Mutation of sec63 in zebrafish causes defects in myelinated axons and liver pathology

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Running title: Mutation of sec63 in zebrafish
Abstract

Mutations in SEC63 cause polycystic liver disease in humans. Sec63 is a member of the endoplasmic reticulum (ER) translocon machinery, although it is unclear how mutations in SEC63 lead to liver cyst formation in humans. Here, we report the identification and characterization of a zebrafish sec63 mutant, which was discovered in a screen for mutations that affect the development of myelinated axons. Accordingly, we show that disruption of sec63 in zebrafish leads to abnormalities in myelinating glia in both the central and peripheral nervous systems. In the vertebrate nervous system, segments of myelin are separated by the nodes of Ranvier, which are unmyelinated regions of axonal membrane containing a high density of voltage-gated sodium channels. We show that sec63 mutants have morphologically abnormal and reduced numbers of clusters of voltage-gated sodium channels in the spinal cord and along peripheral nerves. Additionally, we observe reduced myelination in both the central and peripheral nervous systems, as well as swollen ER in myelinating glia. Markers of ER stress are upregulated in sec63 mutants. Finally, we show that sec63 mutants develop liver pathology. As in glia, the primary defect, detectable at 5 dpf, is fragmentation and swelling of the ER, indicative of accumulation of proteins in the lumen. At 8 dpf, ER swelling is severe, other pathological features include disrupted bile canaliculi, altered cytoplasmic matrix, and accumulation of large lysosomes. Together, our analyses of sec63 mutant zebrafish highlight the possible role of ER stress in polycystic liver disease and suggest that these mutants will serve as a model for understanding the pathophysiology of this disease and other abnormalities involving ER stress.
Introduction

In eukaryotic cells, most proteins destined for membrane insertion or secretion are first processed in the endoplasmic reticulum (ER). Nascent polypeptide chains, synthesized by cytoplasmic ribosomes, enter the ER lumen at specialized sites in the ER membrane called translocons, which are complexes of several ER membrane proteins that associate to form a pore (Schnell and Herbert, 2003). Sec61α, β, and γ form the pore, and this trimeric complex is associated with other proteins including ERj1, Sec62, and Sec63 in mammals (Meyer et al., 2000; Zimmermann et al., 2006). Mutations in SEC63 cause polycystic liver disease (PCLD) in humans, a progressive disorder characterized by the presence of many (>20) cysts throughout the liver (Davila et al., 2004; Everson et al., 2004). PCLD often co-occurs in patients with autosomal dominant polycystic kidney disease (PCKD), but can also exist as a separate disease without kidney cysts (Torres et al., 2007). Polycystic livers can grow up to 10 times their normal size, resulting in significant patient morbidity. Although a few therapeutic interventions are available to slow cyst growth, only liver transplantation can change the disease course (Drenth et al., 2010). It remains unclear how mutations in SEC63 cause liver cysts, but possibilities include disrupted trafficking of vital proteins such as Polycystin-1, an integral cilia membrane protein mutated in PCKD (Fedele et al., 2011) and disrupted tethering of proteins to the cytosolic face of the ER (Muller et al., 2010). Another possibility is that disruption of SEC63 triggers ER stress that contributes to the pathophysiology of PCLD.

Nascent polypeptides are transported across the ER translocon for processing, folding, and maturation (Rapoport, 2007). An imbalance between the load of unfolded
preproteins that enter the ER and the capacity of this organelle to properly process the load results in ER “stress”: in this case an accumulation of misfolded proteins in the ER lumen (Ron and Walter, 2007). This activates the unfolded protein response (UPR), a conserved cellular homeostatic mechanism, in an attempt to reconcile the imbalance; if the imbalance persists, the UPR can ultimately lead to cell death (Ron and Walter, 2007). Not surprisingly, ER stress elevation and activation of the UPR are implicated in the pathology of many diseases, including myelin disorders such as multiple sclerosis and Charcot-Marie-Tooth disease (D’Antonio et al., 2009; Lin and Popko, 2009).

Myelin is a multilayered membrane formed by the wrapping of glial cells around axons that allows for efficient conduction of action potentials in the vertebrate nervous system (Nave and Trapp, 2008). Specialized glial cells generate the myelin sheath: oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). Myelin is formed as an elaboration of the plasma membrane of the glial cells, which must generate tremendous amounts of membrane proteins and lipids (Anitei and Pfeiffer, 2006). Segments of myelin are separated by the nodes of Ranvier, which are unmyelinated regions of axonal membrane containing a high density of voltage-gated sodium channels (NaV; Salzer 2003; Salzer et al., 2008). These channels propagate the action potential by generating current in response to membrane depolarization (Ritchie, 1995).

To discover genes required for the development and organization of myelinated axons, we performed a genetic screen in zebrafish to identify mutants with disruptions in
the node of Ranvier (Voas et al., 2007; Voas et al., 2009; M.G.V. and W.S.T., unpublished). One mutation identified in this screen disrupts the zebrafish ortholog of the translocon protein Sec63. We show that the UPR is active in zebrafish sec63 mutants and that sec63 mutant axons in the CNS and the PNS are hypomyelinated, with reduced and abnormal NaV clusters. Given the role of Sec63 in human PCLD, we also examined the livers of zebrafish sec63 mutants. We show that pathology develops in this organ, with unusual accumulations of enlarged ER cisternae, disrupted bile canaliculi, and accumulation of large, debris-laden lysosomes. These results raise the possibility that ER stress contributes to PCLD caused by SEC63 mutations and offer a new model for diseases involving protein trafficking and ER stress.
Results

Identification and analysis of st67 mutants

In order to understand the genetic mechanisms underlying the organization of myelinated axons, we performed a genetic screen in zebrafish to identify mutations that affect the development of the nodes of Ranvier (Voas et al., 2007; Voas et al., 2009; M.G.V. and W.S.T., unpublished data). In this screen, we examined expression of voltage-gated sodium channels (NaV) along zebrafish axons in whole-mounts of immunostained larvae. A mutation, st67, was identified with a strong defect in NaV clustering along axons. st67 mutants have morphologically abnormal and reduced numbers of NaV channel clusters along the posterior lateral line nerve (PLLn) at 5 dpf (fig. 1D, E). Time course analysis determined that the number of nodal NaV clusters in the mutant PLLn was normal at 3 dpf (fig. 1A, B, E) but was significantly reduced in mutant larvae at 4 dpf and later (fig. 1E). In st67 mutants, axonal acetylated tubulin expression in the PLLn appeared to be normal (fig. 1B, D), and the number of Schwann cells along the PLLN at 40 hours postfertilization (hpf) was also similar to wildtype by in situ hybridization for sox10 (data not shown). The sec63st67 mutation is lethal; mutant larvae fail to inflate their swimbladders (fig. S1) and most do not survive past 14 dpf.

