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A novel *keratin18* promoter that drives reporter gene expression in the intrahepatic and extrahepatic biliary system allows isolation of cell-type specific transcripts from zebrafish liver

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Abstract

Heritable and acquired biliary disorders are an important cause of acute and chronic human liver disease. Biliary development and physiology have been studied extensively in rodent models and more recently, zebrafish have been used to uncover pathogenic mechanisms and potential therapies for these conditions. Here we report development of novel transgenic lines labeling the intrahepatic and extrahepatic biliary system of zebrafish larvae that can be used for lineage tracing and isolation of biliary-specific RNAs from mixed populations of liver cells. We show that GFP expression driven by a 4.4 kilobase promoter fragment from the zebrafish *keratin18* (*krt18*) gene allows visualization of all components of the developing biliary system as early as 3 days post-fertilization. In addition, expression of a ribosomal fusion protein (EGFP-Rp110a) in *krt18:TRAP* transgenic fish allows for enrichment of translated biliary cell mRNAs via translating ribosome affinity purification (TRAP). Future studies utilizing these reagents will enhance our understanding of the morphologic and molecular processes involved in biliary development and disease.

Keywords

liver; gallbladder; translation; gene profiling

1. Introduction

Transcriptional profiling and lineage tracing of specific cell types is essential for understanding basic mechanisms of organ development and disease. Recovery of uniform populations of cells from complex tissues is difficult however, and presents a significant

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challenge for cell-type specific experimental analyses in model organisms. In the absence of surface markers that can be used to recover cells from dissociated tissues, genetic labeling is generally required for efficient recovery of cells within a given lineage.

Cholestatic liver diseases are a group of disorders in which there is altered production or secretion of bile. These conditions typically arise from defects in one of the two main liver cell lineages – epithelial cells lining the biliary ductular network (also referred to as cholangiocytes), or hepatocytes. Although cholestasis is often a benign condition, some forms can progress to biliary cirrhosis and liver failure. Many cholestatic diseases begin in infancy or childhood (Santos et al., 2010), and both hepatocyte-specific defects and primary biliary defects occur. The most common form of infantile cholestasis is extrahepatic biliary atresia (BA), a disease of unknown etiology that results in destruction of the extrahepatic biliary tree and gallbladder, with subsequent damage to intrahepatic ducts and eventually hepatocytes. This leads to fibrotic injury and cirrhosis in nearly all patients. As a result, BA is the most common indication for liver transplantation in the pediatric population.

Given its predilection for the extrahepatic biliary system, molecular characterization of the epithelial cells lining the extrahepatic ducts is essential for understanding the origin of BA and developing new treatment strategies for this important pediatric liver disease. Two major obstacles however, complicate the selective profiling of these and other biliary cells within the liver. The first is that the majority of liver cells are hepatocytes. The second is that hepatocytes are one of the most synthetically active cell types in any organism. Thus, any “stream” of biliary RNA and protein production exists in a background “flood” of concomitant hepatocyte synthetic function. Robust methods are therefore needed to acquire biliary-specific data sets.

We and others have been using the zebrafish to study BA and other forms of infantile cholestasis (Wilkins and Pack, 2013). One limitation to these studies has been the absence of a simple and reliable method to transcriptionally profile biliary epithelial cells of developing larvae. One approach that is feasible is to recover RNA from biliary cells of transgenic fluorescent reporter lines using fluorescence activated cell sorting (FACS). However, this approach is technically challenging and limited to cells of the intrahepatic system because promoters that drive gene expression in extrahepatic biliary cells have not been isolated (Lorent et al., 2010; Parsons et al., 2009). To address this issue, we first cloned a novel promoter fragment from the zebrafish *keratin18* (*krt18*) gene that selectively drives transgene expression in all biliary cells. We use this promoter fragment to generate a transgenic line expressing GFP in the gallbladder and extrahepatic bile ducts, and a second line expressing a ribosomal fusion protein that can be used for molecular profiling of biliary cells via the translating ribosome affinity purification (TRAP) methodology (Heiman et al., 2008).

