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

Kelly R. Monk1,2, Matthew G. Voas1,*, Clara Franzini-Armstrong3, Ian S. Hakkinen2 and William S. Talbot1,‡

SUMMARY
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 peripheral nerves. Additionally, we observed 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, Sec61β and Sec61γ form the pore, and this trimeric complex is associated with other proteins including ERβ1, 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 ten 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 course of the disease (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 (Fedeles et al., 2011) and disrupted tethering of proteins to the cytosolic face of the ER (Müller 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, elevation of ER stress 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

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Results
In a forward genetic screen for zebrafish mutants with abnormal sodium channel clustering in myelinated axons, the authors identified sec63<sup>mut</sup>, a missense mutation in sec63. Mutant larvae showed multiple defects in myelinated axons in the peripheral nervous system (PNS) and central nervous system (CNS), including disruptions in myelination. Additionally, liver development was abnormal in the sec63<sup>mut</sup> mutants; however, cysts were not observed in the liver or kidneys. Swelling and fragmentation of the ER was observed in cells of the PNS, CNS and liver, and multiple molecular markers of ER stress were activated in sec63<sup>mut</sup> mutants.

Implications and future directions
These findings introduce the sec63<sup>mut</sup> mutant as a new model for studying the function of a gene implicated in PCLD, as well as the role of ER stress in disorders of disrupted myelination. Given that myelinating glia and hepatocytes synthesize large amounts of membrane and secreted proteins during development, they are highly susceptible to disruptions in the translocon machinery. This report advances our understanding of how ER stress contributes to disease processes and provides a new model for investigating the underlying mechanisms.

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, 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 data). One mutation identified in this screen disrupts the zebrafish ortholog of the translocon protein Sec63. We show that 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 canalici 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.

Clinical issue
Mutations in human SEC63 cause polycystic liver disease (PCLD). There are few treatment options for PCLD; only invasive surgery or liver transplantation can change the course of the disease. Although it is known that SEC63 is part of the endoplasmic reticulum (ER) translocon complex, which transports nascent polypeptides across membranes for folding and maturation in the ER, how mutations in SEC63 cause PCLD has been unclear. Among many hypotheses, it has been proposed that SEC63 mutations might trigger ER stress, which occurs when the capacity of the ER to process nascent or damaged proteins is overloaded. Many studies have linked ER stress with myelin disorders, including multiple sclerosis and peripheral neuropathy.

Implications and future directions
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RESULTS
Identification and analysis of st67 mutants
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The sec63<sup>st67</sup> mutation is lethal; mutant larvae fail to inflate their swim bladders (supplementary material 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 wild-type PLLn (Fig. 2F,G). By contrast, at 5 dpf, fewer axons were sorted and myelinated in the st67 mutant PLLn compared with wild type (Fig. 2A-D,F,G), and those axons that were myelinated in st67 mutants were surrounded by fewer myelin wraps compared with wild type (wild type, 6.17±0.98 wraps; n=6 axons from four siblings; st67 mutant, 4.5±0.55 wraps; n=6 axons from three 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 wild-type and st67 mutants at 5 dpf (supplementary material Fig. S2). One notable difference, however, between mutant and wild-type siblings was that Schwann cell nuclei were often directly over or in close proximity to nodes of Ranvier in st67 mutants (supplementary material Fig. S2; 4 of 8 mutant nodes examined and 0 of 12 wild-type nodes). 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 the wild type 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 using in situ hybridization for sox10 (data not shown). TEM analysis at 5 dpf showed that axons in the spinal cord...
were more thinly myelinated in the st67 mutant than in the wild type (Fig. 3C,D; wild type, 3.16±0.85 wraps, n=25 axons from four siblings; st67 mutant, 2.53±0.64 wraps, n=15 axons from three mutants; P=0.01). Similarly 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 mRNA encoding wild-type sec63. Injection of 150 pg of sec63 mRNA into wild-type and heterozygous embryos did not affect the number or morphology of NaV clusters in the ventral spinal cord at 3 dpf (Fig. 4D,F), st67 mutants injected with control solution showed a significant reduction in the number of NaV clusters in the ventral spinal cord at 3 dpf compared with wild type (Fig. 4C,F), whereas NaV clustering was rescued in mutants injected with 150 pg of wild-type sec63 mRNA (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; Müller 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; Müller et al., 1999; Schnell and Hebert, 2003). RT-PCR showed that sec63 is expressed at all stages examined in wild-type and st67 mutants. (supplementary material Fig. S3, data not shown), and whole-mount in situ hybridization showed that sec63 is broadly expressed (supplementary material Fig. S3B,C) (Thiisse and Thissee, 2004), with strong staining in the liver and pancreas (supplementary material Fig. S3C).

