Heme Regulates Exocrine Peptidase Precursor Genes in Zebrafish

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We previously determined that *yquem* harbors a mutation in the gene encoding uroporphyrinogen decarboxylase (UROD), the fifth enzyme in heme biosynthesis, and established zebrafish *yquem* (*yqe*^{tp61}) as a vertebrate model for human hepatoerythropoietic porphyria (HEP). Here we report that six exocrine peptidase precursor genes, *carboxypeptidase A*, *trypsin precursor*, *trypsin like*, *chymotrypsinogen B1*, *chymotrypsinogen 1-like*, and *elastase 2 like*, are downregulated in *yquem/urod* (−/−), identified initially by microarray analysis of *yquem/urod* zebrafish and, subsequently, confirmed by *in situ* hybridization. We then determined downregulation of these six zymogens specifically in the exocrine pancreas of *sauternes* (*sau*^{tb223}) larvae, carrying a mutation in the gene encoding δ-aminolevulinate synthase (ALAS2), the first enzyme in heme biosynthesis. We also found that *ptf1a*, a transcription factor regulating exocrine zymogens, is downregulated in both *yquem/urod* (−/−) and *sau/alas2* (−/−) larvae. Further, hemin treatment rescues expression of *ptf1a* and these six zymogens in both *yquem/urod* (−/−) and *sauternes/alas2* (−/−) larvae. Thus, it appears that heme deficiency downregulates *ptf1a*, which, in turn, leads to downregulation of exocrine zymogens. Our findings provide a better understanding of heme deficiency pathogenesis and enhance our ability to diagnose and treat patients with porphyria or pancreatic diseases. Exp Biol Med 232:1170–1180, 2007

Key words: heme; zymogens; pancreas; porphyria; zebrafish

Introduction

Heme (ferroprotoporphyrin IX) is widely known to be the prosthetic group for a number of proteins and enzymes that play critical roles in oxygen delivery and mitochondrial function, such as hemoglobin, catalases, and cytochromes. Heme also serves as a signaling molecule that controls numerous molecular and cellular processes (1). For instance, heme is essential for differentiation of mammalian erythroid, hepatic and nervous cells (2–5) and also suppresses the apoptosis of human neutrophils (6), PC12 neurons (7), and HeLa cells (8).

Heme biosynthesis is catalyzed by a cascade of eight enzymatic reactions, which is highly conserved from bacteria to mammals. Defective enzymatic activities of these enzymes result in heme deficiency and human anemia or porphyrias (1, 4). Four mutants with deficient enzymes in heme biosynthesis, δ-aminolevulinate synthase (ALAS2), δ-aminolevulinic acid dehydratase (ALAD), uroporphyrinogen decarboxylase (UROD), and ferrochelatase (FCH), have been studied in zebrafish and medaka fish (9–12). These mutants all exhibit heme deficiencies resulting from their defective enzymes in the heme biosynthetic pathway (9–12). Zebrafish *sauternes* (*sau*^{tb223}) was modeled for human congenital siderbalstic anemia (CSA; Online Mendelian Inheritance in Man [OMIM] 301300, John Hopkins University, Baltimore, MD) because of its microcytic and hypochromic phenotype, derived from a missense mutation (V249D) in the gene encoding, the first and rate-liming erythroid-specific δ isoform of ALAS2 (EC 2.3.1.375) in the heme biosynthetic pathway (10). Medaka *whiteout* (*who*) is reminiscent of human ALAD porphyria (ADP; OMIM 125270) because of a missense mutation (L251Q) in the gene encoding δ aminolevuninic acid dehydratase (ALAD; EC 4.2.1.24), the second enzyme in the pathway. Zebrafish *yquem* (*yqe*^{tp61}) was established as a vertebrate model for human hepatoerythropoietic porphyria (HEP; OMIM 176100) as a result of a missense mutation (M38R) in the gene encoding UROD (EC 4.1.1.37), the fifth enzyme in the pathway (9). Finally, zebrafish *dracula* (*dra*^{m248}) represents an animal model of human erythropoietic protoporphyria (EPP; OMIM 177000) that resulted from a slice donor mutation in the gene encoding FCH (EC 4.99.1.1), the final enzyme in the pathway (11). These fish mutants are invaluable resources for elucidating molecular

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genetic mechanisms underlying human anemia and porphyria diseases (9–12).

In this study, we performed microarray analysis of *yquem*/*urod* and wild-type control zebrafish and observed downregulation of six exocrine peptidase precursor genes, including *carboxypeptidase A* (*cpa*), *trypsin precursor* (*try*), *trypsin like* (*tryl*), *chymotrypsinogen B1* (*ctrb1*), *chymotrypsinogen 1-like* (*ctr1l*), and *elastase 2 like* (*ela2l*), in *yquem*/*urod* (–/–). We then found downregulation of these six zymogens in zebrafish *sauternes* (sau^{tb223}). We also determined downregulation of *ptf1a*, a transcription factor regulating exocrine zymogens, in both *yquem*/*urod* (–/–) and *sauternes*/*alas2* (–/–). Additionally, hemin treatment was able to restore expression of *ptf1a* and these six zymogens in both *yquem*/*urod* (–/–) and *sauternes*/*alas2* (–/–) larvae. Hence, heme deficiency appears to downregulate *ptf1a*, which may result in downregulation of exocrine zymogens. These findings provide a better understanding of heme deficiency pathogenesis and enhance our ability to diagnose and treat patients with porphyria or pancreatic diseases.

Materials and Methods

Fish Husbandry and Embryo Production. All animal protocols were approved by the University of Oklahoma's Institutional Animal Care and Use Committee (IACUC). Zebrafish (*Danio rerio*), wild-type AB strain, and mutant lines *yquem* (yqe^{tp61}) (9) and *sauternes* (sau^{tb223}) (10) are raised at our fish facility according to standard protocols (13). Wild-type (WT) and mutant larvae were produced by pair mating, collected for RNA isolation, and fixed for *in situ* hybridization experiments at specified stages. Homozygous mutants *yquem*/*urod* (–/–) were obtained by mating heterozygous fish (*yquem*, +/–) and then identified under a microscope with UV light. Homozygous mutants *sau*/*alas2* (–/–) were identified under a light microscope after crossing heterozygous fish (*sauternes*, +/–).

RNA Isolation. Total RNAs from approximately 50 of the homozygous *yquem*/*urod* and wild-type larvae were extracted using TRIZOL (Invitrogen, Carlsbad, CA) at 56, 72, 84, and 96 hrs postfertilization (hpf). RNA quality was analyzed by capillary gel electrophoresis with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), and RNA quantity was measured by a UV spectrophotometer. One zebrafish larvae pool was used for each stage of the mutant and WT control larvae examined.

