg(lyzc:egfp)* and *hi559Tg(lyzc:egfp)* larval intestines, showing leukocyte aggregation (*arrow*) and bar-chart of leukocytes in wild-type and hi559 intestines at 6-dpf (*n*=12). (F) IPA analysis of microarray profile showing most significantly upregulated pathways in hi559 larvae (*n*=3, *P*≤0.01). Es, esophagus; Ib, intestinal bulb; P, pancreas; L, liver; *asterisks* indicate luminal bacterial plaques. **P*<0.01, ***P*<0.001. Scale bar: 20 µM.

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**Fig. 4: ER stress and ultrastructural pathology of IECs.** (A) Anti-HSPA5 immunofluorescence assay (green) shows robust enrichment of Hspa5 protein in the GCs (*arrow, left panel*) and the IECs along the epithelial lining (*arrow, right panel*) of hi559 intestine. (B-D) TEM comparison of wild-type (*left panels*) and hi559 IECs (*middle and right panels*). (B) ER-golgi compartments are grossly expanded in 5-dpf hi559 IECs. Large double-membranous autophagic vacoules (*red outline*) and pre-autophagosome structures (*red arrow*), containing ER fragments (*black arrow*) are apparent in 5.5-dpf hi559 IECs (*right panel*). (C) Wild-type IECs have abundant mitochondria, while hi559 IECs have depleted, abnormal mitochondria and increased mitophagy at 6-dpf (*red arrow*). Multi-lamellar autophagic bodies (*red outline*), engulfing organelles, occur frequently in hi559 IECs at 6-dpf. (D) Secretory granule-rich enteroendocrine cells show ER luminal swelling (*asterisk*) and autophagic vesicles in hi559. Nu, nucleus; Er: endoplasmic reticulum; Ga, Golgi-apparatus; Au, autophagosome, Sg, secretory granules. Mt, mitochondria. Scale bars: A, 20 µM; B-D, 500 nm. (E) Bar-chart of mitochondrial (*left*) and autophagosome (*right*) counts in IECs (**P*<0.01, ***P*<0.001, *n*=7).

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**Fig. 5: Intestinal pathologies of tunicamycin-treated wild-type larvae.** (A) ISH with *fabp2* shows smaller intestine in tunicamycin-treated larvae. (B) Confocal projections of
Tg(fabp2:egfp) intestine (white outline) showing disrupted intestinal architecture of tunicamycin-treated larvae. (C) H&E-stained sections shows abnormal GCs (arrow) and increased luminal bacteria (arrowhead) in tunicamycin-treated larvae. (D) Bar-charts showing reduced gut size, GC depletion and increased intestinal bacteria in tunicamycin-treated larvae (n≥10). (E) Confocal projection of Tg(lyzc:egfp) larval intestine shows increased macrophage aggregation (arrow) in tunicamycin-treated larvae (n=15). (F) Confocal projection of Tg(lc3:gfp) intestine shows increased autophagosomes in tunicamycin-treated larvae (n=7). *P<0.05, **P<0.01, ***P<0.001. TN, tunicamycin. Scale bars: B & E, 100 µM; C, 20 µM; F, 5 µM.

**Fig. 6: Suppression of inflammation by antibiotics and anti-inflammatory drugs.** Larvae were treated with antibiotics (abx) and anti-inflammatory (ai) drugs from 3.5-dpf to 6-dpf. (A) ISH with fabp2 shows improvement of intestinal architecture in hi559 larvae treated with combined antibiotics and anti-inflammatory drugs. (B) ISH with mpo shows reduction of intestinal neutrophil infiltration (arrow) in drug-treated hi559 larvae. (C) H&E-staining shows reduction of luminal bacteria and inflammation in drug-treated hi559 larvae. IECs vacuolation (arrowhead), apoptosis and shedding (arrows) are seen in both DMSO and drug treated larvae. (D) Fluorescent micrograph of drug-treated hi559Tg(lyzc:egfp) mutant larvae shows reduced intestinal leukocyte infiltration. (E) Bar-charts showing gut size, percentages of apoptotic IECs, autophagic IECs, intestinal leukocyte counts, and esophageal GCs in DMSO- or drug-treated hi559 larvae (*P<0.05, **P<0.01, ***P<0.001, n≥12). Scale bar: C, 20 µM; D, 100 µM.

