Sec13 safeguards the integrity of the endoplasmic reticulum and organogenesis of the digestive system in zebrafish

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A B S T R A C T

The Sec13-Sec31 heterotetramer serves as the outer coat in the COPII complex, which mediates protein trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus. Although it has been studied in depth in yeast and cultured cells, the role of COPII in organogenesis in a multicellular organism has not. We report here that a zebrafish sec13sec198 mutant, which exhibits a phenotype of hypoplastic digestive organs, has a mutation in the sec13 gene. The mutant gene encodes a carboxyl-terminus-truncated Sec13 that loses its affinity to Sec31a, which leads to disintegration of the ER structure in various differentiated cells in sec13 mutant. Our data provide the first direct genetic evidence that COPII function is essential for the organogenesis of the digestive system.

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I n t r o d u c t i o n

The vertebrate digestive system is formed by the alimentary tract and digestive glands that originate from the endoderm germ layer ( Wells and Melton, 1999 ). In zebrafish, endoderm cells are distributed in a salt–pepper pattern by the end of gastrulation ( Field et al., 2003; Ng et al., 2005; Ober et al., 2003; Shin et al., 2007 ). The signaling molecules Bmp2a/b, Fgf, and Wnt2bb are apparently crucial factors for the development of the liver and pancreas ( Huang et al., 2008; Ober et al., 2006; Shin et al., 2007 ).

In eukaryotes, protein trafficking in the secretory pathway is essential for normal cellular activities, and is mediated by vesicular carriers (or vesicles), which are composed by the delicately regulated assembly of coat protein complexes. The COPII complex is a specialized coat protein complex that is responsible for transporting newly synthesized secretory and membrane proteins from the endoplasmic reticulum (ER) to the Golgi apparatus in eukaryotic cells (Antony and Schekman, 2001). There are five core COPII components: the small GTPase Sar1, the Sec13/Sec31 heterotetramer and the Sec23/Sec24 heterodimer (Barlowe et al., 1994; Huh et al., 2003). Biogenesis of the COPII complex begins with the activation of Sar1 from a GDP-bound to a GTP-bound form by its guanine-nucleotide-exchange factor Sec12 at the ER exit site (ERES) (Nakano et al., 1988; Nakano and Muramatsu, 1989). The GTP-bound Sar1 then serves as an anchor to recruit the Sec23/Sec24 complex through its carboxyl-terminal region interaction with Sec23 (Bi et al., 2002).
The Sar1/Sec23/Sec24 complex enriches cargo proteins and helps to deform the membrane into a tubule-like pre-budding complex. The Sec13/Sec31 complex is then recruited through a physical interaction between Sec23 and Sec31, which results in the transformation of the pre-budding complexes into COPII transport vesicles (Antonny, 2006; Kuehn et al., 1998).

Although COPII components are found in all vertebrates and plants, their functions have mainly been investigated in yeast and mammalian cells and rarely in whole organisms. One report showed that the loss-of-function mutation F382L in human SEC23A leads to human cranio-ventral-sutural dysplasia. Mutant cells carrying the F382L mutation exhibit mislocalization of Sec31 and gross dilatation of the ER lumen (Boyadjiev et al., 2006). It was also found that the zebrafish crusher mutant carries a mutation in the sec23a gene and exhibits a phenotype that is comparable with cranio-ventral-sutural dysplasia. Chondrocytes in crusher accumulate proteins in bloated ER, resulting in a severe reduction in extracellular matrix (ECM) deposits in the cartilage (Lang et al., 2006). Recently, Townley et al. (2008) reported briefly that zebrafish Sec13 morphants also exhibited a cranial facial defect but they did not characterize the Sec13 morphants in detail. Disruption of the COPII complex not only blocks protein trafficking but also activates the unfolded protein response (UPR). In metazoa, the UPR is mediated by three distinct sensors: IRE1, ATF6, and PERK (Hetz and Glimcher, 2009; Wiseman et al., 2010). The UPR is a cellular response to cope with ER stress (Higashio and Kohno, 2002; Saito and Glimcher, 2009; Wiseman et al., 2010). The UPR is a cellular response to cope with ER stress (Higashio and Kohno, 2002; Saito et al., 2011), such as that caused under pharmacological or pathological condition (Cinaroglu et al., 2011; Patyi et al., 2011; Thakur et al., 2011). However, a prolonged UPR usually triggers cell apoptosis by activating pro-apoptotic factors such as the CCAAT enhancer-binding homologous protein (CHOP) (Pino et al., 2009; Yamaguchi and Wang, 2004).

We report here the identification and characterization of a zebrafish mutant, sec13sq198, which hypoplasia in major digestive organs, including the liver, exocrine pancreas and intestine. The mutation is caused by a thymidine (T) to adenine (A) substitution that creates a new splicing acceptor site in intron 7 of the sec13 gene, which encodes the zebrafish Sec13 protein. This mutation leads to disruption of the ER structure and secretory pathway in mutant cells. We show that the defective secretory pathway in this mutant activates the UPR pathway and up-regulates pro-apoptotic factors such as CHOP, which is associated with cell-cycle arrest and cell apoptosis, causing under-development of the digestive organs and branchial cartilage.