Oligonucleotide Array Production. A zebrafish oligonucleotide library containing gene-specific 50-mer oligonucleotides representing approximately 14,067 transcripts was used. The library was originally generated by MWG Biotech and is now owned by Ocimum Biosolutions (Indianapolis, IN). The oligonucleotide probes were spotted onto Corning UltraGAPS amino-silane coated slides (Corning, Acton, MA) and covalently fixed to the surface of the glass using UV radiation in a UV Stratalinker model 1800 (Stratagene, La Jolla, CA). The printed slides then were blocked with succinic anhydride/sodium borate solution (Sigma, St. Louis, MO).

cDNA Labeling. cDNA was labeled with direct incorporation of Cy3-dUTP (Amersham Biosciences, Piscataway, NJ) from 2 μg of total RNA using Qiagen OmniScript reverse transcriptase (Qiagen). RNA was mixed with 500 ng of anchored oligo-dT primer, brought to 13.5 μl volume with diethypyrocarbonate water and heated to 65°C for 5 mins. Then, this RNA and oligo-dT primer mix was added to 6.5 μl solution containing 2 μl of 10× OmniScript RT buffer (Qiagen), 0.5 nmole Cy3-deoxyuridine triphosphate (dUTP), 2.5 m*M* each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP), 1.5 m*M* TTP, 40 units of ribonuclease (RNase) inhibitor, and 4 U OmniScript reverse transcriptase (Qiagen). The labeling reactions were performed at 37°C for 2 hrs using a Gene Amp PCR System 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA) and terminated by adding 2 μl of 2.5 *N* sodium hydroxide (NaOH) and incubating at 37°C for 15 mins. The final cDNA solution was neutralized with 10 μl of 2*M* HEPES. cDNA was purified with a Montage 96-well format vacuum system (Millipore, Billerica, MA).

Hybridization and Data Acquisition. The purified Cy3-labeled cDNA was then mixed with ChipHybe hybridization buffer (Ventana Medical Systems, Tucson, AZ) containing Cot-1 DNA (0.5 mg/ml), yeast tRNA (0.2 mg/ml), and poly$(dA)_{40–60}$ (0.4 mg/ml). Hybridization was conducted on a Ventana Discovery system for 9 hrs at 58°C. Each labeled cDNA was hybridized to a separate array. Hybridized microarrays were washed and then scanned at 5μm resolution with an Agilent fluorescent scanner (Agilent Technologies, Santa Clara, CA). Fluorescent intensity was measured and analyzed by Imagene software (BioDiscovery, El Segundo, CA).

Normalization of Microarray Data. Microarray data normalization was conducted as previously described (14). Briefly, the procedure assumes fluorescent signals from genes not expressed by the larvae are normally distributed, and these fluorescent signal values were used to calculate their mean (S_0) and standard deviation (SD_0) using an iterative, nonlinear curve-fitting procedure. Genes significantly expressed above background (>3 SD above background) were selected for further normalization, and their gene expression values were log-transformed with substitution of negative values by the lowest positive logarithmic value. Adjustment of the expression profiles of those genes that were significantly expressed above background to each other was conducted using a robust regression procedure (14). In addition, the raw microarray data of this study were deposited into Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/projects/geo/; accession GSE8651).

Determination of Differentially Expressed Genes. To determine differentially expressed genes, both

Table 1. Exocrine Peptidase Precursor Genes Downregulated In *yqe/urod* Revealed By Microarray Analysis[a]

Gene chip ID	Gene name	Genbank accession	Pa[b]	Ratio of WT/*yqe*[c] 72 hpf	84 hpf	96 hpf	Function	Chromosomal location
#07482	*cpa*	AF376130	0.04	1.11	2.70	4.40	carboxypeptidase A activity	25
#04804	*try*	AJ297822	0.009	1.43	3.12	4.69	chymotrypsin activity	17
#01831	*tryl*	BC055625	0.05	3.21	19.77	12.98	trypsin activity	16
#09049	*ctrb1*	BC055574	0.02	1.63	4.68	11.34	chymotrypsin activity	7
#01114	*ctr1l*	NM_001004582	0.002	1.04	9.00	2.21	chymotrypsin activity	15
#06255	*ela2l*	AY179345	0.007	1.25	0.92	2.39	chymotrypsin activity	20

[a] Microarray analysis was performed following the procedures as described (see Materials and Methods). Among more than 14,000 genes examined, 14 downregulated genes and 12 upregulated genes were revealed in *yqe/urod* larvae by microarray analysis (Supplemental Tables 1 and 2, available online). Six downregulated exocrine peptidase precursor genes are listed in the table. BLAST and phylogenetic analyses were used to annotate all the six genes (see Materials and Methods). Gene names and putative functions are from ZFIN (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg). The genomic locations of these genes are from ensembl (http://www.ensembl.org/Danio_rerio/index.html). The raw microarray data was submitted into Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/projects/geo/; GEO accession GSE8651).
[b] Pa, the probability for Associative *T* test. Genes with Pa < 0.05 are potentially differentially expressed.
[c] At least 2-fold ratio of WT to *yqe*, i.e., reduced expression in *yqe/urod* (–/–) relative to WT controls in at least two of 72, 84, and 96 hpf, except for *ela2l*, which only has 2-fold mutant reduction at 96 hpf.

Student's *t* test and Associative *T* test were computed with the normalized genes. The ratio of WT versus *yqe*/*urod* was also calculated for 56, 72, 84, and 96 hpf developmental stages. Although genes selected by Student's *t* test with $P < 0.05$ alone are likely false positives for differential expression, genes selected by Associative *T* test with $P < 0.05$ alone are potentially real positives that require independent experimental confirmation (14). For our study, only genes with significant Associative *T* test (Pa < 0.05) and $\geq$2-fold changes between WT and *yqe*/*urod* in at least two of the four developmental stages examined were selected as potential differentially expressed genes (Supplemental Tables 1 and 2, available in the online version of the journal).

Annotation, Sequence Alignment, and Phylogenetic Analysis. We used the Ocimum sequences to Basic Local Alignment Search Tool (BLAST) against NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and ensembl (http://www.ensembl.org/) databases to obtain full-length cDNA sequences and genomic locations, when available. The full-length cDNA sequences were re-BLASTed against the NCBI database to ascertain their identities. For these peptidase precursor genes, except for *cpa*, homologous and orthologous sequences of the genes from *Takifugu rubripes*, *Tetraodon nigroviridis*, *Homo sapiens*, *Rattus norvegicus*, *Mus musculus*, and two bacteria, *Methanosarcina barkeri* and *Bdellovibrio bacteriovorus* HD100, were also obtained. Multiple sequence alignments were generated using ClustalX (15) and viewed with BioEdit (16). Phylogenetic analyses were then performed using amino acid alignments with the neighbor-joining method (1000 bootstraps) by MEGA 3.1 (17). Genbank accession numbers of proteins used are listed in the online Supplemental Table 3.