2. Results

2.1 keratin18 is expressed in the developing biliary system

Keratins are a large family of intermediate filament proteins that are an important component of the cell's cytoskeleton. In mammals, Keratin protein synthesis is a hallmark of

epithelial cells, including hepatocytes and biliary cells. In teleost fish, Keratins are more widely expressed including some mesenchymal cell types, however they are primarily epithelial cell markers (Schaffeld et al., 2002; Thisse et al., 2001; Thisse and Thisse, 2004; Wong et al., 2009). Previous experiments from our laboratory have shown that a monoclonal antibody directed against one of the 2 Keratin genes expressed in human hepatocytes and biliary cells, *KERATIN-18* (*KRT18*) labels only biliary epithelial cells in larval and adult zebrafish (Lorent et al., 2004). Because there is strong conservation between teleost and mammalian Keratin-18 orthologs, we hypothesized that a zebrafish *krt18* promoter fragment would direct gene expression in the biliary cells of larval zebrafish. To test this hypothesis, we first confirmed *keratin18* expression in the developing biliary system using whole mount RNA in situ hybridization. This showed strong gallbladder expression at 5 days post-fertilization (Fig. 1A). Expression in the intrahepatic bile ducts was suspected but difficult to confirm in this study. Similar results were obtained with fluorescent whole-mount RNA in situ hybridization (Fig. 1B). In this experiment, the pattern of *krt18* expression was compared with the distribution of EGFP protein present in intrahepatic ducts of a Notch-responsive transgenic reporter line *Tg(tp1bglob:egfp)* (Lorent et al., 2010; Parsons et al., 2009). This confirmed strong *krt18* expression in the developing gallbladder and cystic duct of the transgenic larvae, neither of which expressed EGFP, as previously reported. *krt18* expression in intrahepatic ducts was difficult to appreciate in this study.

2.2 *Tg(krt18:egfp)* is expressed throughout the developing intrahepatic and extrahepatic biliary system

Because a previously published zebrafish *krt18* transgenic reporter line did not show liver or gallbladder expression (Wang et al., 2006), we postulated that an enhancer element needed for biliary expression was located outside the 2.9 kilobase (kb) promoter fragment used to generate this line. We therefore amplified a 4.4 kb zebrafish *krt18* promoter fragment and used it to drive EGFP expression in transgenic zebrafish larvae. Larvae injected with the construct showed robust EGFP expression in the gallbladder, intrahepatic biliary cells, and throughout the extrahepatic biliary system (cystic duct, hepatic duct, and common bile duct) at 5 days post-fertilization (dpf) (Fig. 1C–D). In addition to the biliary system, EGFP expression was observed in skin, isolated cells of the intestinal epithelium, and proximal kidney tubules (weak expression) as previously reported (Fig. 1D and data not shown) (Wang et al., 2006). A related *krt18* promoter construct, containing the identical 4.4 kb fragment plus 1.8 kb of *krt18* intron 1, was also tested and showed similar results (data not shown).

Fluorescence microscopy of live *Tg(krt18:egfp)* larvae shows detectable biliary GFP expression within the liver and gallbladder (Fig. 2A), as well as in skin, intestine, and proximal tubules. While live imaging of some GFP-positive cells can be impaired by overlying skin expression, similar analysis of a transgenic line expressing a GFP-ribosomal protein fusion, (*Tg(krt18:TRAP)*; Section 2.3), shows minimal skin expression and better visualization of intrahepatic and gallbladder biliary cells (Fig. 2B–C).

Confocal microscopy showed that expression of the *krt18:egfp* transgene was first observed in the liver at 3 dpf, highlighting the extrahepatic duct system, rudimentary gallbladder, and

a small number of intrahepatic biliary cells (Fig. 3A). As the liver grew between 3 and 5 dpf, the EGFP-positive biliary cells proliferated and remodeled into a functional network (Fig. 3B–C), similar to what we have previously observed in fixed specimens immunostained with the anti-KRT18 antibody or live *Tg(tp1bglob:egfp)* larvae (Lorent et al., 2010; Lorent et al., 2004).

By 5 dpf, the intrahepatic biliary network consists of main ducts, interconnecting ducts, and small terminal ductules that are contiguous with the hepatocyte canaliculi. The *krt18* promoter fragment drove EGFP expression in all of these ducts (Fig. 3D). Overlap of GFP with Mdr, a xenobiotic transporter present in the canalicular membrane confirmed *krt18* expression in biliary cells comprising the terminal ductules (Fig. 3E–F), which are not labeled by the commonly used 2F11 antibody (Dong et al., 2007; Lorent et al., 2010; Wilkins et al., 2013). Taken together, these results show that our enhanced *krt18* promoter construct drives gene expression in all cells of the developing biliary system.