To better understand myelination defects in the st67 mutants, we examined nerve ultrastructure by transmission electron microscopy (TEM). In the developing PNS of mammals and zebrafish, Schwann cells sort axonal segments away from other axons and subsequently myelinate them (Webster et al., 1973; Raphael et al., 2011). At 3 dpf, an early stage of myelination, the extent of axon sorting and myelination by Schwann cells
was similar in st67 mutant PLLN and in wildtype PLLN (fig. 2F, G). In contrast, at 5 dpf, fewer axons were sorted and myelinated in the st67 mutant PLLN compared to wildtype (fig. 2A-D, F, G), and those axons that were myelinated in st67 mutants were surrounded by fewer myelin wraps compared to wildtype (WT = 6.17 wraps ± 0.98, n = 6 axons from 4 siblings; st67 mutant = 4.5 wraps ± 0.55, n = 6 axons from 3 mutants; p = 0.001). In st67 mutants, Schwann cells had abnormally swollen ER (fig. 2D, E), which is indicative of ER stress and ER lumenal protein accumulation (Tsang et al., 2007; Sharma et al., 2011). Thus, the initial stages of myelination and node of Ranvier formation appear normal in st67 mutants at 3 dpf, but myelination and nodal NaV clusters are disrupted by 5 dpf.

Ultrastructural analysis revealed that the nodes of Ranvier are similar in wildtype and st67 mutants at 5 dpf (fig. S2). One notable difference, however, between mutant and wildtype siblings was that Schwann cell nuclei were often directly over or in close proximity to nodes of Ranvier in st67 mutants (fig. S2; n = 4/8 mutant nodes examined, 0/12 WT nodes examined). Schwann cell nuclei are normally located roughly in the middle of a myelin segment (Lubinska, 1959), but have been observed over nodes in mammalian models of peripheral neuropathy (Low, 1976; Robertson et al., 1997).

Examination of NaV expression and myelin in the CNS of st67 mutants revealed that the spinal cord was more severely affected than the PLLN. In contrast to the PLLN, which is indistinguishable from wildtype at 3 dpf, there were fewer NaV clusters in the ventral spinal cord of the mutant at 3 dpf (fig. 3A, B; fig. 4B-F). We did not observe a
reduction in oligodendrocyte number in the spinal cord at 40 hpf by in situ hybridization for sox10 (data not shown). By TEM analysis at 5 dpf, axons in the spinal cord were more thinly myelinated in the st67 mutant than in wildtype (fig. 3C, D; WT = 3.16 wraps ± 0.85, n = 25 axons from 4 siblings; st67 mutant = 2.53 wraps ± 0.64, n = 15 axons from 3 mutants; p = 0.01). Similar to Schwann cells, we also noted swollen and elaborate endoplasmic reticulum in the cytoplasm of oligodendrocytes (fig. 3D, E).

**st67 disrupts zebrafish sec63**

By high-resolution genetic mapping, we determined that st67 disrupts the zebrafish homolog of sec63. In st67, a T to G transversion is predicted to change a highly conserved tyrosine to an aspartic acid at amino acid position 647 (fig. 4A). All mutants tested were homozygous for the T to G mutation (n > 360), demonstrating that sec63 is tightly linked to the st67 mutation. To obtain additional evidence that sec63 is disrupted by the st67 mutation, we rescued the mutants by injecting synthetic wildtype sec63 mRNA. Injection of 150 pg of sec63 RNA into wildtype and heterozygous embryos did not affect the number or morphology of NaV clusters in the ventral spinal cord at 3 dpf (fig. 4D, F). Control injected st67 mutants had a significant reduction in the number of NaV clusters in the ventral spinal cord at 3 dpf compared to wild-type (fig. 4C, F), while NaV clustering was rescued in mutants injected with 150 pg wild-type sec63 (fig. 4E, F). Together, these data indicate that sec63 is disrupted by the st67 mutation.

Sec63 is a member of the ER translocon machinery, and is well conserved from yeast to human (Deshaies et al. 1991; Skowronek et al. 1999; Schnell and Hebert, 2003).
Zebrafish Sec63 is 71% identical and 84% similar to human Sec63. Human Sec63 is predicted to span the ER membrane three times and contain a luminal N-terminus, a cytoplasmic C-terminus, with a coiled-coil region and a luminal DnaJ domain between the second and third transmembrane pass (fig. 4A; Davila et al., 2004; Muller et al., 2010; Waanders et al., 2010). By RT-PCR, sec63 is expressed at all stages examined in wildtype and st67 mutants. (fig. S3, data not shown), and whole mount in situ hybridization showed that sec63 is broadly expressed (fig. S3B, C; Thisse and Thisse, 2004), with strong staining in the liver and pancreas (fig. S3C).

**ER stress response is upregulated in sec63st67 mutants**

To counter the accumulation of unfolded proteins in the ER lumen, genes encoding chaperone proteins are transcriptionally activated during the unfolded protein response (UPR), increasing the protein folding capacity of the ER. One of the best-characterized ER chaperones upregulated by the UPR is BiP, which encodes the immunoglobulin heavy chain-binding protein (Lee, 2005), a chaperone that belongs to the highly conserved hsp70 protein family (Munro and Pelham, 1986; Nicholson et al., 1990). If ER stress cannot be resolved, the UPR leads to transcriptional upregulation of pro-apoptotic genes, such as CHOP (C/EBP-homologous protein), which is downstream of the PERK-eIF2α pathway (Harding et al., 2000). ER transmembrane proteins including Inositol-requiring-protein-1 (IRE1), a kinase that possesses site-specific endoribonuclease (RNase) activity, also control UPR signaling. The only known target of this RNase activity is X-box binding protein-1 (XBP1). Upon accumulation of misfolded proteins in the ER lumen, IRE1 splices the mRNA of XBP-1, excising a 26 nt
fragment. The generation of this noncanonical splice mRNA is specific to UPR activation, and the translated protein is a potent activator of UPR target genes (Yoshida et al., 2001; Calfon et al., 2002).