Generation of RNA Probes. DNA templates for generating RNA probes were first amplified from zebrafish larval RNAs by reverse transcriptase-polymerase chain reaction (RT-PCR) *via* RT-PCR Access (Promega, Madison, WI), with a thermal profile of one cycle of 48°C for 45 mins; one cycle of 94°C for 2 mins; 40 cycles of 94°C for 30 secs, 55°C for 30 secs, and 68°C for 60 secs; and one cycle of 68°C for 10 mins. Primers for the genes examined were designed to cover the partial coding regions and 3′ untranslated regions (UTRs) to increase specificity (See Table 2). The RT-PCR products were then subcloned into the pCR4-TOPO vector (Invitrogen). The positive clones identified by colony polymerase chain reaction (PCR) were subsequently sequenced to ascertain the identities and orientations of the genes in the vector. RNA probes were labeled with digoxigenin (DIG) using a RNA labeling kit (Roche, Indianapolis, IN).

Whole-Mount *In Situ* Hybridization. Whole-mount *in situ* hybridization was conducted as previously described (13). Briefly, fixed larvae were incubated in 50% formamide hybridization buffer with a DIG-labeled RNA probe at 70°C for 18–20 hrs. Both nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche) were used for colorimetric detection. For each *in situ* hybridization, 10–15 larvae were used. At least three independent *in situ* hybridization experiments were conducted for each gene with an antisense probe, and at least one was conducted for each gene with a sense probe as control (not shown).

***In Situ* Hybridization Imaging Acquisition and Analysis.** Following whole-mount *in situ* hybridization, larvae were placed in 4% methyl cellulose and observed under a dissecting stereoscope. The *in situ* hybridization images were acquired with a Leica MZ FLIII stereomicroscope and a Magnafire-cooled charge-coupled device camera and processed with Image Pro Plus (MediaCybernetics, Bethesda, MD), NIH ImageJ (http://rsb.info.nih.gov/ij/, National Institutes of Health, Bethesda, MD), and Adobe (San Jose, CA) Photoshop. Identical microscopic and camera settings were used for each experiment. The optical

Table 2. Primer Sequences for RT-PCR Amplification[a]

Gene name	Primer sequence
cpa	ZF151FOR, 5′-TCGTCTACACCCACACCAAA-3′
	ZF151REV, 5′-CTTTCCGTCTGAAATGTTGCT-3′
try	ZF111FOR, 5′-TACAACAGCAACACCCTGGA-3′
	ZF111REV, 5′-TGCTTTGCCAGATGGTATTG-3′
tryl	ZF117FOR, 5′-GTTTGGGCGAACACAACATC-3′
	ZF117REV, 5′-TTCACAAGTGTTATCTCCAAAACAA-3′
ctrb1	ZF118FOR, 5′-CCTTCTTTGGTGCAGCCTAT-3′
	ZF118REV, 5′-ATGACAGGATTCATTGCTGCT-3′
ctr1l	ZF155FOR, 5′-TGGATCCCTGATCAACCAGT-3′
	ZF155REV, 5′-TTTTATTGGCATTTTCTTCAGAGAG-3′
ela2l	ZF152FOR, 5′-ACTTGCGGTGGAAGCCTTAT-3′
	ZF152REV, 5′-AAGGCATCGATGATACAAATCC-3′
ptf1a	ZF179FOR, 5′-GAGGGACTGCGATCTCACAT-3′
	ZF179REV, 5′-GGCTGAAACACAGATAGTCACAA-3′

[a] To generate RNA probes, we amplified DNA fragments *via* RT-PCR for these seven genes (see Materials and Methods). The primer sequences are listed in the table.

density (O.D.) for the area stained (S) and the neighboring background (B) were measured using ImageJ software. The staining O.D. $= S - B$. Differences in signal intensities between treatments were analyzed by analysis of variance (ANOVA) or t test. Results are expressed as mean optical density $\pm$ standard error (SE).

Hemin Treatment. Hemin solution was prepared as previously described (18). Hemin (Sigma) was dissolved in 0.2 ml of 1 N NaOH, then 1 ml of 0.2 M Tris-HCl (pH 8), distilled, and deionized water was added to yield the desired concentration. The pH was adjusted to 7.8 with 1 N HCl.

Results

Downregulation of Six Peptidase Precursor Genes in the Zebrafish *yquem/urod* Exocrine Pancreas. The six peptidase precursor genes, *cpa*, *try*, *tryl*, *ctrb1*, *ctr1l*, and *ela2l*, are downregulated in the zebrafish *yquem*/*urod* mutant as shown by microarray analysis of *yquem*/*urod* and the control zebrafish ($Pa < 0.05$; see Table 1). Whole-mount *in situ* hybridization was performed to confirm that all six peptidase precursor genes have significantly reduced expression domains, specifically in the zebrafish *yquem*/*urod* pancreas ($P < 0.05$, t test: see Fig. 1). Because the heme deficiency resulted from defective UROD in this fish mutant, we hypothesized that heme might be required for transcription of these digestive enzyme genes. We also examined expression of *insulin*, *glucagon*, and *somatostatin* in both *yquem*/*urod* and control larvae at 92 hpf. No significant differential expression for these three endocrine genes was observed in *yquem*/*urod* or the control larvae ($P > 0.05$, t test; see online Supplemental Fig. 1). Hence, heme appears to exert its effect on exocrine genes *per se* without affecting endocrine genes.

