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Mutations in vacuolar H^+-ATPase subunits lead to biliary developmental defects in zebrafish

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ABSTRACT

We identified three zebrafish mutants with defects in biliary development. One of these mutants, *pekin* (*pn*), also demonstrated generalized hypopigmentation and other defects, including disruption of retinal cell layers, lack of zymogen granules in the pancreas, and dilated Golgi in intestinal epithelial cells. Bile duct cells in *pn* demonstrated an accumulation of electron dense bodies. We determined that the causative defect in *pn* was a splice site mutation in the *atp6ap2* gene that leads to an inframe stop codon. *atp6ap2* encodes a subunit of the vacuolar H^+-ATPase (V-H^+-ATPase), which modulates pH in intracellular compartments. The Atp6ap2 subunit has also been shown to function as an intracellular renin receptor that stimulates fibrogenesis. Here we show that mutants and morphants involving other V-H^+-ATPase subunits also demonstrated developmental biliary defects, but did not demonstrate the inhibition of fibrogenic genes observed in *pn*. The defects in *pn* are reminiscent of those we and others have observed in class C VPS (vacuolar protein sorting) family mutants and morphants, and we report here that knockdown of *atp6ap2* and *vps33b* had an additive negative effect on biliary development. Our findings suggest that pathways which are important in modulating intracompartmental pH lead to defects in digestive organ development, and support previous studies demonstrating the importance of intracellular sorting pathways in biliary development.

Introduction

Understanding biliary development is vital to determining the pathogenesis of hepatobiliary diseases that affect infants and newborns. Infantile biliary diseases result in cholestasis, or poor bile flow, which may lead to cirrhosis and eventual need for liver transplantation. While only a minority of these conditions have a clear genetic etiology, elucidation of the important genes, proteins, and pathways involved in biliary development will lead to greater understanding of the disease processes and may lead to novel treatments.

We use the zebrafish to model hepatobiliary development and disease. There is generally a strong conservation in terms of the overall developmental process and in the importance of specific genetic pathways. In mammals, bipotential hepatoblasts differentiate into hepatocytes and bile duct cells, which form the intrahepatic bile ducts along the developing portal veins, resulting in an arborizing network that drains the liver (Lemaigre and Zaret, 2004). The process of biliary development in mammals continues after birth. In zebrafish, intrahepatic bile ducts lengthen and form connections, leading to a lattice of ducts that drains the liver by around 5 dpf (days post fertilization) (Lorent et al., 2004; Matthews et al., 2004), although the ducts continue to grow and remodel after that time. Mediators of intrahepatic bile duct formation in mammals, such as *Onecut* family members (Clotman et al., 2002, 2005), the homeodomain transcription factor *Hnf1b* (Coffinier et al., 2002), and *Jagged* and *Notch* (Kodama et al., 2004; Lozier et al., 2008), have generally conserved function in zebrafish biliary development (Lorent et al., 2004; Matthews et al., 2004, 2008).

Mutagenesis screens in zebrafish allow unbiased discovery of genes important in developmental processes and disease pathogenesis. Numerous investigators have utilized mutagenesis screens to uncover genes involved in gut development (Pack et al., 1996), the effect of maternally expressed genes on early development (Dosch et al., 2004; Wagner et al., 2004), pigment formation (Pickart et al., 2004), and multiple other developmental processes. The fluorescent lipid reporter PED-6 has been utilized in a previous mutagenesis screen to uncover *fat-free* (*ffr*), which demonstrates abnormal lipid processing and defective intrahepatic biliary development (Ho et al., 2006). PED-6 is a quenched phospholipid that is activated by phospholipase A2, absorbed by the enterohepatic circulation, processed by the liver

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and excreted into intrahepatic bile ducts, and is then excreted into the extrahepatic biliary tree and will accumulate in the gallbladder (Farber et al., 2001). In this study, we used PED-6 to screen for defects in intrahepatic biliary development.

Here we report the identification of three mutants with defects in bile duct development. These mutants — *pekin* (*pn*), *rouen* (*rou*), and *cayuga* (*cay*) — are phenotypically distinct. We report that *pn* is caused by a mutation in *atp6ap2*, which encodes an accessory protein to the vacuolar (V)-H^+-ATPase. The V-H^+-ATPase is a multisubunit complex that is critical in lowering pH in lysosomes, endosomes and synaptic vesicles (Forgac, 2007). It also acts at the plasma membrane, lowering extracellular pH within the renal distal tubule (Saroussi and Nelson, 2009). V-H^+-ATPase activity and vesicular acidification are required for normal intracellular vesicular sorting (Mellman et al., 1986; Sun-Wada et al., 2004). Other roles for intravesicular acidification include release and intracellular processing of endocytosed ligands, as well as mediating or preventing viral invasion of cells (Toei et al., 2010). Others have reported zebrafish mutants involving V-H^+-ATPase subunits, which all demonstrate hypopigmentation and share defects in eye development (Amsterdam et al., 2004; Gross et al., 2005; Wang et al., 2008).