Materials and methods

Fish lines

The WT AB strain of zebrafish (Danio rerio) was used in this study. The sq198 small-liver mutant was identified from screening an ethynitrosourea-induced mutated AB population (Huang et al., 2008). Heterozygous sq198 were mated with the polymorphic ecotype WIK to generate a mapping population. Zebrafish were raised and maintained in a standard Zebrafish Unit (produced by Aisheng Zebrafish Facility Manufacturer Company, Beijing, China).

Whole-mount in-situ hybridization (WISH)

WISH was performed as described previously (Mayer et al., 2005). mRNA probes prox1, fabp10a, trypsin, insulin, fabp2, foxa1, foxa3, gata6 and hhex were labeled with digoxigenin (DIG, Roche Diagnostics) and used as previously described (Chen et al., 2005; Huang et al., 2008). For WISH using fabp10a, fabp2 and trypsin three probes together, fabp10a and fabp2 were labeled with DIG and trypsin was labeled with fluorescein.

Detection of alkaline phosphatase activity and Alcian blue staining

Embryos were fixed in 4% paraformaldehyde/phosphate buffered solution (PBS) and washed in PBS before being assayed for alkaline phosphatase activity using nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate staining buffer or for Alcian blue staining as described previously (Chen et al., 2005).

BrdU incorporation assay, phosphorylated histone 3 (pH3) immunostaining and TUNEL assay

For BrdU incorporation assay, embryos at 3.5 dpf were injected with 1 nanolitre (nl) of 10 mM BrdU solution. Four hours after injection embryos were harvested for immunostaining of BrdU positive cells using an anti-BrdU antibody (AbD serotec, OBT030) in dilution 1:200 as described. TUNEL assay was performed using the In Situ Cell Death Detection Kit, TMR (Roche) and pH3 immunostaining using the monoclonal antibody against pH3 (Santa Cruz, SC-8656-R) in dilution 1:200 as previously described (Chen et al., 2005).

Rescue and morpholino mimicking of sec13sq198 phenotype

WT sec13 and mutant sec13sq198 coding regions were cloned into a pCS2+ vector. mRNAs were synthesized by mMessage mMachine SP6 Kit (Ambion). A 0.5-ng aliquot of in-vitro transcribed WT or mutant sec13siRNA mRNA was injected into one-cell-stage embryos. Injected embryos were subjected to WISH using a digoxigenin-labeled fabp10a probe at ~3.5 dpf.

Morpholinos were ordered from Gene Tools (Philomath, USA). The sec13 sp2 morpholino (5'-TTATTCGCTGTGATACTCGAGGC-3') was designed to target the junction of exon 7 and intron 7 of sec13 and 1 nl (0.2 nmol/µl) was injected into one cell-stage embryos. The sec31a morpholino (Sec31a-ATG MO) (5'-CGTTT-AATTTCCTTGCTGTTATCC-3') was designed to target the sec31a translation start site, and 1 nl (0.75 nmol/µl) was injected into one cell-stage embryos. A human β-globin antisense morpholino (5’-CTTCTACCTCGTTACATT-3’) was used in parallel as the standard control (st-MO).

RNA and protein analysis

RNA sample preparation and northern blot hybridization were performed as described previously (Cheng et al., 2006), as were total protein extraction and western blotting analysis (Chen et al., 2009). The mouse anti-Sec31a antibody was generated by the laboratory of Prof. Wanjin Hong. The mouse anti-sec13 antisera was generated by immunizing mice with full-length zebrafish Sec13 over-expressed in bacteria. Rabbit anti-Bip (Sigma, C9043; 1:1000 dilution), rabbit anti-Chop (Sigma, G6916; 1:1000 dilution), rabbit anti-eIF2a (Cell Signaling Technology, 9720 S; 1:2000 dilution), and rabbit anti-P-eIF2a (Cell Signaling Technology, 9721 S; 1:2000 dilution) antibodies were used in western blotting analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Embryos were collected at 3, 4, and 5 dpf and genotyped. Total RNA was extracted and purified, cDNA synthesis was performed using a reverse transcriptase kit (Invitrogen). Gene specific primers were designed through DNASTar software. Aliquots of 0.5 µl of each prepared cDNA and corresponding primers were
added to SsoFast EvaGreen Super mix (Biorad). The CFX96 Real-Time System (Biorad) was used to obtain the threshold cycle (Ct) value. The relative expression of each gene was determined after being normalized to elongation factor 1a (elf1a) (10680.1 RPKM). Primer sequences were:

\texttt{atf6 (NM_001105199) forward: Ctg- AgggCtcTtgAAAATg; reverse: CgcACgggAgTTgggAAC; bip (NM_213058) forward: gsTccggTCCtCtCtCAT; reverse: Ag- CcggTtCtcTTgTgTgTAAg; chop (NM_001082825) forward: ggAgGCcAgTTTTgATgT; reverse: AgTgCggCgcCtCgTT- CTT; grp4d (NM_1982120) forward: TCCcAgCcAgTGcAg; reverse: gTAgAAgCCcAcAcCcAgtAc; ire1a (NM_001020530) forward: ATtgCgggzeAgTgTgC; reverse: gTATTCTgTgTgCggCCAAgTAA; perk (NM_001077149) forward: CgcGgCggCAACAACagTGT; reverse: gTgTgg-CcAGATATCagaAgAT.}

\textbf{Sec13 and Sec31a interaction assay}

Coding sequences of WT and mutant sec13 were cloned into PDHA and PDMyc, respectively. HA-tagged WT and mutant Sec13 were expressed in HEK-293 T cells. The co-immunoprecipitation study was performed using the ProFound™ HA system (Pierce) following the manufacturer’s instructions. Myc-tagged WT and mutant Sec13 were expressed in HeLa cells grown on coverslips in a six-well dish for the immunostaining assay.

\textbf{Immunofluorescence microscopy}

Zebrafish embryos were fixed, embedded and sectioned as described (Huang et al., 2008). Primary antibodies against PDI (Sigma) and Collagen II (Lifespan) were used at a 1:100 dilution. Polyclonal antibody against Laminin (Sigma, L9393) was used at 1:50 dilution. Secondary antibodies conjugated with Alexa Fluor 488 and 647 were used at a 1:400 dilution. 4′,6-Diamidino-2-phenylindole (DAPI) was used to label nuclei. Images were taken with a Leica TCS SP5 confocal microscope.

\textbf{Transmission electron microscopy (TEM)}

5 dpf zebrafish embryos were fixed in 2% glutaraldehyde and 1% paraformaldehyde in PBS for 2 h followed by post-fixation in 1% osmium tetroxide for 1 h at room temperature. Fixed embryos were gradually dehydrated with ethanol and embedded individually into resin blocks; 50-nm cross-sections were obtained with a Leica TCS SP5 confocal microscope.

\textbf{Results}

\textbf{The sq198 mutation causes growth arrest but does not affect functional differentiation of digestive organs}

The sq198 small-liver mutant was identified during screening an ethynyliditosauraea-induced mutated population (Supplemental Fig. 1A) (Huang et al., 2008). Under a dissection microscope, the sq198 mutant embryo is visibly indistinguishable from a wild-type (WT) embryo before 2 day post-fertilization (dpf). However, at 5 dpf, the sq198 mutant can be readily identified by the lack of an inflated swim-bladder, fully expanded intestinal bulb and mouth opening, and also by a bigger yolk (Supplemental Fig. 1B).

The liver, pancreas and intestine all originate from the endoderm. In zebrafish, the lineage specification and budding of these organs are accomplished by 2 dpf. To determine at which stage the sq198 mutation starts to affect development, we performed whole-mount RNA in-situ hybridization (WISH) with various early endoderm markers. Because the sq198 mutant was characterized by its small-liver phenotype at 3 dpf (Supplemental Fig. 1A), we first compared the liver specification and budding in sq198 mutants and WT embryos by examining the expression patterns of two early hepatic markers, prox1 and hex, at 30 dpf, a stage at which hepatoblasts are specified and a discrete liver bud starts to form. No discernible differences were revealed by these two markers within the progenies from crosses of sq198 heterozygotes (Supplemental Fig. 1C and D). We then investigated the phenotypes of the liver, pancreas and intestine using pan-endoderm markers (foxa1, gata6, and shh), a liver marker (prox1), and a pancreatic marker (pdx1) in sq198 mutants and WT embryos at 2 dpf. No obvious differences between the homoyzogous mutants and WT embryos were revealed at this stage (Supplemental Fig. 1E–I). These results suggest that the sq198 mutation does not impair specification and early morphogenesis of the digestive organs.

After 2 dpf, zebrafish digestive organs undergo rapid cell proliferation and differentiation, which is reflected by significant organ expansion and expression of genes essential for digestive functions. We examined the expression of the hepatocyte marker, fatty acid-binding protein 10a (fabp10a), the exocrine pancreas marker, trypsin, the islet marker, insulin, and the intestine marker, fabp2 using WISH in sq198 mutants and siblings at 3 and 4 dpf. Results revealed that expansion of both the liver and exocrine pancreas was arrested in homozygous sq198 mutant embryos (Fig. 1A and B). At 3 and 4 dpf, the mutant intestine was less expanded at the intestinal bulb region while the gut-looping process appeared to be relatively normal (Fig. 1C). Interestingly, the mutant islet grew normally (Fig. 1D; Supplemental Fig. 2). In addition to defective digestive functions, the sq198 mutant exhibits malformed skeleton cartilage as revealed by Alcian blue staining in 4 and 5 dpf embryos (Fig. 1E). Staining 3- dpf sq198 mutant embryos for internal alkaline phosphatase activity showed that blood vessels were normal (Fig. 1F), whereas the head region of the pronephric ducts was thinner (Fig. 1G), suggesting that the sq198 mutation differentially affects the development of mesoderm-derived tissues. Altogether, these data demonstrated that the expansion, but not the cell differentiation, of the liver, exocrine pancreas, and intestine and the development of the skeleton cartilage are impaired by the sq198 mutation.