To test the hypothesis that the sec63<sup>st67</sup> mutation activates the UPR, we examined xbp-1 splicing as well as bip and chop expression levels in sec63<sup>st67</sup> mutants. The UPR-specific spliced form of xbp-1 was upregulated in sec63<sup>st67</sup> embryos and larvae at all time points examined (fig. 5A). By quantitative real-time PCR, we also found that bip was upregulated in sec63<sup>st67</sup> mutants at 3 dpf and 5 dpf, and that chop is upregulated in sec63<sup>st67</sup> mutants at 5 dpf (fig. 5B, C). Together, these data show an upregulation of multiple UPR markers in sec63<sup>st67</sup> mutants, suggesting that ER stress levels are elevated, as one might expect in mutants with disruptions in sec63.

**sec63<sup>st67</sup> mutant livers develop abnormally**

Mutations in SEC63 cause polycystic liver disease in humans, an inherited, progressive disorder characterized by the presence of numerous cysts throughout the liver (Everson et al., 2004). Although disease symptoms appear in adulthood, the cysts are thought to arise during embryonic development from intralobular bile ducts (Desmet 1992, Qian et al., 2003). Interestingly, one of the disease-causing mutations in patients with polycystic liver disease affects a tryptophan residue near the st67 lesion (*, fig. 4A; Waanders et al. 2010). To determine if the sec63<sup>st67</sup> mutation causes liver pathology in zebrafish larvae, we examined the ultrastructure of this organ at 5 dpf and at 8 dpf. As in glia, at 5 dpf, we observed swelling and fragmenting of the ER, again indicative of ER
stress (fig. S4). At 8 dpf the changes are even more obvious: fragmentation and swelling of the ER with accumulation of a dense matrix in the ER lumen (fig. 6A, B) are accompanied by regions of empty cytoplasm (fig. 6C, D), smaller mitochondria with a dense matrix and wider cristae (fig. 6; fig S2), as well as disrupted and disorganized bile canaliculi (fig. 6D). Finally, we observed multiple regions of sec63^{st67} mutant livers laden with large lysosomes filled with debris and these are more frequent and prominent at 8 dpf than at 5 dpf (fig. 6F). This phenotype was also observed in the intestine of the mutants (data not shown), but never in wild-type siblings.

Additionally, ER stress has been linked to fatty liver disease in humans (Asselah et al., 2010; Hotamisligil 2010) and to steatosis liver pathologies in zebrafish (Cinaroglu et al., 2011). Therefore, to assess steatosis in sec63^{st67} mutant livers, we performed Oil Red O stain at 3, 5, and 8 dpf. No changes in stain intensity were observed in sec63^{st67} mutants at 3 dpf (data not shown; n = 11 WT, 22 heterozygotes, 8 sec63^{st67} mutants) or at 5 dpf (fig. 7; n = 6 WT, 12 heterozygotes, 7 sec63^{st67} mutants). However, at 8 dpf, all sec63^{st67} mutant livers examined showed strong Oil Red O stain compared to wildtype and heterozygous siblings (fig. 7, n = 14 WT, 34 heterozygous, and 8 sec63^{st67} mutants), indicative of liver steatosis. Together, our analyses show that the sec63^{st67} mutation disrupts liver development, in addition to myelinating glia in the PNS and CNS.
Discussion

Starting with a genetic screen for mutants with abnormal nodes of Ranvier, we identified a mutation in sec63 that disrupts nodal NaV clusters in the PNS and the CNS of zebrafish larvae. The specific disruption of myelination and liver development in sec63<sup>st67</sup> mutants is consistent with the requirements of myelinating glia and hepatocytes to synthesize very large amounts of membrane and secreted protein during development, making these cells especially sensitive to perturbations of the secretory pathway (Wrabetz et al., 2004; Saher et al., 2005; Suter and Scherer, 2005; Anitei and Pfeiffer, 2006; Oratz et al., 1975; Crane and Miller, 1977). We show that multiple markers of the unfolded protein response are upregulated in sec63<sup>st67</sup> mutants, and we hypothesize that the pathologies we observe in myelinating glia (fragmented and swollen ER, disrupted NaV clustering, and hypomyelination) are a general consequence of ER stress and disruption of the secretory pathway, and not a specific function for Sec63 in node of Ranvier formation or myelination. Similarly, the liver steatosis and the pathologies we observe in hepatocytes (fragmented and swollen ER, smaller mitochondria with denser matrices and wider cristae, disrupted bile canaliculi, and debris-laden lysosome accumulation) could also be caused by general ER stress in addition to disrupted protein trafficking. In this model, other cells with very active secretory pathways should be preferentially affected by the sec63<sup>st67</sup> mutation. Indeed, we also observe debris-laden lysosome accumulation in the mutant intestine (data not shown).
Comparison with previous analysis of Sec63 in yeast and mammals suggests a number of ways in which the missense mutation in the cytosolic region of Sec63 observed in sec63\textsuperscript{st67} mutants could disrupt translocon function and protein folding. In yeast and mammals, the cytosolic region of Sec63 interacts with Sec62 (Panzer et al., 1995; Tyedmers et al., 2000; Wittke et al., 2000; Willer et al., 2003; Wang and Johnsson, 2005); in yeast, this interaction is essential for protein transport into the ER and is thought to play the same role in higher vertebrates (Muller et al., 2010). In this way, the sec63\textsuperscript{st67} mutation may lead to decreased preprotein translocation into the ER. The lumenal J-domain of Sec63 is required for interactions with the chaperone BiP (Brodsky and Schekman, 1993; Scidmore et al., 1993; Lyman and Schekman, 1995; Corsi and Schekman, 1997; Tyedmers et al., 2000), and although the sec63\textsuperscript{st67} mutation is located in the cytosolic region, the lesion may also lead to a change in Sec63 structure or function that disrupt BiP interactions and therefore protein folding in the lumen of the ER. Finally, in yeast, the Sec61p-Sec63p-BiP translocation complex can also retrogradely transport of misfolded proteins out of the ER for cytosolic proteasome degradation (Plemper et al., 1997). This function of the complex remains to be described in higher eukaryotes, but if it is conserved, the sec63\textsuperscript{st67} mutation may also inhibit transport and degradation of misfolded proteins. In each of these scenarios, a combination of UPR activation and reduced secretion of essential proteins may underlie the pathologies in sec63\textsuperscript{st67} mutants.