Interestingly, in mammals, Atp6ap2 also acts as an intracellular renin receptor that activates ERK1 and ERK2 (Nguyen et al., 2002) and also directly interacts with the transcription factor promyelocytic leukemia zinc finger (PLZF, *Zbtb16*) (Schefe et al., 2006). Prior to the discovery of a distinct renin receptor, renin was known as a protease that activates angiotensin, which upon further modification acts to increase blood pressure by vasoconstriction and by stimulating the release of aldosterone and anti-diuretic hormone. The physiologic actions of the renin receptor (Atp6ap2) overlap some of the effects of renin itself, including increasing blood pressure (Burckle et al., 2006) and activating fibrosis (Jan Danser et al., 2007).

Our results demonstrate that the V-H^+-ATPase mutant *pn* exhibits developmental biliary defects, most likely via effects on the generation of intracellular pH and vesicular trafficking, and not via effects on renin receptor activity. Knockdown or mutation of genes in intracellular trafficking pathways has been shown previously to lead to abnormal biliary development in zebrafish (Matthews et al., 2005; Sadler et al., 2005; Schonthaler et al., 2008), and patients with a homozygous mutation in the intracellular trafficking gene *VPS33B* develop severe cholestasis (Gissen et al., 2004). These findings, along with those presented here, stress the importance of intracellular trafficking pathways in biliary development and disease pathogenesis.

Materials and methods

Mutagenesis screen for biliary defects

A chemical mutagenesis screen using ethylnitrosourea (ENU) was performed essentially as described previously (Dosch et al., 2004; Wagner et al., 2004), screening the equivalent of 600 genomes. We used PED-6 to screen for families in which 1/4 of the F3 members demonstrated diminished gallbladder uptake, similar to the previous (Farber et al., 2001; Ho et al., 2006). PED-6 is a fluorescent lipid compound that is activated in the intestine and absorbed via the enterohepatic circulation into the liver, where it is processed in the hepatocyte and excreted into the bile, accumulating in the gallbladder (Farber et al., 2001). Lack of gallbladder accumulation suggests possible abnormalities in intrahepatic biliary development. All fish were cared for in the zebrafish facility at the University of Pennsylvania or The Children's Hospital of Philadelphia Research Institute, in accordance with Institutional Animal Care and Use Committee guidelines of both institutions.

Genetic identification of *pn* was performed on F2 progeny, to take advantage of the generation of mutant carriers in AB/Tü strain hybrids. A large panel of standard z-markers was used to establish linkage on chromosome 22, and finer mapping was performed using primers listed in Supplemental Table 1. Candidate genes were identified based on available sequence data (www.sanger.ac.uk) and sequenced using primers designed on the available in silico sequence. Screening for carrier heterozygotes was performed using TaqMan primers containing the single nucleotide polymorphism (SNP) constituting the mutation (Table S1). The altered transcript in the mutant was confirmed using standard and quantitative PCRs, with primers shown in Table S1.

Additional mutant lines were obtained from the zebrafish international resource center (ZIRC). These lines were originally uncovered in an insertional mutagenesis screen in the Hopkins laboratory (Amsterdam et al., 1999). The lines included hi3681 (*atp6ap2*), hi112 (*atp6ap1*), and hi2188b (*atp6v0d1*). In addition, we used morpholinos directed against *atp6v1a* (Supplemental Table 2) to establish additional models of V-H^+-ATPase subunit mutants.

In situ hybridization

In situ hybridizations were performed as described previously (Cui et al., 2011a), except that 0.25% acetic anhydride was added after proteinase K fixation to reduce background (Westerfield, 2000). Primers for synthesis of the *foxa3, atp6ap2* and *dct* probes are noted in Table S2. As a control, larvae with no riboprobe added were processed identically to the samples with riboprobes.

Tissue histology and immunostaining

For experiments involving conventional stainings such as hematoxylin and eosin, 5 dpf larvae were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Slides were treated as per standard protocol and then stained with hematoxylin and eosin. Staining for Atp6ap2 was performed on unstained sections from wild-type 5 dpf larvae in accordance with standard techniques, using an antibody against the renin receptor (H-85; sc-67390; Santa Cruz Biotechnology). As a negative control, sections were treated using the same protocol but without primary antibody.

Whole-mount cytokeratin and 2F11 staining were performed as previously described, after fixation in methanol/DMSO or paraformaldehyde (Matthews et al., 2008). Duct quantification was performed identically to the previous one (Cui et al., 2011a).

Electron microscopy

Samples for electron microscopy were obtained at 5 dpf. Larvae were fixed in buffered glutaraldehyde and prepared as previously described (Matthews et al., 2005). Sections were examined at the Penn Bio-Imaging Core, as previously done.

Non-quantitative PCR and quantitative real-time PCR

Samples for examination of *atp6ap2* expression were obtained by dissecting the liver out of 5 dpf larvae. RNA was isolated from the liver and from separate whole wild-type larva in pooled batches of 5 larvae, reverse transcribed and PCR was performed with primers shown in Supplemental Table 3.

Samples for quantitative real-time PCR were obtained at 5 dpf. RNA was isolated from the whole larvae and was reverse transcribed as per standard protocols, and QPCR was performed generally in accordance with standard protocols, using a StepOne Plus from ABI. Primers are depicted in Supplemental Table 3. Normalization was performed using *hprt*. Graphs depicted are representative experiments comparing 4 individual biological replicates per condition, in quadruplicate.