RESEARCH ARTICLE

Heme acts through the Bach1b/Nrf2a-MafK pathway to regulate exocrine peptidase precursor genes in porphyric zebrafish

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ABSTRACT

Using a zebrafish model of hepatoerythropoietic porphyria (HEP), we identify a previously unknown mechanism underlying heme-mediated regulation of exocrine zymogens. Zebrafish bach1b, nrf2a and mafK are all expressed in the zebrafish exocrine pancreas. Overexpression of bach1b or knockdown of nrf2a result in the downregulation of the expression of the exocrine zymogens, whereas overexpression of nrf2a or knockdown of bach1b cause their upregulation. In vitro luciferase assays demonstrate that heme activates the zymogens in a dosage-dependent manner and that the zymogen promoter activities require the integral Maf recognition element (MARE) motif. The Bach1b-MafK heterodimer represses the zymogen promoters, whereas the Nrf2a-MafK heterodimer activates them. Furthermore, chromatin immunoprecipitation (ChIP) assays show that MafK binds to the MARE sites in the 5′ regulatory regions of the zymogens. Taken together, these data indicate that heme promotes the exchange of Bach1b for Nrf2a at MafK-occupied MARE sites and is responsible for the exchange of Bach1b for Nrf2a at MafK-occupied MARE sites and the dynamic exchange of BTB and cap'n'collar homolog 1 (Bach1) and cap'n'collar (CNC) bZIP proteins, including Nrf2 and Bach1 (Shuqing Zhang et al., 2007; Balwani and Desnick, 2012). We have previously established a heme-deficient zebrafish that is homozygous for a mutation in uroporphyrinogen decarboxylase (urod; also known as yquem) as a model for studying human hepatoerythropoietic porphyria (HEP) (Online Mendelian Inheritance in Man number 176100) and heme deficiency pathogenesis (Wang et al., 1998). Our microarray and in situ hybridization analyses of this zebrafish HEP model revealed downregulation of six peptidase precursor genes – including carboxypeptidase A5 (cpa5), chymotrypsinogen 1 like (ctrl1), chymotrypsinogen B1 (ctrb1), elastase 2 like (el2l), trypsin precursor (try) and trypsin like (tryl) – specifically in the exocrine pancreas of the HEP zebrafish (yquem/urod, −/−) (Wang et al., 2007). Of these six zymogens, cpa5 (previously called cpa) belongs to the MEROPS peptidase family M14, whereas the other five peptidases contain a trypsin-like serine protease (tryp_SPc) domain and are members of the serine peptidase chymotrypsin family S1 (chymotrypsin A, clan PA) (http://merops.sanger.ac.uk/) (Wang et al., 2007). These serine peptidases function extracellularly to aid food digestion (Hedstrom, 2002; Wang et al., 2007). However, the molecular mechanism underlying how these exocrine zymogens regulate heme remains poorly understood (Wang et al., 2007). Heme also serves as a signaling molecule and modulates a number of molecular and cellular processes (Sassa and Nagai, 1996; Sun et al., 2004; Mense and Zhang, 2006). Several studies have shown that heme regulates gene expression by mediating the dynamic exchange of BTB and cap’n’collar homolog 1 (Bach1) and nuclear factor erythroid 2 p45-related factor 2 (Nrf2) in the small musculoaponeurotic fibrosarcoma (Maf) oncogene transcription factor network (Igarashi et al., 1998; Ogawa et al., 2001; Kitamura et al., 2003; Brand et al., 2004; Sun et al., 2004; Marro et al., 2010). MafK is a member of the small Maf protein family that binds to the Maf Recognition Element (MARE) motif [TGCTGA(C/G)TCAGCA] through its DNA-binding basic leucine zipper (bZIP) domain (Toki et al., 2005; Igarashi and Sun, 2006; Blank, 2008). However, because MafK lacks a transactivation domain, it regulates transcription through interaction with the cap’n’collar (CNC) bZIP proteins, including Nrf2 and Bach1 (Toki et al., 2005; Igarashi and Sun, 2006; Blank, 2008). The Nrf2-MafK heterodimer serves as an activator (Pratt et al., 2002; Kobayashi and Yamamoto, 2006), whereas the Bach1-MafK heterodimer functions as a repressor because Bach1 harbors a transcription-repressing BTB/POZ domain (Toki et al., 2005; Igarashi and Sun, 2006; Blank, 2008). Moreover, heme binds to Bach1 through its cysteine-proline (CP) dipeptide-containing heme regulatory motifs (HRMs) (Yanqing Zhang et al., 2003). Heme-binding dissociates Bach1 from MafK, thereby abolishing repression of its downstream genes.