Given the essential function of Sec63 in the secretory pathway, it is perhaps surprising that the sec63\textsuperscript{st67} mutants survive for approximately 14 days. Indeed, germline deletion of Sec63 in mammals results in fully penetrant early embryonic lethality.
(Fedele et al., 2011). In zebrafish, maternally supplied transcripts or compensation by another translocon protein, ERj1, may account for the absence of early, general defects observed in the sec63<sup>st67</sup> mutants. ERj1 is an Hsp40 family member related to Sec63 that also provides a luminal J-domain for BiP interactions. Importantly, expression of human ERj1 in yeast can complement mutations in sec63<sup>p</sup> (Kroczynska et al., 2004). Another, not mutually exclusive, possibility is that the mutant protein encoded by the sec63<sup>st67</sup> allele retains enough activity to support some essential functions of the Sec63 protein.

**The unfolded protein response in myelinating disorders**

After UPR was first described in *Saccharomyces cerevisiae* (Kozutsumi et al., 1988; Patil and Walter, 2001), a growing body of evidence has implicated this evolutionarily conserved pathway as a causative factor in many human diseases including liver disease, renal disease, diabetes, cancer, neurodegenerative disease, heart disease, and myelin disorders (Kaufman, 2002; Ron and Walter, 2007; Austin, 2009; D’Antonio et al., 2009; Lin and Popko, 2009). The biogenesis of myelin requires the synthesis of extremely large amounts of myelin lipids and proteins (Wrabetz et al., 2004; Saher et al., 2005; Suter and Scherer, 2005; Anitei and Pfeiffer, 2006). Accordingly, the UPR has been implicated in the pathogenesis of myelin disorders, including Pelizaeus-Merzbacher disease (PMD), vanishing white matter disease, multiple sclerosis, and peripheral neuropathies (for example, van der Voorn et al., 2005, Lin et al., 2005, Lin et al., 2006; Mhaille et al., 2008; Wrabtez et al., 2006; Pennuto et al., 2008).
In PMD, for example, several studies have shown that the mutant forms of the structural myelin proteins Proteolipid protein [PLP] and its alternatively spliced isoform DM20 accumulate in the ER of oligodendrocytes. This activates the UPR and leads to oligodendrocyte death (Gow et al., 1994; Gow and Lazzarini, 1996; Southwood et al., 2002; Swanton et al., 2005; Dhaunchak and Nave, 2007; Dhaunchak et al., 2011). Although, a priori, one might postulate that the pathology in PMD results from reduced levels of PLP in the myelin, a phenotypic comparison of different Plp mutant alleles provides strong evidence against this possibility. Point mutations that cause accumulation of mutant PLP in the ER cause more severe phenotypes than null mutants that lack PLP entirely (Hodes et al., 1993; Klugmann et al., 1997; Swanton et al., 2005). Interestingly, although upregulation of CHOP during the UPR leads to apoptosis in most cell types (Ron and Walter, 2007), it appears to be protective in oligodendrocytes. Analysis of double mutant CHOP null;PLP mutant mice showed that the absence of CHOP exacerbated the clinical phenotype of PLP mutant mice (Southwood et al., 2002). The mechanisms of CHOP’s protective effect on oligodendrocytes are not known, but these genetic studies emphasize the connection between UPR and diseases of myelin. Furthermore, there is substantial evidence from numerous studies that the UPR plays a role in other myelin disorders affecting oligodendrocytes and Schwann cells (reviewed by D’Antonio et al., 2009; Lin and Popko, 2009).

Sec63 in polycystic liver disease

A number of mutations in SEC63 have been identified in patients with autosomal dominant polycystic liver disease (PCLD; Waanders et al. 2010). PCLD is a progressive
disorder characterized by many (>20/liver) fluid-filled liver cysts that may or may not co-occur with autosomal dominant polycystic kidney disease (Torres et al., 2007). PCKD is caused by mutations in \textit{PKD1} and \textit{PKD2}, which respectively encode Polycystin-1 and Polycystin-2 (Ward et al., 1994; Mochizuki et al., 1996). PCLD without PCKD is caused by mutations in \textit{SEC63} (Davila et al., 2004) or \textit{PRKCSH} (Drenth et al., 2003; Li et al., 2003; discussed below). Although polycystic livers retain normal function, cysts lead to increased liver volume, which causes significant morbidity as the enlarged liver impinges upon other organs and impairs their functions. Currently, there are no therapeutic interventions for PCLD, and invasive surgery to remove or aspirate cysts or liver transplant represent the only treatment options (Drenth et al., 2010; Janssen et al., 2010).

\textit{SEC63} mutations in PCLD patients include a T > G missense mutation that changes a conserved tryptophan to a glycine at amino acid position 651 (Waanders et al. 2010). This mutation is located in the same cytosolic region of Sec63 as the \textit{st67} mutation (fig. 4A). This region of Sec63 is located between two \(\beta\)-sheets, and in humans, the W651G mutation is predicted to profoundly alter the structure and therefore function of Sec63 (Waanders et al. 2010).