2.3 Biliary and hepatocyte gene expression using translating ribosome affinity purification

One major goal for developing a robust biliary promoter fragment is to use it to isolate and/or molecularly characterize biliary cells during normal development and at specific time points in disease models. To this end, we generated transgenic lines that express a ribosomal protein fusion (EGFP-Rpl10a) in either biliary cells or hepatocytes. The transgenic lines can be used to isolate cell-type specific populations of polysomes using the translating ribosome affinity purification methodology (TRAP) (Heiman et al., 2008), thus facilitating gene profiling from these cells without having to first isolate cells using FACS. At least 4 independent transgenic lines were generated for each construct; the distribution of fusion protein expression was similar in each line, with moderate variation in intensity seen in *Tg(krt18:egfp-rpl10a)* lines (referred to hereafter as *Tg(krt18:TRAP)*) and minimal intensity variation in *Tg(lfabp:TRAP)* lines (data not shown). Fluorescence intensity was stable in subsequent generations of larvae analyzed. Confocal microscopy showed that *Tg(krt18:TRAP)* larvae expressed the ribosomal fusion protein in biliary cells of the intrahepatic and extrahepatic ducts and the gallbladder, as evidenced by EGFP colocalization with the 2F11 biliary epitope (Fig. 4A–D). In contrast, *Tg(lfabp:TRAP)* larvae show expression of the ribosomal fusion protein in hepatocytes, but not 2F11-positive biliary cells (Fig. 4E–H). In all lines the TRAP fusion protein is localized to the cytoplasm and nucleus (Fig. 4D,H), with nucleolar enrichment as expected for a ribosomal protein. Consistent with their greater synthetic capacity, hepatocytes have more prominent nucleoli than biliary cells, and more prominent nucleolar localization of the TRAP fusion protein.

The TRAP methodology is based on the isolation of mRNAs bound to EGFP-labeled ribosomes (Fig. 5A). To test its applicability in the liver, we raised TRAP transgenic fish to two different developmental time points and recovered polysome RNA, utilizing the highest-expressing TRAP lines in order to maximize the percentage of labeled ribosomes per cell. Livers and gallbladders (15–25 per line) were dissected from one month-old transgenic fish, and translated mRNAs were isolated using published TRAP protocols (Heiman et al., 2008). RT-PCR for selected cell type-specific genes was performed to test the specificity of each transgenic line. The biliary-specific genes *krt18* and *cfr* were

selectively amplified from *Tg(krt18:TRAP)* livers and gallbladders, while the hepatocyte-specific genes *transferrin* and *lfabp* were selectively amplified from *Tg(lfabp:TRAP)* livers and gallbladders (Fig. 5B–C). The same tissues were dissected from 6–7 dpf larvae (400 *Tg(krt18:TRAP)* larvae, 50 *Tg(lfabp:TRAP)* larvae) and processed as for the one month-old fish. Again, selective amplification of biliary-specific genes (*cftr*, *krt18*, and *sox17*) was observed from *Tg(krt18:TRAP)* livers and gallbladders, while hepatocyte-specific genes (*transferrin*, *lfabp*, and *alpha2-macroglobulin-like*) were selectively amplified from the *Tg(lfabp:TRAP)* material (Fig. 5D). Hepatocyte transcripts were amplified in both the adult and larval biliary trap experiments. We speculate this is caused by carry-over of hepatocyte mRNA, which is synthesized in far greater quantities than biliary mRNA (Watson et al., 2012).