**Fig. 7: Chemical chaperone 4-PBA rescues the hi559 GI phenotype.** (A) Brigh-field image of DMSO and 4-PBA treated hi559 larvae at 6.5-dpf, showing amelioration of gross intestinal structure (red outline). (B) H&E-stained sagittal sections shows improved villous architecture (small arrow), GCs (arrow) and reduction of mucosal necrosis (arrowhead) in 4-PBA treated larvae. (C) Confocal projection of hi559Tg(lyzc:egfp) larval intestine showing reduction of intestinal leukocyte infiltration in 4-PBA treated larvae. (D) Bar-charts of hi559 larval gut size (n=15), intestinal leukocyte counts in hi559Tg(lyzc:egfp) larvae (n=12), and intestinal autophagosomes (lc3:gfp punctates) in δ-HCH-treated wild-type Tg(lc3:gfp) larvae (n=7) exposed to DMSO or 4-PBA. (E)
Survival curve of hi559 larvae treated with DMSO or 4-PBA from 3 through 9-dpf. Error bars indicate standard deviations. **P<0.01; ***P<0.001. Scale bar: B, 20 µM; C, 50 µM.

**TRANSLATIONAL IMPACT BOX**

*Clinical issue:*

Intestinal epithelial disruption and inflammation is a hallmark feature of several chronic GI diseases, including IBD and GI cancer. IBD is a debilitating chronic disorder with a peak incidence in early adult life, often requiring lifetime prescriptions of treatment with significant side-effects. Although IBD is believed to result from an inappropriate inflammatory response to commensal microbes in a genetically susceptible host, we only have limited insights into its pathogenesis, underlining the importance of finding novel genes and pathways that may contribute to GI inflammation. Notably, genes that affect the cellular stress response pathway have recently been implicated in IBD pathogenesis. To explore the underlying mechanism of cellular stress and inflammation, the authors have used a novel zebrafish genetic model linking PI signaling to these processes. Zebrafish provide an attractive tool for unraveling such mechanisms due to the similarity in basic GI architecture, cell types, and function with the mammalian system and as they offer effective in vivo imaging and drug screening.

*Results:*

Phosphoinositide (PI) signaling is associated with GI diseases and malignancies. However, there is little information on the mechanisms through which PI may impart its pathophysiological functions in the intestine. This study elucidates the significance of PI in the GI epithelium by a whole organism in vivo approach, using zebrafish insertional mutant and pharmacological models. Analyses of the cdiphti559 mutant revealed that deficient de novo PI synthesis elicits persistent ER stress and affects intestinal architecture, epithelial restitution and homeostasis. The unresolved ER stress sequentially leads to reduced mucosecretion, goblet cell apoptosis, autophagy, bacterial overgrowth, and myeloid inflammation in the mucosa, resembling IBD pathologies. Pharmacological
induction of ER stress is sufficient to elicit similar inflammatory pathologies. Suppression of inflammation by anti-inflammatory drugs fails to resolve the ER stress pathologies, whereas, ER stress alleviation by chemical chaperones, resolves the mutant phenotype.

**Implications and future directions:**

Using a whole organism in vivo approach, this study unravels a novel mechanism whereby lack of de novo PI causes ER stress-mediated intestinal mucosal injury and inflammation. The data presented here suggests that modulation of PI signaling and ER stress components may alleviate GI inflammation, thus offering new avenues for therapeutic strategies against IBD and associated diseases. The zebrafish genetic and pharmacological model presented here is amenable to commonly tested anti-inflammatory drugs and chemical chaperones, thus providing a preclinical platform to develop molecularly targeted therapies for GI inflammatory diseases and cancer.