\textbf{Hypoplasia of the liver, exocrine pancreas and intestine in sq198 is a result of cell-cycle arrest and abnormal cell apoptosis}

To learn more about the nature of hypoplastic digestive organs in sq198, we studied the fate of cells in the liver, pancreas and intestine by analyzing the cell cycle and cell apoptosis status in WT and mutant embryos. We first performed immunofluorescence staining using an anti-phosphorylated histone 3 (pH3) antibody, a cell-cycle marker of G2 to M transition (Supplemental Fig. 3A and B). By examining transverse cryosections from three embryos in each case (Supplemental Fig. 3C, Supplemental Table 1), we found that the ratio of pH3-positive cells (16 positive cells out of 997 cells counted at 3 dpf; 11 out of 1666 counted at 4 dpf) in the mutant intestine was 2.6- and 2.8-fold lower, respectively, than that in WT intestine at the same stage (54 positive cells out of 1289 cells counted at 3 dpf; 16 out of 997 counted at 4 dpf). The ratio of pH3-positive cells in the mutant pancreas (1 positive cell out of 724 cells counted) was significantly lower (~4.7-fold) at 4 dpf compared with that in wildtype (13 out of 1963 cells counted) but not at 3 dpf (Supplemental Fig. 3C). Interestingly, we observed no significant reduction in the ratio of pH3-positive cells between WT and mutant liver (Supplemental Fig. 3C). We also performed BrdU labeling experiment and...
found that the ratio of BrdU positive cells were markedly reduced in the mutant liver (14.6% versus 22.8% in WT), intestine (13.3% versus 36.1% in WT) and pancreas (11.7% versus 32.3% in WT) at 3.5 dpf while the ratio in the somites did not show drastic difference between 3 dpf wildtype (WT) and mutant (mu) embryos. (E) Alcan blue staining showing the branchial arches 1–7 in WT and sq198 (mu) at 4 dpf. (F,G) Histochemical staining of alkaline phosphatase (AP) did not reveal obvious difference between 3 dpf wildtype (WT) and mutant (mu) blood vessels (F) whilst the head region of the pronephric ducts appears thinner in the mutant (G).

**sq198 mutation disrupts the integrity of ER structure and impairs protein secretion**

We have shown that sq198 suffers from hypoplasia of digestive organs (Fig. 1A) and exhibits a deformed skeletal cartilage phenotype (Fig. 1E) which is more severe than that observed in the *crusher* mutant (Lang et al., 2006). These observations prompted us to compare the intestinal epithelium and cartilage of 5-dpf sq198 and WT embryos at the subcellular level. Transmission electron microscopy (TEM) results revealed that microvilli in the intestinal epitheliu were reduced in number, shorter in length and less organized in sq198 (Supplemental Fig. 4A and B). More importantly, the mutant epithelial cells were no longer tightly linked to each other like those in the WT, but had many gaps in between, apparently due to the lack of junction proteins between epithelial cells (Fig. 3A and B; Supplemental Fig. 4C and D, white arrows). Co-immunostaining of the basal layer component Laminin and the ER resident protein disulfide isomerase (PDI) revealed that Laminin forms a nice lining (yellow arrows) that separates the intestine and the liver at 5 dpf and is not accumulated in the ER (Fig. 3C, E, G). In contrast, although Laminin secretion did happen in the mutant (likely due to the function of the maternal Sec13) the regular pattern of Laminin is disrupted and Laminin is clearly accumulated in the ER (white arrowheads) in the mutant (Fig. 3D, F, H) at 5 dpf. Interestingly, it appeared that Laminin is also expressed in the mutant hepatocytes (Fig. 3H).

TEM results also revealed that the chondrocytes were deformed and disorganized (Fig. 4A, B) while the ECM was significantly less dense in sq198 (Fig. 4 A and B’). It was also evident that the mutant chondrocytes accumulated dilated vacuole-like structures in the cytoplasm (Fig. 4B, green asterisks). These results suggest that the mutant cells suffered severe defects in exporting secretory and membrane proteins from the ER (Bonfanti et al., 1998). Normal chondrocytes are known to secrete matrix protein collagen II (Col2a). Double immunostaining using antibodies against PDI (Fig. 4C and F) and Col2a (Fig. 4E and H) showed that almost all of the Col2a was secreted in the ECM in 5-dpf WT chondrocytes (Fig. 4D). In contrast, the Col2a was retained in the discrete ER vacuoles in sq198 (Fig. 4G).