3. Discussion

The biliary system is comprised of a network of intrahepatic ducts that transport bile secreted by hepatocytes to the large extrahepatic ducts that are contiguous with the gallbladder and intestine. During development, biliary epithelial cells undergo extensive proliferation and remodeling, and may undergo further structural or functional changes during pathologic processes. In order to understand the molecular basis of these developmental and disease processes, it is essential to be able to isolate biliary cells within the complex cellular milieu in which they normally reside. Here, we describe a zebrafish *keratin18* (*krt18*) transgenic promoter that can selectively drive gene expression in all biliary epithelial cells of the intrahepatic and extrahepatic ductular system, and the gallbladder. This is in contrast to a previously reported Notch-responsive transgenic line that only labels cells of the intrahepatic biliary system, *Tg(tp1bglob:egfp)* (Lorent et al., 2010; Parsons et al., 2009), and a previously published transgenic line *Tg(krt18:rfp)* that did not have biliary expression, owing to the absence of a presumptive enhancer in the additional 1.5 kb of sequence in the 4.4 kb fragment we recovered (Wang et al., 2006). The ability to track GFP expression throughout the developing biliary system is a distinct advantage of this new transgenic line (Fig. 2A). Although *krt18* expression in the overlying skin interferes with using *Tg(krt18:egfp)* for real-time analyses of biliary development, as reported for the *Tg(tp1bglob:egfp)* line (Lorent et al., 2010), biliary visualization is sufficient in live animals for phenotypic analyses. The subcellular localization of EGFP reporter largely alleviates this problem, however duct morphology is less well delineated. In the future it may be possible to design reporter lines with selective biliary reporter gene expression using a minimal promoter together with the presumptive biliary enhancer that resides within our *krt18* promoter construct.

Gene expression from the *krt18* promoter fragment was sufficient for recovery of an enriched fraction of biliary RNA using the translating ribosome affinity purification (TRAP) methodology. Our findings show that TRAP can be used for cell-type specific molecular profiling in the zebrafish liver (section 2.3). TRAP was first described as a method to profile selective populations of neurons in the brains of transgenic mice (Doyle et al., 2008; Heiman et al., 2008), and has since been shown to be efficacious in other model organisms, including *X. laevis* (Watson et al., 2012), *Drosophila* (Thomas et al., 2012), and zebrafish (Tryon et al., 2013). As discussed previously (section 1), molecular profiling of biliary cells is

technically challenging due to the overwhelming cell mass and synthetic activity of neighboring hepatocytes. Indeed, we recovered nearly 40-fold more total RNA from each *Tg(lfabp:TRAP)* fish as compared to *Tg(krt18:TRAP)* fish at one month of age, and approximately 7-fold more at larval stages (see section 4.4). While some of this discrepancy is certainly due to increased ribosomal RNA recovery from the more synthetically-active hepatocytes, it also reflects the differences between hepatocyte and biliary cell census in individual fish (Fig. 4). TRAP yield also varies from experiment to experiment; using larval-stage *Tg(krt18:TRAP)* livers and gallbladders, we have occasionally obtained up to 3-fold more RNA per fish than in the current experiment (X Zhao, W Gong, and M Pack, unpublished data). We are continuing to screen for new *Tg(krt18:TRAP)* lines with increased EGFP-Rp110a expression. We anticipate that this will increase the efficiency of the GFP pulldown, thus enhancing RNA recovery and hopefully avoiding what appears to be low levels of hepatocyte RNA contaminating the biliary RNA preps (Fig. 5B–D).

TRAP has several advantages over other potential methods for profiling biliary cells. Given that the biliary network extends throughout the liver, manual dissection of biliary tissue from livers of live fish is not feasible. Fluorescence-activated cell sorting (FACS) of labeled biliary cells is technically feasible, but requires liver dissection with cellular dissociation, followed by sorting of live cells. Compared to TRAP, this method is slower, technically demanding, and is more likely to introduce methodological error in transcript levels (Okaty et al., 2011). TRAP can be performed on whole embryos or crudely dissected tissues (as in our study), and generates a profile of actively translated mRNAs in the cell type of interest, which may not be reflected in overall mRNA levels (Heiman et al., 2008). Indeed, we detected mRNA for *sox17*, a transcription factor important for biliary and gallbladder development (Spence et al., 2009), in larval stage *Tg(krt18:TRAP)* fish (Fig. 5D), but were unable to amplify it from older animals (data not shown). TRAP can also identify low-copy and high-copy mRNAs from a given cell type (Watson et al., 2012). One drawback of this method is the inability to recover a full complement of non-coding RNAs, such as miRNAs or lncRNAs. However, the ability to independently assess the role of biliary cells and hepatocytes will undoubtedly be useful in future studies. As an example, our laboratory has developed a novel zebrafish model of toxin-induced biliary atresia (Lorent K et al, manuscript in preparation), and TRAP provides an unbiased method for assaying the molecular mechanisms involved in development of the atresia phenotype by RNA sequencing. In addition, the TRAP procedure described here and elsewhere could also be used to profile other defined liver cell populations, such as stellate cells (Yin et al., 2012) and sinusoidal endothelial cells (Korzh et al., 2008; Sakaguchi et al., 2008).