**ER stress response is upregulated in sec63<sup>st67</sup> 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 XBP1, excising a 26-nucleotide fragment. The generation of this noncanonically spliced 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). Using quantitative real-time PCR (qRT-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 PCLD 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 PCLD affects a tryptophan residue near the st67 lesion (Waanders et al., 2010). To determine whether 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 (supplementary material Fig. S4). At 8 dpf, the changes were even more obvious: fragmentation and swelling of the ER with accumulation of a dense matrix in the ER lumen (Fig. 6A,B) were accompanied by regions of empty cytoplasm (Fig. 6C,D), smaller mitochondria with a dense matrix and wider cristae (Fig. 6; supplementary material Fig. S2), as well as disrupted and disorganized bile canaliculi (Fig. 6D). Finally, we observed multiple regions of sec63<sup>st67</sup> mutant livers laden with large lysosomes filled with debris and these were 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<sup>st67</sup> mutant livers, we performed Oil Red O staining at 3, 5 and 8 dpf. No changes in stain intensity were observed in sec63<sup>st67</sup> mutants at 3 dpf (data not shown; sample sizes were 11 wild type, 22 heterozygotes and 8 sec63<sup>st67</sup> mutants) or at 5 dpf (Fig. 7; sample sizes were 6 wild type, 12 heterozygotes and 7 sec63<sup>st67</sup> mutants). However, at 8 dpf, all sec63<sup>st67</sup> mutant livers examined showed strong Oil Red O stain compared with wild-type and heterozygous siblings (Fig. 7; sample sizes were 14 wild type, 34 heterozygous and 8 sec63<sup>st67</sup> mutants), which is indicative of liver steatosis. Together, our analyses show that the sec63<sup>st67</sup> 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 proteins during development, making these cells especially sensitive to perturbations of the secretory pathway (Wrabetz et al., 2004; Saher et al., 2005; Suter and Scherer, 2003; 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 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 of Sec63 in node of Ranvier formation or myelination. Similarly, the liver steatosis and the pathologies we observed 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 observed debris-laden lysosome accumulation in the mutant intestine (data not shown).

Comparison with previous analyses 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<sup>st67</sup> mutants could disrupt translocon function and protein folding. In yeast and mammals, the cytosolic region of Sec63 interacts with Sec62 (Panzner 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 (Müller et al., 2010). In this way, the sec63<sup>st67</sup> mutation could lead to decreased preprotein translocation into the ER. The luminal 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). Although the sec63<sup>st67</sup> mutation is located in the cytosolic region, the lesion might also lead to a change in Sec63 structure or function that disrupts BiP interactions and therefore protein folding in the lumen of the ER. Finally, in yeast, the Sec61p-Sec63p-BiP translocation complex can also retrogradely affect 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<sup>st67</sup> mutation might also inhibit transport and degradation of misfolded proteins. In each of these scenarios, a combination of UPR activation and reduced secretion of essential proteins might underlie the pathologies in sec63<sup>st67</sup> mutants.

Given the essential function of Sec63 in the secretory pathway, it is perhaps surprising that the sec63<sup>st67</sup> 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, might account for the absence of the 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 sec63p (Kroczyńska 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., 1998; 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, 2003; Anteix 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 (van der Voorn et al., 2005; Lin et al., 2005; Lin et al., 2006; Mhaille et al., 2008; Wrabetz et al., 2006; Pennuto et al., 2008).

In PMD, for example, several studies have shown that the mutant forms of the structural myelin protein 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 that showed that the absence of CHOP exacerbated the clinical phenotype of PLP mutant mice (Southwood et al., 2002). The mechanisms of the protective effect of CHOP on oligodendrocytes are not known, but these genetic studies emphasize the connection between UPR and diseases of the 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 PCLD (Waanders et al., 2010). PCLD is a progressive disorder characterized by many (>20/liver) fluid-filled liver cysts that might or might not co-occur with autosomal dominant PKCD (Torres et al., 2007). PKCD is caused by mutations in PKD1 and PKD2, which respectively encode polycystin-1 and polycystin-2 (The European Polycystic Kidney Disease Consortium, 1994; Mochizuki et al., 1996). PCLD without PKCD is caused by mutations in SEC63 (Davila et al., 2004) or PRKCSH (Drenth et al., 2003; Li et al., 2003). Although polycystic livers retain normal function, cysts lead to enlarged 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). SEC63 mutations in PCLD patients include a T-to-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 s667 mutation (Fig. 4A). This region of Sec63 is located between two β-sheets; 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 PRKCSH (Drenth et al., 2003; Li et al., 2003), which encodes the β-subunit of glucosidase II (protein kinase C substrate 80K-H, also called hepatocystin), an enzyme involved in the oligosaccharide processing of newly synthesized glycoproteins (Drenth et al., 2004). PCLD patients are heterozygous for mutations in PRKCSH or SEC63, and loss of 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 PRKCSH 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 (Fedele et al., 2011). In addition, our analysis shows that homozygous sec63Δ67 mutants develop liver pathology. We noted multiple abnormalities in hepatocytes of sec63Δ67 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 shows robust expression of Sec63 in human fetal hepatocytes (Waanders et al., 2008). It is possible that the pathologies that we observed 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 suggested that this is a key contributing factor in cyst formation (Fedele 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 (Müller et al., 2010), which interacts with Dishevelled to negatively regulate the Wnt–β-catenin and Wnt–planar cell polarity signaling pathways (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 sec63Δ67 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, Fedele et al. reported that treatment with a proteasome inhibitor ameliorated kidney cyst pathology in mouse models of PCKD (Fedele et al., 2011). 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>1667</sup> mutant zebrafish described in this study will prove useful in future efforts to dissect the pathogenesis of PCDL as well as the consequences of UPR induction in myelinating glia. Future chemical modifier screens might uncover small molecules that alter liver and myelin phenotypes in sec63<sup>1667</sup> mutants and perturbation of ER stress or unbiased screens in heterozygous larvae and adults could 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 published methods (Kimmel et al., 1995). N-ethyl-N-nitrosourea (ENU) mutagenesis and rearing of fish for screening were performed as described (Pogoda et al., 2006).