KEY WORDS: Bach1b, Nrf2a, MafK, Heme, Porphyria, Zymogens, Zebrafish

INTRODUCTION

As the prosthetic moiety for numerous proteins and enzymes – such as hemoglobin, catalases and cytochrome – heme is essential for nearly all forms of life through oxygen transport, respiration, detoxification and other important processes (Padmanaban et al., 1989; Igarashi and Sun, 2006; Mense and Zhang, 2006; Balwani and Desnick, 2012). A cascade of eight highly conserved enzymatic reactions controls heme biosynthesis (Kappas et al., 1995; Ryter and Vajda, 2008; Cappellini et al., 2010; Puy et al., 2010; Siegesmund et al., 2010; Balwani and Desnick, 2012). We have previously established a heme-deficient zebrafish that is homozygous for a mutation in uroporphyrinogen decarboxylase (urod; also known as yquem) as a model for studying human hepatoerythropoietic porphyria (HEP) (Online Mendelian Inheritance in Man number 176100) and heme deficiency pathogenesis (Wang et al., 1998). Our microarray and in situ hybridization analyses of this zebrafish HEP model revealed downregulation of six peptidase precursor genes – including carboxypeptidase A5 (cpa5), chymotrypsinogen 1 like (ctrl1), chymotrypsinogen B1 (ctrb1), elastase 2 like (el2l), trypsin precursor (try) and trypsin like (tryl) – specifically in the exocrine pancreas of the HEP zebrafish (yquem/urod, −/−) (Wang et al., 2007). Of these six zymogens, cpa5 (previously called cpa) belongs to the MEROPS peptidase family M14, whereas the other five peptidases contain a trypsin-like serine protease (tryp_SPc) domain and are members of the serine peptidase chymotrypsin family S1 (chymotrypsin A, clan PA) (http://merops.sanger.ac.uk/) (Wang et al., 2007). These serine peptidases function extracellularly to aid food digestion (Hedstrom, 2002; Wang et al., 2007). However, the molecular mechanism underlying how these exocrine zymogens regulate heme remains poorly understood (Wang et al., 2007). Heme also serves as a signaling molecule and modulates a number of molecular and cellular processes (Sassa and Nagai, 1996; Sun et al., 2004; Mense and Zhang, 2006). Several studies have shown that heme regulates gene expression by mediating the dynamic exchange of BTB and cap’n’collar homolog 1 (Bach1) and nuclear factor erythroid 2 p45-related factor 2 (Nrf2) in the small musculoaponeurotic fibrosarcoma (Maf) oncogene transcription factor network (Igarashi et al., 1998; Ogawa et al., 2001; Kitamura et al., 2003; Brand et al., 2004; Sun et al., 2004; Marro et al., 2010). MafK is a member of the small Maf protein family that binds to the Maf Recognition Element (MARE) motif [TGCTGA(C/G)TCAGCA] through its DNA-binding basic leucine zipper (bZIP) domain (Tokie et al., 2005; Igarashi and Sun, 2006; Blank, 2008). However, because MafK lacks a transactivation domain, it regulates transcription through interaction with the cap’n’collar (CNC) bZIP proteins, including Nrf2 and Bach1 (Toki et al., 2005; Igarashi and Sun, 2006; Blank, 2008). The Nrf2-MafK heterodimer serves as an activator (Pratt et al., 2002; Kobayashi and Yamamoto, 2006), whereas the Bach1-MafK heterodimer functions as a repressor because Bach1 harbors a transcription-repressing BTB/POZ domain (Toki et al., 2005; Igarashi and Sun, 2006; Blank, 2008). Moreover, heme binds to Bach1 through its cysteine-proline (CP) dipeptide-containing heme regulatory motifs (HRMs) (Fig. 1A) (Kitamura et al., 2003). Heme-binding dissociates Bach1 from MafK, thereby abolishing repression of its downstream genes.