4. Materials and Methods

4.1 Generation and maintenance of transgenic fish lines

A 4.43 kb *krt18* upstream fragment (NC_007134.5, nt 10449727–10454157) was amplified from zebrafish genomic DNA using primers GGCCGGCCTTGTGTACGCCTAGTTGATGAGGA and GGCGCGCCCTG-GTGTAAGTGAGCAGACGAGTGA. One hundred (100 ng) of genomic DNA was used in a 50 µl PCR reaction with pfuTurbo DNA polymerase (Stratagene). PCR conditions were: a

3 min denaturation step at 95°C followed by 30 cycles of, denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 5 min, and a final 10 min extension at 72 °C. The purified PCR product was digested with FseI and AscI and ligated to the predigested FseI and AscI sites of P5E-Fse-Asc vector. The resulting 5' entry clone and p3E-egfp clone (Invitrogen) were recombined via Gateway cloning (Invitrogen) into a Tol2 plasmid backbone (Kawakami, 2004; Kwan et al., 2007; Villefranc et al., 2007).

For the *krt18* promoter-intron-GFP construct, the 1.83 kb first intron of *krt18* was amplified using primers GGAAACGCCAAGAGAAGCTTTG and AGAGCTTCCCATGAGGTCAAAATG and cloned into PCR8/GW/TOPO vector (Invitrogen).

TRAP transgenic lines were generated by PCR amplifying an *egfp-rpl10a* fusion gene from pMlc2-EGFP-Rpl10a (gift of Dr. Kenneth Poss) using primers GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGTGAGCAAGGGCGAGGAG and GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTAGAGGCGCTGTGGTTT. The purified PCR product was recombined into pDONR221 (Invitrogen) via Gateway cloning. Confirmed clones were recombined with either p5E-krt18 or p5E-lfabp, and p3E-pA into the Tol2 destination vector pDestTol2pA2 to create transgenic constructs.

To generate transgenic zebrafish, DNA was prepared using QIAprep midiprep kit (Qiagen). Plasmid (10–25 ng/μl) was co-injected into one-cell stage Top Long Fin (TLF) embryos along with mRNA encoding the Tol2 Transposase (20 ng/μl) (Kawakami, 2004). At least four independent transgene insertions (lines) were screened for each construct.

Methods for general zebrafish maintenance and breeding have been previously described (Pack et al., 1996). All animal experiments have been approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

4.2 Whole mount color and fluorescence in situ hybridization

To minimize cross-reactivity, the 3'UTR sequence of *krt18* was used to design primers to generate a digoxigenin-labeled riboprobe using PCR-based strategy. The forward and reverse primer sequences are: GCAGACAAATTCAGACATTGGAGAT and CACTAGTAATACGACTCACTATAGGGAGCGTAGTGTCTGTGACCTTTGGTCT. Color in situ hybridization was performed in fixed 5 dpf TLF embryos, and the protocol was described in detail elsewhere (Thisse and Thisse, 2008; Yee and Pack, 2005). Fluorescence in situ hybridization was performed on 5 dpf *Tg(tp1b:glob:egfp)* larvae, hemizygous for a Notch-responsive fluorescent reporter gene (GFP) (Parsons et al., 2009). The procedure used followed the protocol published (Welten et al., 2006).

4.3 Immunohistochemistry

Detailed protocols for antibody staining have been published previously (Lorent et al., 2004). Primary antibodies included rabbit anti-GFP-Alexa 488 (1:400, except 1:200 following fluorescence in situ hybridization) (Invitrogen), rabbit anti-Mdr (1:100, Santa Cruz Biotechnology), and mouse monoclonal 2F11 (1:1000, gift of Julien Lewis). Primary antibody incubations were performed overnight at 4°C. Secondary antibodies included goat

anti-rabbit IgG-Alexa 568 and goat anti-mouse IgG-Alexa 568 (Invitrogen), and were diluted 1:600 for incubation at room temperature for 2–3 h. For confocal microscopy, livers and attached gastrointestinal tracts were dissected from immunostained larvae and mounted in Vectashield with DAPI (Vector Industries), coverslipped, and imaged using a Zeiss LSM 700 confocal microscope.