PCLD can also be caused by mutations in \textit{Protein Kinase C Substrate 80K-H} (\textit{PRKCSH}, also called Hepatocystin; Drenth et al., 2003; Li et al., 2003), which encodes the beta-subunit of Glucosidase II, an enzyme involved in the oligosaccharide processing of newly synthesized glycoproteins (Drenth et al., 2004). PCLD patients are heterozygous for mutations in \textit{PRKCSH} or \textit{SEC63}, and loss of \textit{PRKCSH} heterozygosity has been observed in cells obtained from liver cyst biopsies (Janssen et al., 2011). Loss
of heterozygosity (LOH) for SEC63 mutations remains to be described, but given the observed LOH in PRKSC mutations, a similar model has been proposed for SEC63 (Zimmermann et al., 2006). In line with this model, a recent report in mouse shows that tissue-specific homozygous loss-of-function mutations in Sec63 result in kidney and liver cyst formation (Fedeles et al., 2011). In addition, our analysis shows that homozygous sec63<sup>st67</sup> mutants develop liver pathology. We noted multiple abnormalities in hepatocytes of sec63<sup>st67</sup> mutants, although we did not observe cysts in mutant livers or kidneys. Although human PCLD cysts are thought to arise in bile ducts, our observations of pathology in hepatocytes are consistent with a previous report that showed robust expression of Sec63 in human fetal hepatocytes (Waanders et al., 2008). It is possible that the pathologies that we observe in hepatocytes precede cyst formation in bile ducts, and that cysts might become evident at later stages than we were able to analyze.

It is unclear how mutations in SEC63 lead to PCLD in humans, but there are at least three possible, non-mutually exclusive models. One possibility is that reduced expression of a protein(s) trafficked through the ER causes overgrowth and cyst formation in the liver. In accordance with this model, a recent study provided evidence that levels of Polycystin-1 protein are reduced in Sec63 mutant mouse cells and proposed that this is a key contributing factor to cyst formation (Fedeles et al., 2011). A second possibility is that Sec63 binds to and retains specific proteins at the cytosolic face of the ER, so that Sec63 mutations mislocalize these proteins. This model is supported by the finding that Sec63 physically interacts with the cytosolic protein Nucleoredoxin (Muller et al., 2010), which interacts with Dishevelled to negatively regulate Wnt/beta-cateninin
and Wnt/planar cell polarity pathway (Funato et al., 2006; Funato et al., 2010). Despite this possible connection to Wnt signaling, we did not observe defects in the expression of Wnt targets *axin2* and *naked1* in *sec63*<sup>st67</sup> mutants at 24 hpf (data not shown). Our results highlight a third possible contributing factor, ER stress.

It is likely that a combination of these factors contributes to the final disease outcome. For example, Fedeles et al. (2011) reported that treatment with a proteasome inhibitor ameliorated kidney cyst pathology in mouse models of PCKD. The authors proposed a model in which this treatment raised the levels of Polycystin-1 and also killed cells under severe ER stress. Future work will define the relationship between UPR, the trafficking of specific proteins such as Polycystin-1, and cyst formation. We expect that the *sec63*<sup>st67</sup> mutant zebrafish described in this study will prove useful for future efforts to dissect the pathogenesis of PCLD as well as the consequences of UPR induction in myelinating glia. Future chemical modifier screens may uncover small molecules that alter liver and myelin phenotypes in *sec63*<sup>st67</sup> mutants, and perturbation of ER stress or unbiased screens in heterozygous larvae and adults may shed light on the pathways that drive pathologic alterations in liver, myelinated axons, and other organs.
Methods

Fish strains

Zebrafish embryos were raised at 28.5°C and staged according to (Kimmel et al., 1995). ENU mutagenesis and rearing of fish for screening were performed as described (Pogoda et al., 2006).

Genetic mapping and positional cloning of sec63<sup>st67</sup>

Wild-type and st67 mutants at 5 dpf were sorted by their NaV-clustering phenotype. The st67 mutation was genetically localized via bulked segregant analysis with PCR-based simple sequence length polymorphisms (SSLPs) by standard methods (Talbot and Schier, 1999). Fully sequenced BACs within the st67 interval were identified from the zebrafish fpc database (http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish) and the Ensembl genome browser (http://useast.ensembl.org/Danio_rerio/Info/Index). Predicted coding regions in these BAC sequences were used in additional mapping experiments to further refine the st67 interval. These experiments placed the st67 mutation in a region of LG20 between the clones CH211-278N15 (GenBank accession #BX927123.8) and CH211-260K9 (GenBank accession #BX649264.4). We sequenced the coding regions of the genes in the interval to find the lesion in sec63.

Genotyping

To genotype individual larvae in the phenotypic analyses, the st67 mutation was scored in genomic DNA samples via a TaqαI restriction enzyme recognition site introduced by the lesion. We used the following primers to amplify fragments from genomic DNA
templates: 5’-GGTCACTCTGTACGTTCT-3’ and 5’-TGTGTGATGCTGTTTTGC-3’. To genotype individual larvae in the QRT-PCR analysis (see below), the st67 mutation was again scored via TaqαI cleavage. We used the following primers to amplify cDNA generated from single larvae: 5’-ACAAAGGCAGCGAATCAGAC-3’ and 5’-TGGAGCAGGGAATTTCAGTT-3’. The PCR products were digested with TaqαI to generate a smaller fragment from the mutant allele.

**In situ hybridization and fluorescent antibody labeling**

In situ hybridization was performed with standard protocols (Thisse and Thisse, 2008). To synthesize the sec63 riboprobe, we used the following primers to amplify an 886 bp fragment from a full-length zebrafish sec63 cDNA (Accession #BC076198) obtained from Open Biosystems: 5’-TAAAGGCGGAGATGAGGCTA-3’, 5’-CTTCTCCTCCCGAGGAGTGGCTA-3’, 5’-CTTCTCCTCCCGAGGAGTGGCTA-3’. The PCR product cloned into pCR II-TOPO vector (Invitrogen) and sequenced. This construct was linearized with NotI and transcribed with SP6 for antisense and linearized with BamHI and transcribed with T7 for sense. Antibody labeling and image acquisition was performed as described for anti-panNaV, anti-acetylated tubulin, and anti-Mbp (Voas et al., 2007; Voas et al., 2009; Monk et al., 2009). For NaV cluster quantification in the PNS, we counted the total number of NaV clusters labeled by the anti-panNaV antibody along the entire length of the posterior lateral line nerve. For NaV cluster quantification in the CNS, we counted the number of NaV clusters labeled by the anti-panNaV antibody in two hemisegments (~200 µm) of
ventral spinal cord. Genotypes were determined by PCR assay after image acquisition (IHC) or from larval tail clips after in situ hybridization.