Carboxypeptidase A (EC 3.4.17.1), one of the six peptidases studied, belongs to the MEROPS (http://merops.sanger.ac.uk/) peptidase family M14 (Carboxypeptidase A, clan MC). It is an exo-peptidase and can remove all C-terminal amino acids with the exception of Arg, Lys, and Pro (19). The remaining five peptidases contain a trypsin-like serine protease (tryp_SPc) domain and belong to the serine peptidase chymotrypsin family S1 (chymotrypsin A, clan PA). Phylogenetic analysis using the tryp_SPc domains of peptidases (online Supplemental Fig. 2) showed that the serine peptidases of fishes and mammals form monophyletic groups, suggesting that these serine peptidase genes are highly conserved throughout evolution (Fig. 2). Fish *try* and

Table 3. The Number of E-Box (CANNTG) Within the −6000 nt Upstream of the Transcription Initiation Site of These Six Exocrine Peptidase Precursor Genes[a]

Gene name	Ensembl accession	5′-predicted E-boxes (number)
cpa	ENSDARG00000021339	CAGCTG (2)/CACCTG (1), CATGTTG (1)
try	ENSDARG00000042993	CAGCTG (1), CACGTG (1)
tryl	ENSDARG00000040390	CAGCTG (5)/CACCTG (4), CATGTTG (3)
ctrb1	ENSDARG00000039728	CAGCTG (2)
ctr1l	ENSDARG00000053900	CAGCTG (1)/CACCTG (2)
ela2l	ENSDARG00000041954	CAGCTG (1), CACGTG (2)
ptf1a	ENSDARG00000014479	not detected

[a] The genomic sequences of the seven genes were downloaded from ensembl (http://www.ensembl.org/Danio_rerio/index.html). The putative E-box sequences were searched manually for each gene and listed in the table.

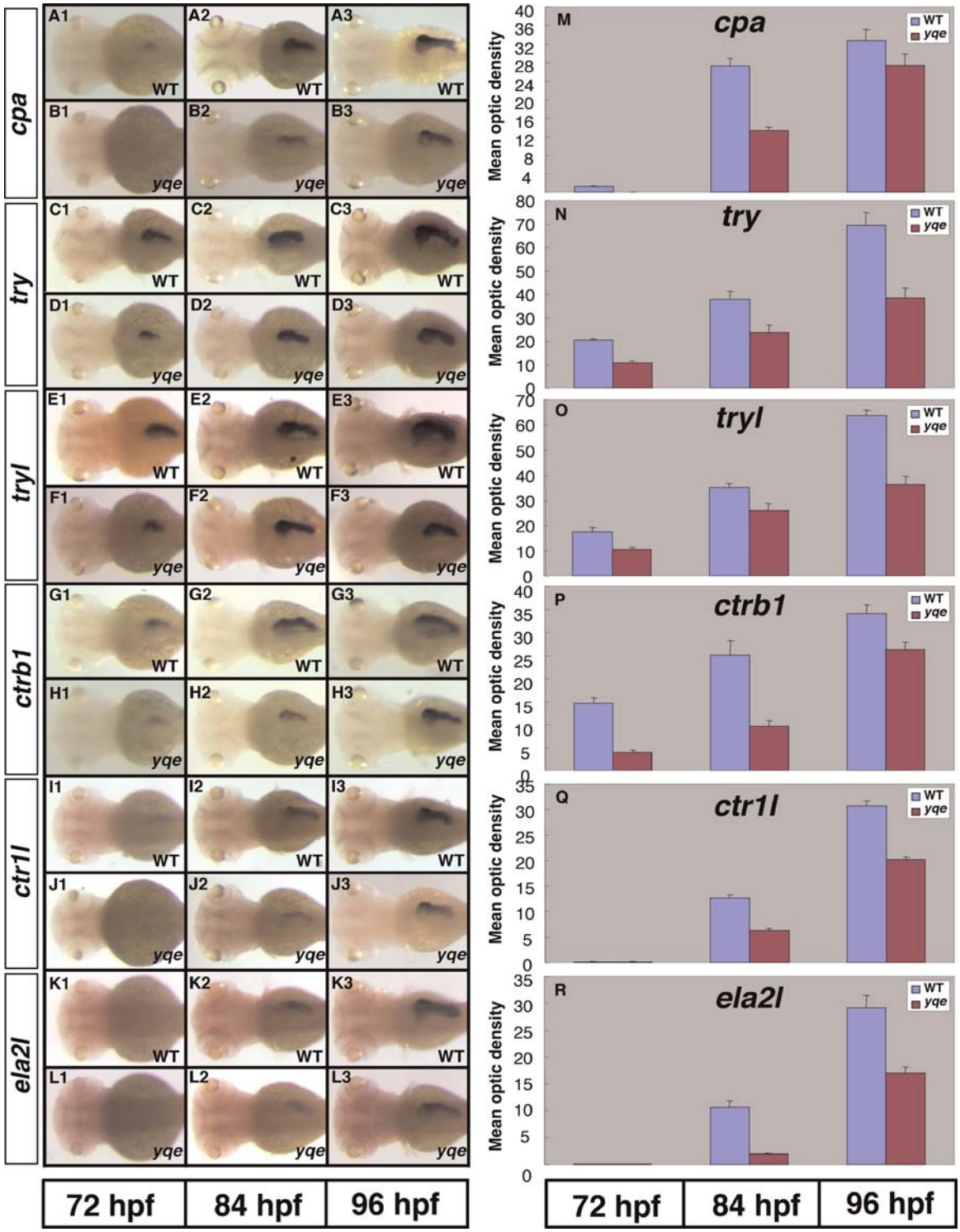

Figure 1. Downregulation of six peptidase precursor genes specifically in exocrine pancreas of the *yquem/urod* mutant shown by (A–L) whole-mount *in situ* hybridization and (M–R) analyzed by ImageJ. All larvae are shown in dorsal view, anterior to the left. The six peptidase precursor genes are (A, B, and M) *cpa*, (C, D, and N) *try*, (E, F, and O) *tryl protein*, (G, H, and P) *ctrb1*, (I, J, and Q) *ctrb1l protein*, and (K, L, and R) *ela2l*. The mean total O.D. $= S - B$ (see Materials and Methods) in 20–50 embryos. Differences between WT and the mutant are statistically significant at all stages ($P < 0.05$, *t* test), except for (M) *cpa* at 96 hpf, where it could result from difficult quantification of deep *in situ* hybridization staining as well as (Q and R) *ctr1l* and *ela2l* at 72 hpf where no *in situ* hybridization signals were detected. Error bars in M–R are standard deviation. Color figures are available in the online version of the journal.