The above results strongly suggest that the ER structure might be affected in sq198. Next, we examined the ER structures in sq198 and WT embryos using TEM. Typical rough ER structures were easily identified in 5-dpf WT chondrocytes, hepatocytes and intestinal epithelial cells (Fig. 5A, C and E, red arrow). In contrast. The normal ER morphology in 5-dpf mutant chondrocytes and hepatocytes was disrupted and appeared as large number of discrete vacuoles containing electron-dense material (Fig. 5B and D, red asterisks), which probably represents undelivered protein cargos accumulated in the dilated ER. The ER in the mutant intestinal epithelial cells did not form discrete vacuoles, but was also severely distended and dilated (Fig. 5F). Interestingly, the distended ER lumen in the intestinal epithelium did not contain electron-dense materials, which may reflect the different nature or amount of the products secreted by the intestinal epithelial cells (Fig. 5F).

**A T to A substitution in sq198 creates a new splicing acceptor site in intron 7 of sec13**

The initial positional cloning of the sq198 mutant gene determined by bulked-segregant analysis (Shimoda et al., 1999) mapped the mutation on chromosome 22 at a position between two existing simple sequence length polymorphism markers, z230 and z10321. Successive use of a series of markers on 1332 individual mutants placed the sq198 mutation between two closely linked markers, 257E and 257H, spanning a 54.3-kb genomic DNA fragment (Fig. 6A). Sequencing analysis of sec13, one of the four genes encompassed by this genomic region, identified a T to A substitution in intron 7 of sec13 that creates a new mRNA splicing acceptor site eight nucleotides away from exon 8 (Fig. 6B and C, panel for genomic DNA sequence). This results in the addition of eight nucleotides of intronic origin into the final transcripts in sq198 (Fig. 6C, panel for cDNA sequence).
This sq198 mutant transcript appears to dominate the native transcript because no WT splicing product was found in the cDNA cloned from nearly 100 5-dpf mutant embryos (data not shown).

To prove that this T to A mutation in sec13 is indeed responsible for the sq198 mutant phenotypes, in-vitro transcribed WT or mutant sec13 mRNA was injected into one cell-stage embryos derived from heterozygote crosses. The injected embryos were analyzed by WISH using a fabp10a probe at /C24 3.5 dpf (Supplemental Fig. 5A). In total, the small-liver in 144/194 mutant embryos was successfully restored to normal by injection of WT sec13 mRNA. In addition, injection of sp2-MO, which specifically targets the splice junction of exon7/intron7 of sec13 (Fig. 6D; Supplemental Fig. 5B), into WT zebrafish embryos resulted in a small-liver phenotype resembling that in sq198 (Fig. 6E). These data demonstrated that the T to A substitution in the sec13 gene in sq198 confers the hypoplastic phenotype to the digestive organs. This mutation was therefore designated as sec13sq198.

sec13 exhibits dynamic expression patterns in zebrafish

We investigated the temporal and spatial expression patterns of sec13 during early embryogenesis by northern blot analysis and WISH hybridization. Northern blot analysis showed that sec13 mRNA is deposited maternally and its expression spans the early embryonic stages from 1 dpf to 5 dpf (Fig. 6F). WISH analysis showed that sec13 is distributed ubiquitously in the unfertilized egg. After fertilization, sec13 is evenly distributed into daughter cells after the first two rounds of cell division. At 2 and 3 dpf, in addition to its continuous expression in the head and trunk, sec13 is also enriched in the developing digestive organs, including the liver, intestine and exocrine pancreas (Fig. 6G). At 4 and 5 dpf, strong sec13 mRNA expression is apparent in the intestine while a weak signal is observed in the liver, exocrine pancreas and skeletal cartilage (Fig. 6G).

We then studied the temporal protein expression pattern of Sec13 and that of its known interaction partner, Sec31a, using...
western blot analysis. Consistent with its mRNA expression dynamics, Sec13 protein is deposited maternally and was detected in all stages investigated (Fig. 6H). However, expression of its interacting partner, Sec31a, was barely detectable before 2 dpf, although it exhibited strong and consistent expression from 3 to 5 dpf (Fig. 6H). These findings imply that the COPII complex functions minimally during the earlier stages of development and becomes very active from 3 dpf onwards by regulating the protein levels of Sec31a and therefore functional Sec13-Sec31a complex.

**Fig. 3.** The sq198 mutation causes loosening of the cell-cell junction in the digestive organs. (A,B) TEM analysis of intestinal epithelium in 5 dpf wildtype and mutant embryos. The epithelial cells are tightly adhered to each other in the wildtype (WT) (A, white arrows) but are separated by clearly visible gaps in the mutant (mu) (B, white arrows). (C–H) Double immunofluorescence staining using the antibodies against the basement component Laminin (red) and the ER marker PDI (green). In the wild type control at 5 dpf, Laminin forms the normal lining along the basement layer (C) that nicely separates the liver and intestine (yellow arrows) (G). No apparent accumulation of Laminin in the ER (EG). In contrast, the mutant did not form an apparent basement layer to separate the liver and intestine (white arrowheads) (D,H) and the mutant cells accumulated Laminin in the ER (white arrowheads) (D,F,H).