**Genetic mapping and positional cloning of sec63<sup>1667</sup>**

Wild-type and st67 mutants at 5 dpf were sorted by their NaV-riboprobe, we again scored via Taq cleavage. We used the following primers to refine the 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 TaqI restriction enzyme recognition site introduced by the lesion. We used the following primers to amplify fragments from genomic DNA templates: 5′-GGTCACCTGTCATCGGT-TCT-3′ and 5′-TGTGTGATGCTCATGTTTGC-3′. To genotype individual larvae in the qRT-PCR analysis (see below), the st67 mutation was again scored via TaqI cleavage. We used the following primers to amplify cDNA generated from single larvae: 5′-ACAAAGCGCAGGATCATCGAC-3′ and 5′-TGAGGACAGGGAATTTCAGGT-3′. The PCR products were digested with TaqI 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 (GenBank accession #BC076198) obtained from Open Biosystems: 5′-TAAAGGC-GGAGATGAGGCTA-3′, 5′-CTTCTCCTCCCCCAGAAATC-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 (immunohistochemistry) or from larval tail clips after in situ hybridization.

**Microinjections**

Full-length zebrafish sec63 cDNA (GenBank accession #BC076198) was obtained from Open Biosystems and subcloned into the pCS2+ expression vector. The sequence was confirmed, and synthetic mRNA encoding sec63 was generated with the SP6 mMessage mMachete 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 1× Danieau buffer with 5 mg/ml Phenol Red. Control embryos were injected with 1.5 nl of 1× 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 wild-type embryos and larvae at the stages indicated in supplementary material 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′-GATTCTCGGGGAGGAGAAG-3′ and 5′-TCCCTCTGTCTCGTGATT-3′ (457 bp); xbp-1, 5′-CTGTT-GCGAGACAAGACGAG-3′ and 5′-GAAGAGCTCGGA-GTCAAGGA-3′ (310 bp unspliced, 275 bp spliced). Either eif4a (Monk et al., 2009) or gapdh: 5′-TGACCCATTCATTGACCTTG-3′ and 5′-GCAATGACCCTATCGACCGAG-3′ (117 bp) were used as normalization standards. As an additional control for genomic DNA contamination, all primer pairs spanned introns. RT-PCR was performed according to standard protocols and cycling conditions.

**qRT-PCR**

For qRT-PCR, total RNA was isolated from individual larvae obtained from a sec63<sup>1667</sup>/+ 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 according to the manufacturer’s instructions. To control for genomic DNA contamination, all primer pairs spanned introns. RT-PCR was performed according to standard protocols and cycling conditions.
contamination, reverse transcription was omitted using the same RNA samples. For qRT-PCR, we used the following primers: bip, 5'-TCAGCGTCAGGCACATAA-3’ and 5'-GTACAGCAG-AAGACAGTCAA-3’ (171 bp); chop, 5'-CGGTTC-CGCGACATCA-3’ and 5'-CACTTTCTCTTTAT-CTCCGTGT-3’ (179 bp). 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 spanned 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^ΔΔCT method (Livak and Schmittgen, 2001).

Tunicamycin administration
Tunicamycin (2.5 μg/ml; Sigma) or an equal volume of DMSO was added to the embryo medium of manually dechorionated embryos from 3 dpf to 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 given 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.

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

AUTHOR CONTRIBUTIONS
M.G.V. and W.S.T. designed and performed the genetic screen, and M.G.V. designed the experiments, and K.R.M. and I.S.H. performed the experiments. C.F.A. obtained live 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.

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SUPPLEMENTARY MATERIAL
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REFERENCES