**Microinjections**

A full-length zebrafish sec63 cDNA (Accession #BC076198) was obtained from Open Biosystems and subcloned into the pCS2+ expression vector. The sequence was confirmed, and synthetic sec63 mRNA was generated with the SP6 mMessage mMachine kit (Ambion) after linearization with ApaI. For the rescue experiments, embryos were injected at the 1-2 cell stage with 150 pg of sec63 mRNA in 1.5 nl of 1X Danieau buffer with 5 mg/ml Phenol red. Control embryos were injected with 1.5 nl of 1X Danieau buffer with 5 mg/ml Phenol red.

**Oil Red O stain**

Oil Red O staining was performed as described (Passeri et al., 2009). Genotypes were determined by PCR from larval tail clips following Oil Red O staining.

**RT-PCR**

For traditional RT-PCR, total RNA was isolated from pooled wildtype embryos and larvae at the stages indicated in fig. S3 using TRIZOL according to standard protocols. cDNA was reverse transcribed using SuperScript II reverse transcriptase and random hexamers according to the manufacturer’s instructions. To control for genomic DNA contamination, reverse transcriptase was omitted using the same RNA samples. For RT-PCR, we used the following primers: sec63: 5’-GATTCTGGGGAGGAGAAG-3’, 5’-
TCCCTCTGCTTCGTCTGATT -3’ (457 bp); \textit{xbp-1}: 5’-

CTGTTGCGAGACAGACGAG-3’, 5’-GAAGAGCTCGGAGTCAAGGA-3’ (310 bp unspliced, 275 bp spliced). Either \textit{ef1a} (Monk et al., 2009) or \textit{gapdh}: 5’-

TGACCCATTTCATTGACCTTG-3’, 5’-GCATGACCATCAATGACCAG-3’ (117 bp)

were used as normalization standards. As an additional control for genomic DNA contamination, all primer pairs span introns. RT-PCR was performed according to standard protocols and cycling conditions.

**QRT-PCR**

For quantitative real-time PCR (QRT-PCR), total RNA was isolated from individual larvae obtained from a \textit{sec63\textsuperscript{st67}/+} intercross at 1, 3, and 5 dpf using the RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. cDNA was reverse transcribed using SuperScript II reverse transcriptase and oligo(dT) primers (QRT-PCR) according to the manufacturer’s instructions. To control for genomic DNA contamination, reverse transcriptase was omitted using the same RNA samples. For QRT-PCR, we used the following primers: \textit{bip}: 5’-TCAGCGTCAGGCCTAA-3’, 5’-

GTCAGCAGAGACAGTCAAA-3’ (171 bp); \textit{chop}: 5’-CGGTCCCGACACATCA-3’, 5’-CATTTTCTTTCTTCTCCTGT-3’ (179 bp). \textit{Gapdh} was used as a normalization standard with the primers listed in RT-PCR. As an additional control for genomic DNA contamination, all primer pairs span introns. QRT-PCR was performed using SYBR Green Master Mix (Applied Biosystems) and a Roche LightCycler 2.0 according to the manufacturers’ instructions. Fold change was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).
Tunicamycin administration

2.5 µg/ml tunicamycin (Sigma) or an equal volume of DMSO was added to the embryo medium of manually dechorionated embryos from 3 dpf – 5 dpf. These embryos were used as a positive control for the ER stress assays (fig. 5 and data not shown).

Transmission electron microscopy

Larval fixation and embedding for electron microscopy was performed as described (Czopka and Lyons, 2011). The number of larvae examined in each experiment are provided in the figure legends. Sections were acquired and stained as described (Czopka and Lyons, 2011), and imaged on a Jeol 1230 or a Philips 410 electron microscope.
Acknowledgements

We thank members of the Talbot laboratory for helpful discussion and Dave Lyons, Florence Marlow, Julie Perlin, and Alya Raphael for critical evaluation of the manuscript; Alison Walker for excellent technical support in the initial mapping of the *st67* mutation; Tuky Reyes and Chenelle Hill for outstanding fish care; and Roel Nusse and Angela Bowman for sharing the Roche LightCycler. This work was supported by grants to W.S.T. from the National Multiple Sclerosis Society (RG3943-A-2) and the National Institute of Health (NIH; NS050223) and to C.F.A from NIH (AR055104 to K.G. Beam). K.R.M. was supported by a National Multiple Sclerosis Society postdoctoral award (FG1719-A-1) and by the Stanford Genome Training Program (NIH/NGHRI). M.G.V. was supported by a fellowship from the NIH.

Competing interests statement

The authors declare no competing financial interests.

Author contributions

M.G.V. and W.S.T. designed and performed the genetic screen, and M.G.V identified the *st67* mutation in the screen. K.R.M. and W.S.T. designed the experiments, and K.R.M. and I.S.H. performed the experiments. C.F.A obtained liver electron micrographs and analyzed the mutant liver pathology. K.R.M. and W.S.T. wrote the manuscript, and all authors read, discussed, and edited the manuscript.
Translational Impact