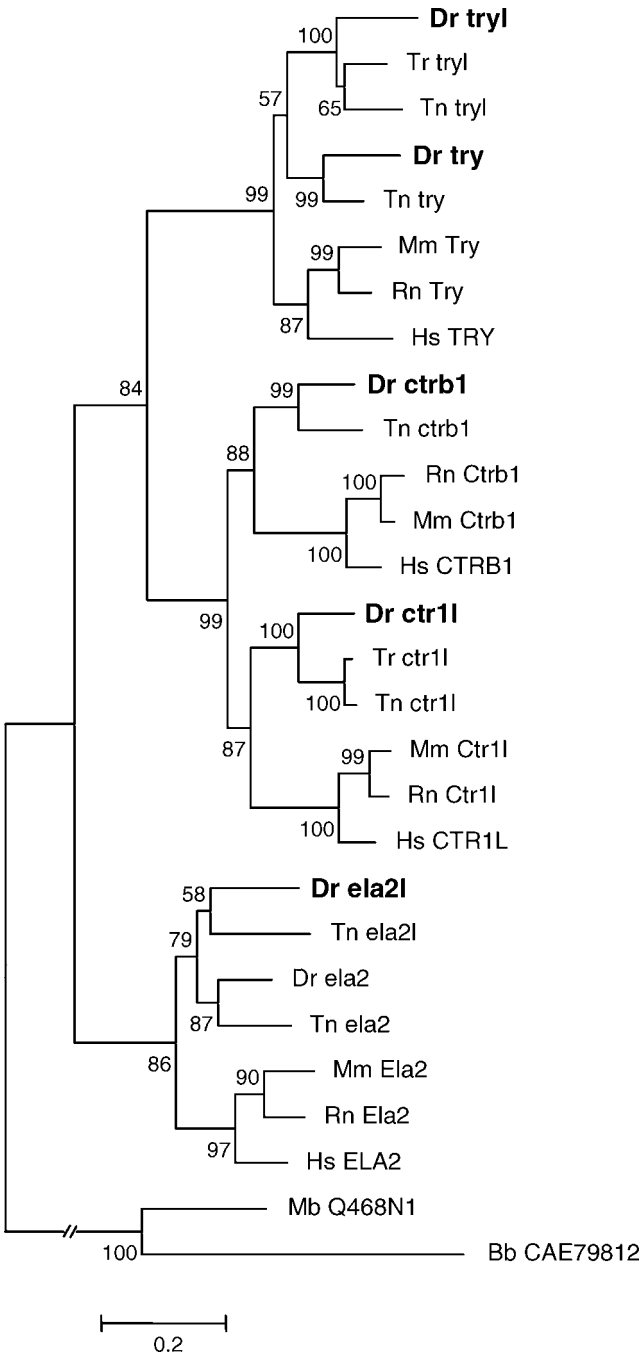

Figure 2. Phylogenetic tree using the tryp_SPc domains of serine peptidases with bacterial homologs as outgroups. The tree was constructed by the neighbor-joining method using MEGA 3.1 (17) and is a consensus derived from a heuristic search of 1000 bootstrap replicates. The numbers indicate the percentage bootstrap support. Try, trypsin precursor; tryl, trypsin-like protein; ctrb1, chymotrypsinogen B1; ctr1l, chymotrypsinogen 1–like protein; ela, elastase; Dr, *Danio rerio*; Tr, *Takifugu rubripes*; Tn, *Tetraodon nigroviridis*; Hs, *Homo sapiens*; Rn, *Rattus norvegicus*; Mm, *Mus musculus*; Mb, *Methanosarcina barkeri*; Bb, *Bdellovibrio bacteriovorus* HD100. Mb_Q468N1 is a hypothetical protein of *Methanosarcina barkeri*, and Bb_CAE79812 is a putative V8-like Glu-specific endopeptidase of *Bdellovibrio bacteriovorus* HD100. The Genbank accession numbers of these serine peptidases are in online Supplemental Table 3.

tryl are co-orthologs (20) of mammalian *Try*, whereas fish *ela2* and *ela2l* are co-orthologs of mammalian *Ela2* (Fig. 2), suggesting that both zebrafish *try*/*tryl*, and *ela2*/*ela2l* likely are ancient duplicates preserved after a genome-wide duplication in the teleost lineage approximately 400 million years ago (21). Further, fish *ctrb1* and *ctrb1l* are orthologs of mammalian *Ctrb1* and *Ctr1l*, respectively (Fig. 2).

All members of the serine peptidase SA clan have a conserved catalytic triad sequence with histidine (His), aspartate (Asp), and serine (Ser; see asterisks in online Supplemental Fig. 2). These serine peptidases function extracellularly, such as in food digestion and fibrinolysis. The preferential cleavages of trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and elastase 2 (EC 3.4.21.71) are arginine (Arg)-unspecified amino acid (Xaa)/lysine (Lys)-Xaa, tyrosine (Tyr)-Xaa > tryptophan (Trp)-Xaa > phenylalanine (Phe)-Xaa > leucine (Leu)-Xaa, and Leu-Xaa/methionine (Met)-Xaa/Phe-Xaa, respectively (22–24).

Identification of Three Zebrafish Novel Exocrine Pancreas-Specific Peptidase Genes. Of the six peptidase genes, *try* (25), *ela2l* (26), and *cpa* (27, 28) were previously shown to be expressed specifically in the pancreas. We now report that *tryl*, *ctrb1*, and *ctr1l* also have pancreas-specific expression in the zebrafish (Figs. 1E, G, and I). Phylogenetic analysis indicated zebrafish *tryl* is a co-ortholog of mammalian *Try*, whereas zebrafish *ctrb1* and *ctr1l* are orthologs of mammalian *Ctrb1* and *Ctr1l*, respectively (see the above and Fig. 2). The *tryl* gene (Genbank accession BC055625) has a predicted 726-nucleotide (nt) open reading frame (ORF) and encodes a 242–amino acid (aa) protein that shares 61.5% identity to the zebrafish Try protein (see online Supplemental Table 4). The *ctrb1* (Genbank accession BC055574) has a predicted 789-nt ORF and encodes a 263-aa protein that shares 65.3% identity to human chymotrypsinogen B1 (29) (see online Supplemental Table 4). The *ctr1l* (Genbank accession NM_001004582) has a 783-nt ORF and encodes a 261-aa protein that shares 60.3% identity to human chymotrypsinogen B1 (29) (see online Supplemental Table 4). All the three predicted proteins contain the tryp_SPc domains (SM00020; online Supplemental Fig. 2) and the typical serine peptidase catalytic triad (see asterisks in online Supplemental Fig. 2). Our whole-mount *in situ* hybridization and bioinformatic analyses suggest that these three novel genes, *tryl*, *ctrb1*, and *ctr1l*, likely contribute to pancreatic functions.