Sec13 and Sec31a are co-localized in a variety of cell types in zebrafish

The most essential function of Sec13 in the COPII complex is to form the outer coat together with Sec31. We investigated the in-vivo cellular localization of Sec13 and Sec31a in zebrafish by immunostaining with anti-Sec13 and anti-Sec31a antibodies in 5 dpf WT cross-sections. The results showed that Sec13 and Sec31a appear to be expressed in a variety of tissues and cells differentially in zebrafish (Supplemental Fig. 5C). For instance, while Sec13 is expressed abundantly in the neural tube, Sec31a is only enriched peripherally to the neural tube (Supplemental Fig. 5C). At the cellular level, the co-localization patterns of Sec13-Sec31a in hepatocytes (Fig. 7A), pancreatic cells (Fig. 7B), intestinal epithelial cells (Fig. 7C), and chondrocytes (Fig. 7D) are strikingly similar to those observed in yeast and human cells (Tang et al., 2000).

sec13<sup>sq198</sup> encodes a carboxyl-terminal-truncated protein that is unable to interact physically with Sec31a

The sec13<sup>sq198</sup> mutant mRNA is predicted to encode a polypeptide with 85 amino acids truncated from the carboxyl-terminus of
WT Sec13 and the addition of 34 new amino acids due to frame-shift in the new open reading frame (Fig. 8A; Supplemental Fig. 6A). Human and zebrafish Sec13 and Sec31a share high sequence homology (Supplemental Fig. 6B; Supplemental Fig. 7). To determine whether the truncated carboxyl-terminus of Sec13 is important for its interaction with Sec31a, we created a human mutant Sec13 equivalent to the zebrafish sec13sq198 by inserting the excess eight nucleotides in the zebrafish sec13sq198 mRNA into the comparable site of human SEC13. Both human SEC13 and SEC13sq198 were tagged with HA epitope and expressed in the HEK-293T cells. An anti-HA monoclonal antibody was used to immunoprecipitate HA-SEC13 or HA-SEC13sq198 from the protein extracts from HEK-293T cells. A rabbit anti-SEC31a antibody was used to detect the co-immunoprecipitated endogenous SEC31a.

The result showed that endogenous SEC31a was efficiently co-immunoprecipitated with HA-SEC13 but not with the mutant HA-SEC13sq198 (Fig. 8B). This result defined the mechanistic basis for the loss of function of the mutant because it was no longer able to interact with Sec31a.

Myc-tagged human SEC13 (Myc-SEC13) and SEC13sq198 (Myc-SEC13sq198) were tagged with HA epitope and expressed in HeLa cells. Immunofluorescence staining was then carried out to investigate the subcellular relationship between endogenous SEC31a and over-expressed Myc-SEC13 and Myc-SEC13sq198. The results showed that Myc-SEC13 was enriched in the perinuclear region and was essentially co-localized with endogenous SEC31a which marked the ERES (Fig. 8C). A similar co-localization pattern was also observed in HeLa cells that expressed Myc-tagged zebrafish Sec13 (Fig. 8D), suggesting that zebrafish Sec13 is a functional ortholog of human SEC13. In contrast, Myc-SEC13sq198 appeared to be uniformly distributed in the cytoplasm, and showed no evidence of co-localization with endogenous SEC31a in the transfected cells (Fig. 8E). This result suggests that loss of interaction with Sec31a resulted in defective recruitment of the mutant Sec13 to the ERES.

Knockdown of Sec31a results in hypoplastic digestive organs

To demonstrate the necessity of the Sec13-Sec31a complex during organogenesis of the digestive system, we designed a morpholino (31a-ATGMO) that specifically targets the translation start region of sec31a. Immunoblots performed with rabbit anti-Sec31a antibody revealed that the level of Sec31a in 4-dpf sec31a-ATGMO morphants was approximately three fold lower than that in the WT, while Sec13 remained unchanged (Fig. 9A). WISH examination of the sec31a-ATGMO morphants using fabp10a, trypsin and fabp2 probes showed that all morphant embryos displayed a phenotype of hypoplastic liver (Fig. 9B), hypoplastic pancreas (Fig. 9C) and intestinal tube (Fig. 9D). In fact, majority of Sec31a-ATGMO morphant embryos exhibited largely diminished digestive organs (Fig. 9B–D).

Fig. 4. The sq198 mutation impairs the protein secretory pathway. (A,A',B,B') TEM analysis. Compared to wildtype (WT), the mutant (mu) exhibited disorganized chondrocytes (compare A and B) and less-accumulated ECM (compare A' and B'). A' and B' represent high resolution pictures of boxed regions in A and B, respectively. e: ECM; green asterisk: unknown vesicles in a mutant chondrocyte. (C–H) Double immunostaining of the ER resident marker PDI and the secretory protein Collagen II (Col2a) in chondrocytes in 5 dpf wildtype (WT) (C–E) and mutant (mu) (F–H) embryos. (CF) Immunostaining of PDI (green). (EH) Immunostaining of Col2a secretion (red); (D,G) Superimpose of PDI and Col2a staining accordingly.