In a forward genetic screen for zebrafish mutants with abnormal sodium channel clustering, we uncovered a mutation in sec63, which encodes a component of the ER translocon. Multiple ER stress markers are activated in the mutants. Many studies link ER stress with myelin disorders, including multiple sclerosis and peripheral neuropathy. Mutations in human SEC63 cause polycystic liver disease (PCLD). The sec63st67 mutants represent a new model to study PCLD as well as the role of ER stress in myelin pathology and other disorders.
Figure 1: Nodes of Ranvier are abnormal in st67 mutant zebrafish at 5 dpf. (A–D) Images of axons from the posterior lateral line nerve (PLLn) in larvae of the indicated genotypes at 3 (A, B) and 5 days post fertilization (dpf; C, D). Axons were double labeled with anti-acetylated tubulin (AcTub, red) and an antibody against the voltage-gated sodium channel (NaV, green). In wildtype (WT) and st67 mutant larvae, sodium channel clusters appear as discrete labeled puncta. At 3 dpf, no differences are observed in either frequency or morphology of NaV clusters in st67 mutants compared to wildtype (A, B). At 5 dpf, st67 mutants have fewer NaV clusters, and many NaV clusters are more diffuse than in WT PLLn (C, D). (E) Quantification of the total number of NaV clusters along the entire length of the PLLn in wildtype and st67/+ larvae (WT, black bars) compared to homozygous st67 mutants (st67, gray bars) at the indicated developmental stages. The P values for unpaired t-test comparisons (two-tailed) are shown, and the error bars show the standard deviation. Sample sizes: at 3 dpf, n = 23 sibling (WT and st67/+) and 9 mutant; at 4 dpf, n = 18 sibling and 8 mutant; at 5 dpf, n = 7 sibling and 9 mutant. Genotypes were assessed by PCR after photography. (A–D) scale bar = 10 µm.
Figure 2: PLLn axons are hypomyelinated in *st67* mutants. (A–E) Transmission electron micrographs showing cross-section through the PLLn at 5 dpf. Sorted axons (s) are completely surrounded by Schwann cells, and some sorted axons are myelinated (*). In WT (A,C), many axons are surrounded by several wraps of myelin at 5 dpf. In *st67* mutants (B, D), fewer axons are myelinated at 5 dpf, and irregular Schwann cell cytoplasm is observed with visibly swollen endoplasmic reticulum (D). (E) Blow up of boxed region in (D) showing swollen ER (arrows). (F,G) A significant decrease in both sorted (F) and myelinated (G) axons is detected in *st67* mutant larvae at 5 dpf, but not at 3 dpf. The P values for unpaired t-test comparisons (two-tailed) are shown, and the error bars show the standard deviation. Sample size: 3 dpf, n = 4 nerves from 4 siblings and 6 nerves from 5 mutants; at 5 dpf, n = 8 nerves from 5 siblings and 6 nerves from 3 mutants. All larvae were imaged at and quantifications made from approximately the same location along the anterioposterior axis, at the level of the 7th hemisegment. (A,B) scale bar = 2 μm (C, D) scale bar = 0.5 μm.
Figure 3: Nodes of Ranvier are disrupted in the spinal cord of st67 mutants, and spinal cord axons are hypomyelinated. (A,B) show Myelin basic protein (Mbp, red) and voltage-gated sodium channel (NaV, green) antibody staining in the ventral spinal cord at 3 dpf. At 3 dpf, no differences are observed in the intensity of Mbp stain in st67 mutants (B) compared to siblings (A), but NaV puncta are greatly reduced in st67 mutants (see also fig. 4). (C–E), Transmission electron micrographs showing cross-sections through the ventral spinal cord at 5 dpf. (C) At 5 dpf, many axons in sibling spinal cord are surrounded by several wraps of myelin (*). (D) Fewer axons are myelinated (*) in st67 mutant spinal cord, and irregular oligodendrocyte cytoplasm is observed with visibly swollen endoplasmic reticulum (ER; arrowheads) in all mutants examined. (E) shows an enlarged view of the boxed region in (D). Arrowheads denote swollen ER. We examined 8 wild type and heterozygous larvae and 3 mutant larvae. (A,B) scale bar = 20 µm; (E,F) scale bars = 1 µm.
Figure 4; *st67* disrupts zebrafish *sec63*. (A) Schematic representation of Sec63 showing functional domains and the location of the lesion in the *st67* mutation. TM, transmembrane domain; DnaJ, DnaJ domain; Sec63, Sec63 domain; CC, coiled-coil region. Also shown are sequence traces from homozygous WT and *st67* mutant larvae. The *st67* mutation changes a conserved tyrosine to an aspartic acid in the Sec63 domain. A comparison of zebrafish and human Sec63 amino acid sequence in the vicinity of the *st67* mutation is also shown. The light blue box indicates the location of the lesion in *st67* zebrafish mutants. The asterisk marks the position of a *SEC63* mutation identified in human patients with polycystic liver disease (W651G; Waanders et al., 2010). (B–E) Representative images of antibody-stained preparations of the spinal cord of larvae of the indicated genotypes and injection treatments at 72 hpf. (B) Siblings injected with control solution show normal NaV clustering (arrowheads). (C) *st67* mutants injected with control solution show aberrant NaV clustering. (D,E) Siblings and *st67* mutants injected with 150 pg of synthetic *sec63* mRNA show normal NaV clustering. (H) Quantification of the total number of NaV puncta in two hemisegments (~200 µm) of ventral spinal cord of sibling (black bars) and *st67* mutant larvae (gray bars) at 72 hpf following the indicated injection regiments. The P values for unpaired t-test comparisons (two-tailed) are shown, and the error bars show the standard deviation. N = 9 control-injected siblings; n = 6 control-injected mutants; n = 12 *sec63*-injected siblings; n = 11 *sec63*-injected mutants. (B–E), scale bar = 20 µm.
Figure 5: Markers of ER stress are elevated in sec63<sup>str67</sup> mutants. (A) RT-PCR showing expression of spliced and unspliced <i>xbp-1</i> in individual larvae of the indicated genotypes and developmental stages. During the UPR, a 26 nt fragment of <i>xbp-1</i> mRNA is spliced. RT-PCR shows that the spliced form of <i>xbp-1</i> (S) is enriched in sec63<sup>str67</sup> mutants compared to wildtype and <i>st67</i>/+ larvae. Unspliced <i>xbp-1</i> (US) is present in all samples. As a positive control for the assay, WT larvae treated with the ER stressor tunicamycin show upregulation of the spliced form of <i>xbp-1</i>. (B,C) Quantitative real time PCR (QRT-PCR) showing relative expression of UPR markers <i>bip</i> (B) and <i>chop</i> (C) in WT (wildtype and heterozygous siblings) vs. sec63<sup>str67</sup> mutant larvae at the indicated stages.
Figure 6: sec63<sup>st67</sup> mutants develop numerous liver pathologies. (A-F) Transmission electron micrographs showing liver ultrastructure at 8 dpf in WT and sec63<sup>st67</sup> mutant zebrafish. (A) WT liver; arrow points to ER with normal morphology. (B) sec63<sup>st67</sup> mutant liver; ER is swollen (arrow) and cytoplasm is disrupted. (C,D) Ultrastructure of bile canaliculi (*) in WT (C) and sec63<sup>st67</sup> mutant (D) livers. sec63<sup>st67</sup> bile canaliculi appear disorganized compared to WT. Arrows in (F) show lysosomes filled with debris in sec63<sup>st67</sup> mutants but not in WT (E). Scale bars, (A-D) = 500nm; (E,F) = 2 μm.
Figure 7: *sec63<sup>st67</sup>* mutants develop liver steatosis. (A-F') Lateral views of larvae stained with Oil Red O of the indicated genotypes and at the indicated developmental stages. (A-C) Arrows indicate the location of the liver in 5 dpf larvae. *sec63<sup>st67</sup>* mutant livers (C, n = 7) are indistinguishable from WT (A, n = 6) or heterozygous (B, n = 12) livers. (D-F) Boxed region denotes the areas enlarged in (D'-F'). (D'-F') Outlines denote the liver in 8 dpf larvae. *sec63<sup>st67</sup>* mutant livers (F, F', n = 8) show stronger Oil Red O stain than WT (D, D', n = 14) or heterozygous (E, E', n = 34) livers. Scale bars = 200 μm.
Figure S1