Downregulation of the Six Peptidase Precursor Genes in *sauternes/alas2*. We also examined expression of the six peptidase genes in zebrafish mutant *sau*/*alas2* (30), wherein a missense mutation (V249D) in the gene encoding the first and rate-liming erythroid-specific δ isoform of ALAS2 (EC 2.3.1.375) in heme biosynthesis results in microcystic and hypochromic anemia (10). Whole-mount *in situ* hybridization showed downregulation of these six peptidase precursor genes in the *sau*/*alas2* mutant ($P < 0.05$, ANOVA; see Fig. 4). Because ALAS2

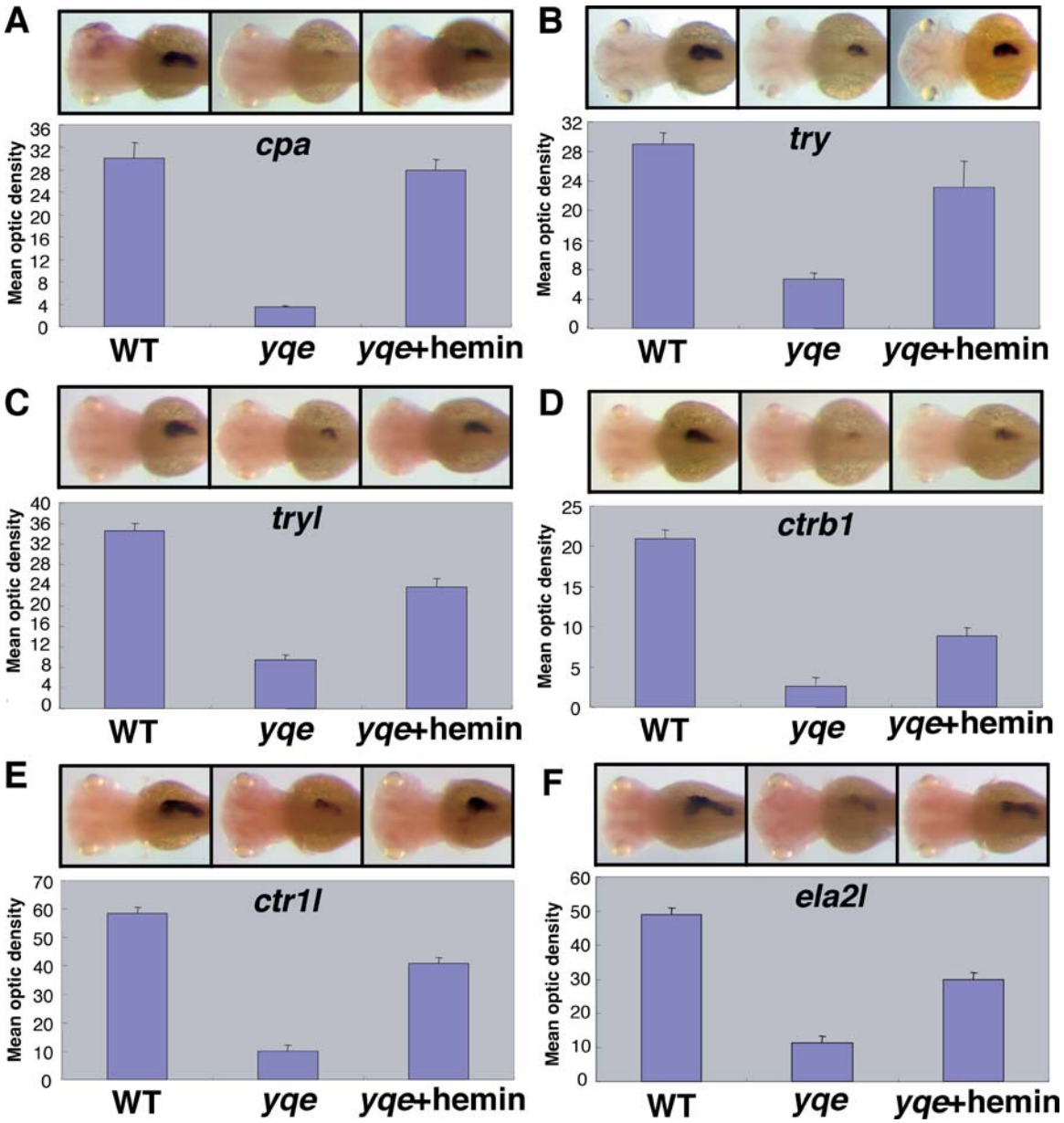

Figure 3. Rescue the expression of the six peptidase precursor genes in *yquem/urod* (–/–) by hemin treatment. All larvae are shown in dorsal view, anterior to the left. Larvae are 74 hpf in (A) *cpa*, (B) *try*, (C) *tryl*, and (D) *ctrb1*, and 97 hpf in (E) *ctr1l* and (F) *ela2l*. The O.D. of staining (*S*) and the neighboring background (*B*) were measured by ImageJ. The mean total O.D. = $S - B$ (see Materials and Methods) in 20–50 embryos. Error bars are standard deviation. (A–F) Differences of signal intensities between hemin-treated and untreated larvae are statistically significant ($P < 0.001$, ANOVA). Color figures are available in the online version of the journal.

(the first enzyme) and UROD (the fifth enzyme) are in the same heme biosynthetic pathway, our results of downregulation of the six zymogens in both *yquem*/*urod* and *sau*/*alas2* strongly suggest that heme is required for expression of these zymogens in zebrafish.

Downregulation of *ptf1a* in the Exocrine Pancreas of Zebrafish *yquem/urod* and *sauternes/alas2*. To investigate the molecular genetic mechanisms underlying heme regulation of exocrine zymogens, we examined the expression of *pancreas transcription factor 1α* (*ptf1a*), which encodes a pancreas-specific basic helix-loop-helix (bHLH) transcription factor. PTF1A, the 48-kDa DNA-binding subunit of the heterotrimeric pancreas transcription factor-1 (PTF1) complex, is required for activating digestive zymogens (31) and zebrafish exocrine acinar cell differentiation and development (32). Whole-mount *in situ* hybridization showed that *ptf1a* is downregulated in both *yquem*/*urod* and *sauternes*/*alas2* mutant larvae ($P < 0.05$, ANOVA; see Fig. 5).