Fig. 5. The sq198 mutation disrupts the ER structure in chondrocytes, hepatocytes and intestinal epithelium. (A–F) TEM analysis of the ER structure in chondrocytes (A,B), hepatocytes (C,D), and intestinal epithelium (E,F) in wildtype (WT) and mutant (mu) embryos at 5 dpf. Rough ER in WT cells are indicated with red arrows (A,C,E), disrupted ER structures in the mutant cells are highlighted with red asterisks (B,D,F).
the Sec31a-ATGMO morphants (Supplemental Fig. 8). We also cross-sectioned the embryos after WISH using fabp10a, fabp2 and trypsin three probes for checking the liver, intestine and exocrine pancreas simultaneously. The result showed that the Sec31a-ATGMO morphants exhibited greatly diminished digestive organs were actually not abolished of endoderm-derived cells (Supplemental Fig. 9A–C). Alcian blue staining showed that the Sec31a-ATGMO morphant embryos also suffered from malformation of the cartilage skeleton (Supplemental Fig. 10). Interestingly, WISH using fabp10a and insulin two probes together showed that, in contrast to the drastic reduction of the liver size, both the Sec31a-ATGMO morphant and sec13sq198 mutant developed a normal islet as that observed in the WT (Supplemental Fig. 11A–D). It was reported that compromised Sec31 function would lead to deformation the ER structure in yeast (Wuestehube et al., 1996). Co-immunostaining of PDI and Col2a revealed that, as observed for the sec13sq198 mutant, the ER structure of chondrocytes in the Sec31a-ATGMO morphants was distended and formed discrete vacuoles (Supplemental Fig. 12). These results demonstrate that the functional Sec13-Sec31a complex is essential for the development of the digestive organs.

sec13sq198 mutation activates the UPR pathway

UPR is a highly regulated process that plays an essential role in cellular protein homeostasis (Lin et al., 2008; Ron and Walter, 2007). Previous reports have shown that ER stress caused by disruption of components of the COPII complex can activate UPR (Chang et al., 2004; Higashio et al., 2002; Saito et al., 2009). Because sec13sq198 mutation causes disintegration of the ER structure and accumulation of secretory cargoes, which are expected to generate intracellular ER stress, we examined the expression of six UPR-related genes (bip, chop, grp94, atf6, ire1a and perk) in mutant embryos at 3, 4, and 5 dpf. All six genes were significantly up-regulated in the mutant, and chop showed the highest magnitude of up-regulation at all three stages examined (Fig. 10A). Using chop as the probe, WISH showed that its expression was mainly elevated in the digestive organs.
We then examined the protein expression of Bip, Chop and phosphorylated eIF2a (p-eIF2a) in the mutant. The results showed that although the protein level of Bip was not obviously altered, the levels of Chop and p-eIF2a were markedly upregulated in the mutant (Fig. 10C).

Discussion

We investigated the zebrafish sec13sq198 mutant, which exhibits digestive organ growth arrest. Positional cloning revealed that sec13sq198 carries a T to A substitution in intron 7 of the sec13 gene, which leads to the addition of eight nucleotides of intronic origin to the mature mRNA and results in a carboxyl-terminal-truncated Sec13 protein. The main function of Sec13 is to form the outer layer of the COPII complex with Sec31. This mutation devastates the integrity of normal COPII formation because Sec13sq198 loses its affinity to Sec31a. All evidence presented demonstrate that sec13sq198 is a loss-of-function mutation: 1) sec13sq198 is a recessive mutant; 2) WT sec13 could rescue the mutant phenotype and 3) knockdown of sec13 expression using sec13 specific morpholino targeting the junction between exon7 and intron7 mimicked the sec13sq198 mutant phenotype. In addition, the fact that the endogenous Sec31 still formed a nice crescent around the nuclei in the presence of Sec13sq198 in the cultured HeLa cells suggests that the Sec13sq198 mutant protein appears not to inhibit the recruitment of Sec31 to the ER exit site. Based on these data we conclude that the mutant protein unlikely contributed to the mutant phenotype.

Immunostaining in 5-dpf WT embryos revealed that Sec13 and Sec31a are expressed and co-localized in chondrocytes, hepatocytes and intestinal epithelial cells. TEM of these cell types in sec13sq198 revealed a dilated ER structure and/or accumulation of dilated vacuoles. Further studies showed that the ECM protein Col2a and Laminin were not correctly transported to the outer surface of mutant chondrocytes and intestinal epithelial cells, respectively, but were retained in the dilated ER lumen. This demonstrated that the COPII export function is deficient in these mutants. Prolonged cargo retention in the ER is expected to induce cellular ER stress that in turn activates the UPR. We found that six hallmark genes for the UPR were all up-regulated in the 5-dpf mutants. More importantly, the pro-apoptotic factor Chop was most notably up-regulated in the digestive organs, suggesting that cells in these organs likely underwent apoptosis.

