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

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ABSTRACT

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 *sec13*^{sq198} 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*^{sq198}, including chondrocytes, intestinal epithelial cells and hepatocytes. Disruption of the ER structure activates an unfolded protein response that eventually causes the cells to undergo cell-cycle arrest and cell apoptosis, which arrest the growth of developing digestive organs in the mutant. Our data provide the first direct genetic evidence that COPII function is essential for the organogenesis of the digestive system.

Introduction

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 (Warga and Nusslein-Volhard, 1999). The salt–pepper patterned endoderm cells migrate medially and form a solid rod along the midline at ~24 h post-fertilization (hpf), from which the alimentary tract and digestive glands are derived. The liver and pancreatic buds are clearly identifiable at the fore-part of the rod at ~50 hpf (Field et al., 2003; Ng et al., 2005; Ober et al., 2003; Wallace et al., 2005; Wallace and Pack, 2003). Morphologically, the liver and pancreas in adult fish are comparable to those in other vertebrates. However, the zebrafish alimentary tract does not develop a distinct stomach but only forms an expanded foregut bulb (Wallace et al., 2005). The nodal factors Cyclop and Squint, and the Sox-type factors, Casanova and Sox17, are essential for the specification of the whole endoderm. In contrast, Gata factors (Gata4/5/6), FoxA factors (FoxA1/2/3), and other factors, such as Def, Npo and Elys, are essential for the normal organogenesis of the entire digestive system (Chen et al., 2005; de Jong-Curtain et al., 2009; Mayer and Fishman, 2003). 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 (Antonny 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).

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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-lenticulo-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-lenticulo-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 membrane-bound stress sensors—IRE1α, 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 et al., 2011), such as that caused under pharmacological or pathological condition (Cinaroglu et al., 2011; Pyati 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, *sec13*^{sq198}. *sec13*^{sq198} is hypoplastic in major digestive organs, including the liver, exocrine pancreas and intestine. The mutation is caused by a thymdine (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 underdevelopment 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 ethylnitrosourea-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., 2003). RNA 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′-indolyphosphate 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, OBT0030) in dilution 1:200 as described. TUNEL assay was performed using the In Situ Cell Death Detection Kit, TMR red (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 sec13^{sq198} *phenotype*

WT *sec13* and mutant *sec13*^{sq198} 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 *sec13*^{sq198} 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′- TATTTGGCTGTGAATACCTGCGAGC-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′-CGGTT-AATTTCTTTCAGCTTCATCC-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′-CCTCTTACCTCAGTTACAATTT-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 rabbit anti-Sec31a antibody was generated by the laboratory of Prof. Wanjin Hong. The mouse anti-Sec13 antiserum was generated by immunizing mice with full-length zebrafish Sec13 over-expressed in bacteria. Rabbit anti-Bip (Sigma, G9043; 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