Rescue of Expression of *ptf1a* and the Six Peptidase Precursor Genes in *yquem/urod* and *sauternes/alas2* by Hemin Treatment. To determine

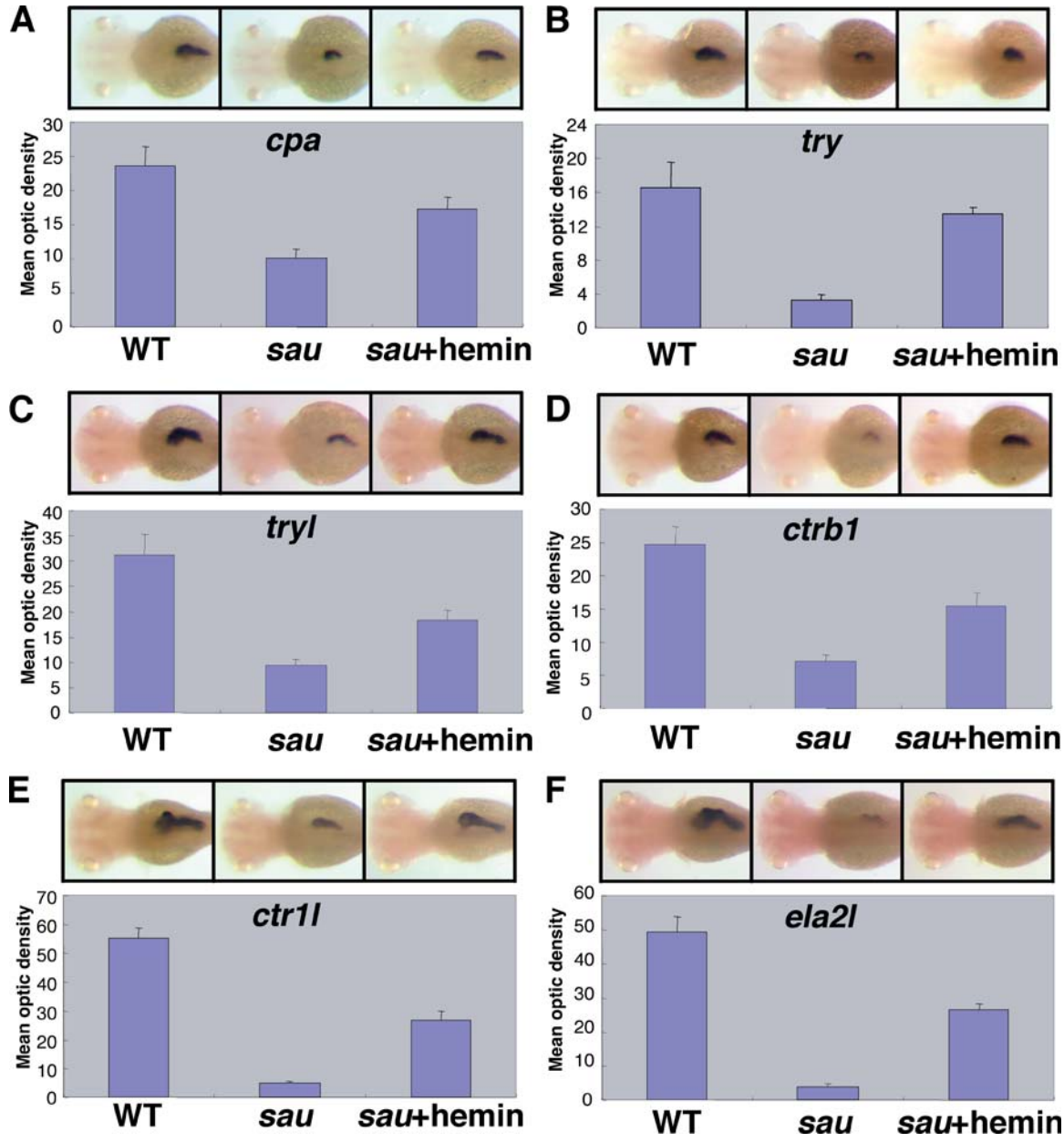

Figure 4. Downregulation of the six peptidase precursor genes in *sau/alas2* (–/–) and rescue of their downregulated expression in *sau/alas2* (–/–) by hemin treatment. All larvae are shown in dorsal view, anterior to the left. Larvae are 74 hpf in (A) *cpa*, (B) *try*, (C) *tryl,* and (D) *ctrb1*, and 97 hpf in (E) *ctr1l* and (F) *ela2l*. The O.D. of staining (*S*) and the neighboring background (*B*) were measured by ImageJ. The mean total O.D. = *S* – *B* (see Materials and Methods) 10–30 embryos. Error bars are standard deviation. (A–F) Differences of signal intensities between hemin-treated and untreated larvae are statistically significant ($P < 0.001$, ANOVA), except for (A) *cpa* ($P < 0.05$, ANOVA). Color figures are available in the online version of the journal.

whether heme regulates zebrafish exocrine zymogens, the *yquem*/*urod* and *sauternes*/*alas2* mutant larvae were treated with 1 μ*M* hemin for 1 hr and then fixed for *in situ* hybridization experiments. Remarkably, the expression of *ptf1a* and the six peptidase precursor genes was rescued in hemin-treated *yquem*/*urod* and *sauternes*/*alas2* mutant larvae ($P < 0.05$, ANOVA; see Figs. 3–5). We did not observe significant differences in the exocrine pancreatic expression domains for all six zymogens and *ptf1a* between hemin-treated WT and untreated WT larvae, nor did we observe any significant differences for them between vehicle solution-treated and untreated *yquem*/*urod* and *sauternes*/*alas2* mutant larvae (data not shown).

Discussion

Our study now provides evidence that heme plays an important role in regulating zebrafish exocrine zymogens. Heme, the prosthetic moiety for a variety of proteins that have critical roles in oxygen transportation, mitochondrial function, and signal transduction, seems to have evolved to