Fig. 8. Sec13sq198 mutant protein is a C-terminal truncated Sec13 which is unable to interact with Sec31a. (A) Alignment of the C-terminal sequences of Sec13 and Sec13sq198. Sec13sq198 mutant protein lacks the C-terminus of Sec13 starting from D236 (blue arrow) and contains 34 new amino acids after Q235 (blue arrow). (B) Co-immunoprecipitation (Co-IP) analysis. HEK-293T cells were transfected with HA-SEC13 or HA-SEC13sq198 plasmids. An anti-HA antibody was used to immunoprecipitate (IP) the tagged proteins. The input (1st and 2nd panels) and the IP products (3rd and 4th panels) were blotted using an anti-SEC31A antibody (2nd and 3rd panels), C-E In human Hela cells, Myc-SEC13 (C) or zebrafish Myc-Sec13 (D) co-localizes with endogenous SEC31a whilst SEC13sq198 is evenly distributed in the cytoplasm (E). (C1,D1,E1) Immunofluorescence staining of endogenous SEC31a (red); (C3,D3,E3) Immunostaining of Myc-tagged hSEC13 (C3) or zSec13 (D3) or hSEC13sq198 (E3) (green). (C2,D2,E2) Superimposed.

Fig. 9. Knockdown of Sec31a resulted in hypoplastic digestive organs. (A) Western blotting analysis of Sec31a and Sec13 in sec31a-ATG MO (31a-ATG MO) and standard control MO (st MO) injected embryos at 4 dpf, respectively. β-Actin was used as the loading control. (B-D) WISH using the fabp10a (B), trypsin (C) and fabp2 (D) probes to examine the development of the liver, exocrine pancreas and intestine in wildtype (WT), standard control MO (st MO), and 31a-ATG MO embryos at 4 dpf. Number of embryos displaying the showed phenotype of the total embryos examined was provided at the bottom right side of each representative embryo.
The BrdU incorporation assay, pH3 staining and TUNEL assay revealed that the progression of the cell cycle was arrested in mutant cells which finally resulted in abnormal apoptotic activities in the mutant digestive organs. These results provide direct evidence to explain the sec13<sup>344758</sup> phenotypes. First, cell-cycle arrest and increased cell apoptosis lead to reduced cell number, as reflected by decreased expansion of the digestive organs and cartilage. Second, compromised transport of membrane and secrective proteins disrupts the normal patterns of organs, such as the intestinal epithelium and cartilage, because insufficient material is available to build up the ECM and tissue boundary. It is worth to point out that trafficking of small molecules does not depend upon the recruitment of Sec13/Sec31 dimer to the ER exit sites. The organogenesis of digestive organs requires secreted signaling molecules such as Fgfs and Bmps. Whether the secretion of these small molecules is affected in the mutant is an interesting question to be addressed in the future.

In addition, Sec13 and Sec31a displayed different spatial and temporal expression patterns during the early stages of development, which raises an interesting possibility that regulated expression of Sec31a or Sec13 may be employed to regulate the capacity of secretory pathway, depending on physiological needs. Sec13 is maternal protein. The sharply increased expression of Sec31a around 3 dpf compared with the low or undetectable levels of expression before this time-point coincides with increased cell proliferation and organ size, implying that the level of Sec31a is regulated to accommodate the needs of organ growth. This is also in agreement with the observation that trafficking of small molecules does not affect the development of digestive organs, with majority of Sec31a morphants showing loss of almost all of their digestive organs suggests that Sec31a is the one to form the COPII complex with Sec13 during early embryogenesis. When cell proliferation and organ growth rely increasingly on COPII from 3 dpf onwards (as shown by the increased level of Sec31a), the function of Sec13 is similarly more important and maternal Sec13 is no longer sufficient, which leads to a defective COPII function that is not able to meet developmental needs in the sec13<sup>344758</sup> mutant.

Our study provides the first genetic evidence that sec13 and the COPII complex safeguard the normal establishment of the digestive organs. In addition to its major function in the COPII complex, Sec13 also plays a role in the nuclear pore complex (Loidioce et al., 2004). Recent studies have shown that the nuclear pore complex plays an important role in regulating the expression of certain developmental induced genes by specifically binding to their regulatory sites in the genome (Capelson et al., 2010). Therefore, whether this function of Sec13 is related to the observed organ-specific subcellular defects in the mutant fish will be of great interest in our future study. Meanwhile, it would be interesting to use sec13<sup>344758</sup> to study the role of the nuclear pore complex structure in organogenesis in the future (de Jong-Curtain et al., 2009). Although the currently known function of Sec31 is solely attributed to form the COPII complex we cannot exclude the possibility that Sec31 might function independently in the organogenesis of the digestive organs. For example, we found that the endocrine pancreas region appeared to be devoid of Sec13, suggesting that Sec31a might play a special role in organogenesis of the endocrine pancreas or the secretion of endocrine hormones. Apparently, it will need extensive future work to unravel this myth.

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Appendix A. Supporting information

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References

Multimammalian orthologues of yeast Sec31p that associate with the COPII coat. Development 129, 1647–1658.