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zymogens. Our studies reveal novel aspects of heme deficiency (ChIP) assays were conducted to elucidate how heme regulates these expression of cpa5 and triggers the dissociation of Bach1 from MafK, allowing Nrf2 to transcription. Conversely, at normal or higher levels of heme as in porphyria, the Bach1b-MafK heterodimer binds to the MARE et al., 2004; Marro et al., 2010).

RESULTS

In this study, the authors use the zebrafish model for HEP and a combination of genetic, embryological, in vitro cell transfection and biochemical approaches to investigate the regulation of pancreatic zymogen production by heme. They show that heme determines pancreatic zymogen production levels by mediating the dynamic exchange of the bZIP transcription factors Bach1b and Nrf2a at Maf recognition element (MARE) sites that are occupied by MafK, another bZIP transcription factor. Specifically, in conditions of heme-deficiency such as porphyria, the Bach1b-MafK heterodimer occupies the MARE sites of the genes encoding pancreatic zymogens and represses their expression. Notably, the authors show that treatment with hemin (a reddish-brown chloride of heme) can dissociate Bach1b from MafK, which allows Nrf2a to enter the MafK-occupied MARE sites. The Nrf2a-MafK heterodimer, which is a transcriptional activator, then restores zymogen production.

Implications and future directions

These findings shed light on the frequently reported acute episodic abdominal pains with co-occurrences of nausea and vomiting in some porphyria individuals, and on the regulation of exocrine function. Specifically, because the findings reported here also show that the roles of the Bach1b, Nrf2a and MafK in heme-mediated regulation of exocrine zymogens appear to be highly conserved between zebrafish and human, the acute episodic abdominal pains in these individuals probably stem from decreased production of pancreatic zymogens. Moreover, these findings provide an explanation for why treatment with hemin can cure these symptoms in individuals with porphyria and identify BACH1 expression in the exocrine pancreas as a potential new therapeutic target.

(Ohawa et al., 2001; Kitamura et al., 2003; Sun et al., 2004; Suzuki et al., 2004; Marro et al., 2010).

Thus, we hypothesize that under heme-deficient conditions, such as in porphyria, the Bach1-MafK heterodimer binds to the MARE sites of exocrine pancreatic zymogens and represses their transcription. Conversely, at normal or higher levels of heme (induced by heme treatment, for instance), heme binds to Bach1 and triggers the dissociation of Bach1 from MafK, allowing Nrf2a to enter the MafK-occupied MARE sites in the regulatory regions of zymogens and then activate them.

To test this hypothesis, zebrafish Bach1b (a co-ortholog of mammalian Bach1) (Fig. 1B) was isolated, its expression, along with the expression of zebrafish nrf2a (a co-ortholog of mammalian Nrf2) (Timme-Laragy et al., 2012) and mafK (Takagi et al., 2004), in the zebrafish exocrine pancreas was determined; the effect of both knockdown and overexpression of bach1b or nrf2a on the expression of cpa5, ctr1l, ctrbl, elal2, try and try1 was examined, and in vitro cell transfection and chromatin immunoprecipitation (ChIP) assays were conducted to elucidate how heme regulates these zymogens. Our studies reveal novel aspects of heme deficiency pathogenesis and should offer new ways to diagnose and treat individuals that have porphyria.

RESULTS

Heme regulation of exocrine peptidase precursor genes

Our previous microarray and whole-mount in situ hybridization analyses of the zebrafish yquem/urod (−/−) HEP model shows that
six peptidase precursor genes – cpa5, ctr1l, ctrb1, ela2l, try and tryl – are significantly downregulated in this zebrafish model (Wang et al., 2007). Quantitative real-time (qRT)-PCR analysis corroborated these previous findings (Fig. 1C). Remarkably, treatment with hemin (a reddish-brown chloride of heme) significantly restored the expression of these six peptidase precursor genes in the zebrafish yquem/urod (−/−) (Fig. 1C) (Wang et al., 2007). These results indicate that heme regulates transcription of these six exocrine zymogens in zebrafish. Here, we aimed to elucidate its underlying molecular mechanism.