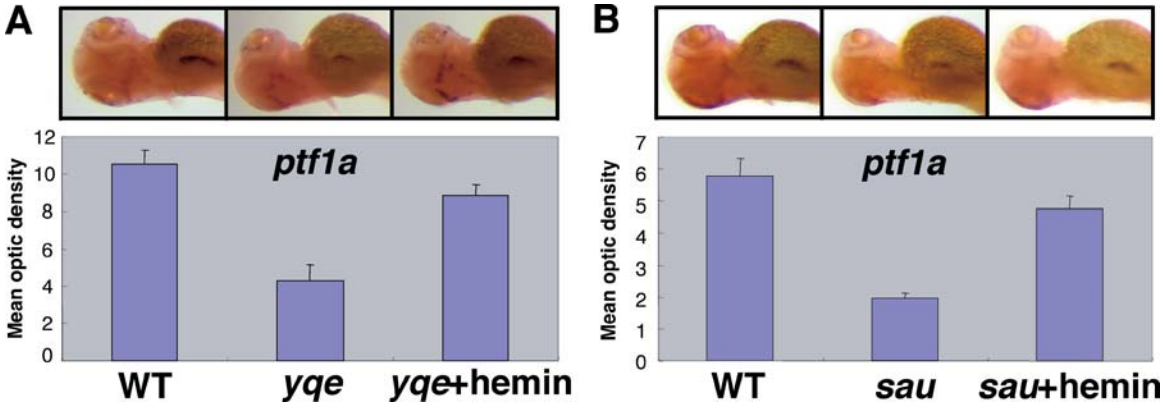

Figure 5. Downregulation of *ptf1a* in both *yquem/urod* (–/–) and *sau/alas2* (–/–) and rescue of its downregulated expression in both *yquem/urod* (–/–) and *sau/alas2* (–/–) by hemin treatment. All larvae are shown as left dorsal oblique view, anterior to the left. Downregulation of *ptf1a* was also observed in the (panel A) *yquem/urod* (–/–) mutant and the (panel B) *sau/alas2* (–/–) mutant and its downregulated expression is rescued by hemin treatment. Larvae are 74 hpf in panels A and B. The O.D. of staining (*S*) and the neighboring background (*B*) were measured by ImageJ. The mean total O.D. = $S - B$ (see Materials and Methods) 30–50 embryos. Error bars are standard deviation. Differences of signal intensities between hemin-treated and untreated larvae in A and B are statistically significant ($P < 0.05$, ANOVA). Color figures are available in the online version of the journal.

have other biological functions in addition to its widely known roles in biosynthesis of hemoglobin, cytochromes, and nitric oxide synthase. In this regard, heme was recently found to differentially modulate expression of *mPer1* and *mPer2* and thereby regulate the circadian clock by controlling BMAL1:NPAS2–mediated transcription activities (33). In addition, heme is required for differentiation of mammalian erythroid, hepatic, and nervous cells (2–5), and it also suppresses the apoptosis of human neutrophils (6), PC12 neurons (7), and HeLa cells (8), whereas hemin activates heme oxygenase-1 (Ho-1)-mediated macrophages recruitment to the pancreas and hence prevents from acute pancreatitis in mouse (34).

Our microarray analysis and *in situ* hybridization experiments consistently showed that these six exocrine peptidase precursor genes are downregulated in *yquem*/*urod* zebrafish, even though expression levels between *yquem*/*urod* and WT zebrafish determined by the two methods may not be the same for some of these six genes at specific developmental stages (Fig. 1 and Table 1). This is likely a result of the differences in detection sensitivity of the two methods. Whereas microarray analysis appears to measure the whole expression level of a gene at a specific developmental stage, the *in situ* hybridization-based Image J analysis allows for detection of a relative expression level of the gene at that stage, in particular the expression of the gene in the deep cells (for instance, inside the exocrine pancreas) may not be estimated precisely. Even so, the independent *in situ* hybridization experiments clearly corroborated the microarray results for most of the stages examined (Fig. 1 and Table 1).

In addition to activating transcription of exocrine peptidase precursor genes (31), PTF1A also is required for zebrafish exocrine acinar cell differentiation and development (32). Thus, it is possible that exocrine pancreatic development is delayed in *yquem*/*urod*, and *sauternes*/*alas2* mutant larvae resulted from downregulation of *ptf1a*, which should also contribute to the observed downregulation of these exocrine zymogens. Further, hemin treatment also induces Ho-1, one of the three heme oxygenase enzymes that catalyze the degradation of heme (34–36). It is also possible that Ho-1 will in turn inhibit heme-mediated transcription of these exocrine zymogens by degrading heme *per se*. In fact, the likelihood of the exocrine pancreatic developmental delay as well as the induction of Ho-1 may have resulted in partial rescue of zymogen transcription by hemin treatment (Figs. 3 and 4). Though not completely rescued, the rapid response to hemin treatment by these exocrine zymogens strongly suggests that heme likely regulates their transcription.

Mammalian studies have implicated important roles for PTF1A in the development of the exocrine pancreas (37). As a member of the bHLH transcription factor family, PTF1A activates the genes by binding the E-box in the 5′ flanking region of the controlled genes (31, 38–40). Interestingly, all six zymogens possess multiple E-boxes (CANNTG) in the 5′ flanking regions (approximately 6000 nt upstream of the transcription site; Table 3), supporting the idea that PTF1A regulates these six zymogens in zebrafish.

Importantly, several heme-responsive transcription factors recently were revealed, such as the iron regulatory regulator (Irr) in the bacterium *Bradyrhizobium japonicum* (41), the heme activator protein (Hap1) in the yeast *Saccharomyces cerevisiae* (5, 42), and the transcriptional repressor Bach1 in mammals (43, 44). All the heme-regulated proteins share cysteine- and proline-containing heme regulatory motifs (HRMs) that heme directly binds to (45). A database search using mammalian homologs identified zebrafish *bach2* (Genbank accession XP_682933), with the predicted zebrafish BACH2 protein containing four heme-binding HRMs and a DNA-binding basic leucine zipper (bZip) domain (SM00338) (data not

shown). It is tempting to imagine that zebrafish heme-responsive proteins, such as BACH2, may be a missing link through which heme exerts its regulatory role on transcription of *ptf1a*, and then, the PTF1A protein regulates these six zymogens in zebrafish. Alternatively, BACH2 or other zebrafish heme-responsive proteins may directly regulate these six zymogens in zebrafish. On the other hand, Ho-1 induced by hemin treatment catalyzes the breakdown of heme into biliverdin, carbon monoxide (CO) and iron (34–36). Biliverdin is subsequently converted into bilirubin by biliverdin reductase (35, 36). All the three heme degradation products, bilirubin, CO and iron, appear to have regulatory or signaling roles in various physiological and cellular processes (35). Further investigation is needed to examine these competing hypotheses concerning how heme and the heme-responsive proteins regulate the *ptf1a* expression as well as whether the heme-responsive proteins, Ho-1, and the three heme degradation products directly regulate zymogens.

Hemin, a heme substrate analog, has been used clinically to treat porphyria patients to alleviate the acute episodic pains, although the mechanism underlying this treatment is still elusive (46–48). Because molecular genetic pathways underlying many zebrafish and human biochemical, developmental, and physiological processes are highly conserved (49, 50), it is tempting to speculate that the acute episodic abdominal pains associated with nausea and vomiting inflicted upon porphyria patients (48) are caused by underproduction of exocrine zymogens resulting from heme deficiency, and hemin treatment alleviate these pains (46–48) by restoring zymogen production levels. Our study suggests that patients with porphyria and a heme deficiency, who also have exocrine pancreatic problems caused by symptomatic underproduction of zymogens, would benefit by hemin treatment (46–48) as it would help increase zymogen production levels.

In summary, our results indicated that heme deficiency results in underproduction of exocrine zymogens and heme regulates exocrine zymogens in zebrafish. These findings add to a growing body of knowledge regarding heme deficiency pathogenesis and should enhance our ability to diagnose and treat human patients with porphyria or pancreatic diseases.

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