4.4 Translating Ribosome Affinity Purification (TRAP) and RT-PCR

All polysome purifications were performed as described (Heiman et al., 2008), with few modifications. Briefly, livers and gallbladders from 23 and 13 one month old *Tg(krt18:TRAP)* and *Tg(lfabp:TRAP)* F1 fish, respectively, were dissected and pooled. Specimens were homogenized in lysis buffer and the post-nuclear supernatant was prepared by centrifugation at 2000 *xg* for 10 min. S20 fraction was prepared by adding 10% NP-40 and DHPC followed by centrifugation at 20000 *xg* for 10 min. Immunoprecipitation (IP) was carried out by incubation with Streptavidin MyOne T1 Dynabeads (Invitrogen) coated by biotinylated protein L (Pierce, Thermoscientific) and anti-GFP monoclonal antibodies (19C8 and 19F7 from Memorial Sloan-Kettering antibody facility) overnight at 4°C. After incubation the beads were collected using a magnet and washed with 0.35 M KCl IP wash buffer. Total RNA was isolated using the Micro RNeasy kit (Qiagen). RNA quantity was measured with a Nanodrop 1000 spectrophotometer. Approximately 12.45 µg (498 ng/µL) of total RNA was isolated from *Tg(lfabp:TRAP)* fish, and 550 ng of total RNA (22 ng/µL) from *Tg(krt18:TRAP)* fish.

For larval stage TRAP experiments, livers and gallbladders from 400 and 50 6–7 dpf *Tg(krt18:TRAP)* and *Tg(lfabp:TRAP)* F1 fish, respectively, were dissected in two batches and pooled. The pooled specimens were processed as described above, except that each RNA isolation column was eluted twice with 10 µL. Approximately 387 ng [(26.4 ng/µL x 10 µL) + (12.3 ng/µL x 10 µL)] of total RNA was isolated from *Tg(krt18:TRAP)* larvae, and 343 ng [(21.2 ng/µL x 10 µL) + (13.1 ng/µL x 10 µL)] from *Tg(lfabp:TRAP)* larvae.

Equal amounts of total RNA (~350 ng) from each line were used for cDNA synthesis with qScript cDNA Supermix (Quanta Bioscience). RT-PCR was performed using GoTaq® Green Master Mix (Promega). PCR conditions were: a 2 min denaturation step at 94°C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53–57 °C for 30 s and extension at 72 °C for 30 s, and a final 10 min extension at 72 °C. Primer sequences were: krt18-F TCACAGAAGAACCTGAAAGGGTCAC, krt18-R TTCAAGAGCATCCTGGAGTTTGAAG, lfabp-F AAAAACCGTCACCAACTCCTTCAC, lfabp-R TGAAACGCTTCAGATCTTCTTGCTC, cftr-F CGCTTCAGAAATGGAGGAAA, cftr-R ACATAACCGCCGTACTCCAG, transferrin-F ACTACGCTGTGGCTGTTGTG, transferrin-R AATCCTTTTGCCAGTCCTTT, sox17-F AGTCCGCTCTCAGACTCCAA, sox17-R CTCCTCAACGAATGGACGTT, a2ml-F GGGTCGATTCCAAAGATGAC, and a2ml-R TGGGAATTAATTTGCCAGA. PCR products were electrophoresed on a 2% agarose/TBE gel and imaged using ethidium bromide (0.5 µg/mL). The gel image was scanned using Quantity One (BioRad), and densitometry was determined using ImageJ.

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Highlights

- A novel *keratin18* promoter marks all biliary cells in transgenic zebrafish.
- TRAP methodology can be used to recover polysome RNA from specific liver cell types.
- These tools allow cell lineage tracing and gene profiling during zebrafish liver development and in zebrafish disease models.