**Expression of bach1b, nrf2a and mafK in the exocrine pancreas**

Because we hypothesized that Bach1, Nrf2 and MafK are involved in heme-mediated regulation of these exocrine peptidase precursor genes, we first examined whether these three genes were expressed in the exocrine pancreas. We have previously examined two genes that were initially called zebrafish bach1 and nrf2. Owing to the teleost genome duplication (Amores et al., 2011), zebrafish possess more copies of certain genes (Postlethwait et al., 2004; Wang, 2008). Through interrogation of fish genome sequences and phylogenetic analysis, we have recently found that zebrafish have two bach1 genes – bach1α and bach1β – which are co-orthologs of mammalian Bach1 (Fig. 1B); Timme-Laragy et al. have also reported that zebrafish have two nrf2 genes – nrf2α and nrf2β – which are co-orthologs of mammalian Nrf2 (Timme-Laragy et al., 2012). Therefore, the two genes that we have examined (here and previously) are actually zebrafish bach1b and nrf2a. Double in situ hybridization analyses showed that bach1b, nrf2a and mafK are all expressed in the exocrine pancreas, as evidenced by colocalization of the transcription of the three genes in the exocrine pancreas with the exocrine pancreas maker gene try (Wang et al., 2007) (Fig. 2); however, they also are expressed in some other tissues in zebrafish larvae. Because these results suggest that bach1b, nrf2a and mafK play regulatory roles in the exocrine pancreas, we focused on these three genes in this study.

**Roles of Bach1b and Nrf2a in regulation of exocrine peptidase precursor genes**

To elucidate the roles of Bach1b and Nrf2a in the regulation of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments. Capped mRNAs of exocrine zymogens, we conducted both overexpression and knockdown experiments.
Fig. 3. Overexpression experiments with bach1b and nrf2α reveal their antagonism in the regulation of zymogen expression. (A) qRT-PCR analysis showed that bach1b overexpression resulted in the downregulation of six peptidase precursor genes — cpa5, ctr1l, ctrb1, ela2l, try and tryl — whereas nrf2α overexpression resulted in their upregulation. Overexpression experiments were performed by microinjecting bach1b or nrf2α capped mRNAs into one-cell stage embryos. The expression levels of the six peptidase precursor genes in the microinjected and control larvae at 84 hpf were determined by qRT-PCR analysis. (B) Representative images of in situ hybridization staining show that downregulation of cpa5 resulted from bach1b overexpression and that its upregulation resulted from nrf2α overexpression, both specifically in the exocrine pancreas. Dorsal view, anterior to the left. (C) Mean optic densities of in situ hybridization staining of a group of larvae (10-12 each) corresponding to Fig. 3B were quantified by using ImageJ. (D) cpa5 morphant phenotypes. For individual larvae (84 hpf) of the bach1b overexpression group, the optic density values lower than the mean optic density value of its own group were marked as ‘weak’, and the optic density values higher than the mean optic density value of the control group were marked as ‘strong’. For individual larvae of the nrf2α overexpression group, the optic density values lower than the mean optic density value of its own group were marked as ‘strong’, the optic density values higher than the mean optic density value of the control group were marked as ‘weak’, and the values of optic density between ‘weak’ and ‘strong’ marked as ‘medium’. The statistical significance of difference between means was determined by one-way ANOVA and Tukey’s multiple comparison test (n=9) by using SPSS10.0.1. *P<0.05, **P<0.01, ***P<0.001.