Figure S1: Morphology of st67 mutant larvae. (A,B) Morphology of sibling (Sib, A) and st67 mutant (B) zebrafish at 7 dpf. st67 mutants are slightly smaller than wildtype and heterozygous siblings and fail to inflate their swim bladders (arrowhead).
Figure S2: Nodes of Ranvier display subtle ultrastructural defects in *st67* mutants. When observed by TEM, *st67* nodes of Ranvier (B,D) are generally indistinguishable from sibling nodes of Ranvier (A,C), although nodal lengths may be slightly longer in *st67* mutants at 5 dpf (WT average length 0.56±0.12 µm, n = 6 nodes from 3 siblings; *st67* mutant average length 0.72±0.06 µm, n = 4 nodes from 2 mutants; *p* = 0.03). Additionally, Schwann cell nuclei (n) are observed near or directly over mutant nodes (A vs. B, 4/8 nuclei over nodes in *st67* mutants vs. 0/12 nuclei over nodes in siblings) and node length appears to be slightly longer in *st67* mutants (D, bracket) compared to siblings (C, bracket). (C,D) show enlarged view of boxed region in (A,B). (A,B) scale bar = 1 µm; (C,D) scale bar = 0.2 µm.
Figure S3: 

**sec63** expression during development (A-C) Expression of **sec63** in developing zebrafish. (A) RT-PCR showing expression of **sec63** in WT embryos at the indicated stages: M, maternal; 70%, 70% epiboly; 25s, 25 somites; 1d – 5d, 1 dpf – 5 dpf. Sample lacking reverse transcriptase is included as a control, and expression of **ef1α** is included as a normalization standard. (B, C) Whole mount in situ hybridization shows **sec63** expression in WT embryos at 22.5 hpf (B) and at 3 dpf (C). In (C), the arrow denotes expression in the liver, and the arrowhead denotes expression in the pancreas.
Figure S4: Liver pathology in sec63<sup>st67</sup> mutants is observed at 5 dpf. (A, B) Transmission electron micrographs showing liver ultrastructure at 5 dpf in WT and sec63<sup>st67</sup> mutant zebrafish. (A) WT liver; arrow points to ER with normal morphology. (B) sec63<sup>st67</sup> mutant liver; some ER is swollen (arrows) Scale bars = 500nm.
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**Figure S3:** *sec63* expression during development (A-C) Expression of *sec63* in developing zebrafish. (A) RT-PCR showing expression of *sec63* in WT embryos at the indicated stages: M, maternal; 70%, 70% epiboly; 25s, 25 somites; 1d – 5d, 1 dpf – 5 dpf. Sample lacking reverse transcriptase is included as a control, and expression of *ef1α* is included as a normalization standard. (B, C) Whole mount in situ hybridization shows *sec63* expression in WT embryos at 22.5 hpf (B) and at 3 dpf (C). In (C), the arrow denotes expression in the liver, and the arrowhead denotes expression in the pancreas.

**Figure S4:** Liver pathology in *sec63*<sup>st67</sup> mutants is observed at 5 dpf. (A, B) Transmission electron micrographs showing liver ultrastructure at 5 dpf in WT and *sec63*<sup>st67</sup> mutant zebrafish. (A) WT liver; arrow points to ER with normal morphology. (B) *sec63*<sup>st67</sup> mutant liver; some ER is swollen (arrows) Scale bars = 500nm.
Figure S1
Figure S2

A. WT 5 dpf

B. st67 5 dpf

C. 5 dpf

D. 5 dpf
Figure S3
Figure S4
Fig. S1. Morphology of st67 mutant larvae. (A,B) Morphology of sibling (Sib, A) and st67 mutant (B) zebrafish at 7 dpf. st67 mutants are slightly smaller than wild type and heterozygous siblings and fail to inflate their swim bladders (arrowhead).
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**Fig. S3. sec63 expression during development.** (A-C) Expression of *sec63* in developing zebrafish. (A) RT-PCR showing expression of *sec63* in WT embryos at the indicated stages: M, maternal; 70%, 70% epiboly; 25s, 25 somites; 1d – 5d, 1–5 dpf. Sample lacking reverse transcriptase is included as a control, and expression of *ef1α* is included as a normalization standard. (B, C) Whole mount in situ hybridization shows *sec63* expression in WT embryos at 22.5 hpf (B) and at 3 dpf (C). In C, the arrow denotes expression in the liver, and the arrowhead denotes expression in the pancreas.
Fig. S4. Liver pathology in sec63st67 mutants is observed at 5 dpf. (A,B) Transmission electron micrographs showing liver ultrastructure at 5 dpf in WT and sec63st67 mutant zebrafish. (A) WT liver; arrow points to ER with normal morphology. (B) sec63st67 mutant liver; some ER is swollen (arrows) Scale bars: 500 nm.