Figure 1.

krt18 and *Tg(krt18:egfp)* are expressed in the developing biliary system. (A) Whole-mount in situ hybridization of 5 dpf wild-type larva (left lateral view), showing gallbladder expression of *krt18* (arrow). (B) Combined immunostaining and fluorescent in situ hybridization of 5 dpf *Tg(tp1bglob:egfp)* larva (left lateral view), showing Notch-responsive GFP protein in intrahepatic bile ducts within the liver (l), but not the *krt18*-expressing gallbladder (g). Red signal anterior to the liver is background staining. (C) Whole-mount right lateral-inferior fluorescent image of 5 dpf wild-type control larva, stained with anti-GFP, showing background autofluorescence in larval tissues. (D) Whole-mount right lateral-inferior fluorescent image of 5 dpf *Tg(krt18:egfp)* larva stained with anti-GFP, showing EGFP expression in the developing biliary system, skin, and proximal tubule. Abbreviations: l=liver, g=gallbladder, i=intestine, t=trunk, cd=cystic duct, chd=common hepatic duct, cbd=common bile duct, pt=proximal tubule.

Figure 2.

krt18-driven reporters allow live detection of the developing biliary system. (A) Fluorescent micrograph of live 6 dpf *Tg(krt18:egfp)* larva, showing EGFP expression in intrahepatic biliary cells (single arrow) and gallbladder epithelium (double arrow), partly obscured by skin expression. (B–C) Fluorescent micrographs of live 4 dpf (B) and 7 dpf (C) *Tg(krt18:TRAP)* larvae, showing visualization of EGFP-Rp110a fusion protein in developing intrahepatic biliary cells (single arrows) and gallbladder epithelium (double arrow). Red dashed line in (B) indicates gallbladder overlying the liver, out of the focal plane.

Figure 3.

Tg(krt18:egfp) is expressed at all levels of the developing intrahepatic and extrahepatic biliary system. (A–C) Confocal projections of dissected livers from 3, 4, and 5 dpf *Tg(krt18:egfp)* fish, indicating biliary proliferation and remodeling (400X magnification). Arrow in panel A indicates rudimentary gallbladder. (D–E) Confocal projections from dissected liver of 5 dpf *Tg(krt18:egfp)* larva stained with (D) anti-GFP and (E) anti-Mdr to mark biliary cells and hepatocyte canaliculi, respectively. (F) Merged projection from panels D and E, demonstrating colocalization of canaliculi with GFP-positive terminal ductules (D–F: 630X magnification).

Figure 4.

Generation of transgenic zebrafish for liver translating ribosome affinity purification (TRAP). (A–C) Confocal projection of dissected liver and gallbladder from a 5 dpf *Tg(krt18:TRAP)* larva, showing EGFP-Rpl10a fusion protein (A) and biliary marker 2F11 (B), which co-localize (C; EGFP - green; 2F11 - red). EGFP is also visualized in *krt18*-expressing cells of the esophagus (e) and skin (s). (D) High-magnification confocal scan of liver from the same larva shows localization of TRAP fusion protein to biliary cell cytoplasm and nucleus, with a subset showing nucleolar enrichment (insets). (E–G) Confocal projection of dissected liver and gallbladder from 5 dpf *Tg(lfabp:TRAP)* larva, showing hepatocyte expression of TRAP fusion protein (E) that is non-overlapping with biliary 2F11 staining (F). (H) High-magnification confocal scan of liver from same larva, showing localization of TRAP fusion protein to the hepatocyte cytoplasm and nucleolus (inset), exclusive of biliary cells stained red with the 2F11 antibody.

Figure 5.

Selective mRNA isolation from biliary cells and hepatocytes by translating ribosome affinity purification (TRAP). (A) Schematic of TRAP methodology, demonstrating selective isolation of mRNAs actively translated by EGFP-Rpl10a-containing ribosomes. (B–C) Gel electrophoresis (B) and band densitometry (C) of RT-PCR products from one month-old *Tg(krt18:TRAP)* and *Tg(lfabp:TRAP)* fish, demonstrating enhanced cell-type specific recovery of biliary (*krt18*, *cftr*) and hepatocyte (*lfabp*, *transferrin*) mRNAs, respectively. (D) Quantitation of RT-PCR products from 6–7 dpf *Tg(krt18:TRAP)* and *Tg(lfabp:TRAP)* livers and gallbladders, demonstrating enhanced cell-type specific amplification of biliary (*krt18*, *cftr*, *sox17*) and hepatocyte (*transferrin*, *lfabp*, *a2ml*) mRNAs, respectively.