Fig. 4. Knockdown experiments with bach1b and nrf2α reveal their antagonism in the regulation of zymogen expression. (A) qRT-PCR analysis showed that bach1b knockdown results in the upregulation of the six peptidase precursor genes that we examined, whereas nrf2α knockdown resulted in their downregulation. Knockdown experiments were performed by microinjecting MOs that targeted bach1b or nrf2α into one-cell stage embryos. For bach1b knockdown experiments, an ATG MO and SPL MO were used. Reverse transcription PCR showed that the SPL MO effectively altered bach1b intron 2 splicing (supplementary material Fig. S1A,B). The ATG MO also effectively knocked down bach1b (supplementary material Fig. S1C). The results using the SPL MO are shown here. For nrf2α knockdown experiments, we used an ATG MO, of which the efficacy of knockdown has been confirmed previously (Kobayashi et al., 2002; Kobayashi et al., 2009; Wang and Gallagher, 2013). The expression levels of the six peptidase precursor genes in the microinjected and control larvae at 84 hpf were determined by using qRT-PCR. (B) Representative images of in situ hybridization staining show that the upregulation of cpa5 resulted from bach1b knockdown and that its downregulation resulted from nrf2α knockdown, both results occurred specifically in the exocrine pancreas. Dorsal view, anterior to the left. (C) Mean optic densities of in situ hybridization staining of a group of larvae (10-12 each) corresponding to Fig. 4C were quantified by using ImageJ. (D) cpa5 morphant phenotypes. For individual larvae (84 hpf) of the bach1b knockdown overexpression group, the optic density values higher than the mean optic density value of its own group were marked as ‘strong’, and the optic density values lower than the mean optic density value of the control group were marked as ‘weak’. For individual larvae of the nrf2α knockdown group, the optic density values lower than the mean optic density value of its own group were marked as ‘weak’, the optic density values higher than the mean optic density value of the control group were marked as ‘strong’, and the values of optic density between ‘weak’ and ‘strong’ marked as ‘medium’. The statistical significance of difference between means was determined by using one-way ANOVA and Tukey’s multiple comparison test (n=9) with SPSS10.0.1. *P<0.05, **P<0.01, ***P<0.001.
Roles of the Bach1b-MafK and Nrf2a-MafK heterodimers in heme-mediated regulation of exocrine peptidase precursor genes

To delineate the roles of the Bach1b-MafK and Nrf2a-MafK heterodimers in heme-mediated regulation of exocrinezymogens, we conducted assays that involved co-transfection of Mafk pathway genes and treatment with heme. Co-transfection of nrf2a and mafk facilitated the activities of the two ctrll and tryl gene promoters (Fig. 5C), whereas co-transfection of bach1b and mafk repressed them (supplementary material Fig. S5C). Moreover, higher doses of Nrf2a outcompeted Bach1b and activated the luciferase reporter constructs for the zymogens, whereas higher doses of Bach1b outcompeted Nrf2a and repressed them, even though the activating effects on these two zymogens, stimulated by higher doses of Nrf2a, were much greater than the repressive effects of higher doses of Bach1b (Fig. 5C; supplementary material Fig. S5C). It is noteworthy that treatment with 10 μM hemin for 1 hour was sufficient to enhance the activity of these two gene promoters in all cases – whether cells were transfected with mafk alone, or co-transfected in combination with mafk and nrf2a or mafk and bach1b (Fig. 5C; supplementary material Fig. S5C).

These results show that the Nrf2a-MafK heterodimer activates these exocrinezymogens, whereas the Bach1b-MafK heterodimer represses them.

The dynamic exchange of Bach1b and Nrf2a is mediated by heme

Previous studies have shown that MafK can form a heterodimer with either Nrf2a or Bach1 (Takagi et al., 2004; Zenke-Kawasaki et al., 2007), and an electrophoretic mobility shift assay was used to show that zebrafish MafK binds to the MARE site (Takagi et al., 2004). To determine whether zebrafish MafK can bind to the MARE-harboring regulatory regions of zebrafish exocrinezymogens, we performed chromatin immunoprecipitation (ChIP) assays. The results showed that zebrafish MafK bound to the MARE-containing promoters of all six zebrafish exocrinezymogens (Fig. 6A). We also conducted ChIP assays to investigate exactly how heme mediates the dynamic exchange of Bach1b and Nrf2a in their heterodimerization.

Conversely, in the heme-deficient HEP larvae, more Bach1b than Nrf2a appeared to be associated with the MARE-containing regulatory regions of the zymogens. In particular, treatment of the HEP larvae with hemin reversed this situation so that more Nrf2a than Bach1b interacted with the MARE-containing regulatory regions of the zymogens.

These data suggest that heme mediates the dynamic exchange of Bach1b and Nrf2a in their heterodimerization with MafK, which in turn exerts regulatory effects on the exocrinezymogens, in a manner similar that in which they regulate their other downstream genes (Ogawa et al., 2001; Sun et al., 2004; Marro et al., 2010).
**DISCUSSION**

Zebrafish possess duplicate genes *bach1a* and *bach1b* (Fig. 1B), and *nrf2a* and *nrf2b* (Timme-Laragy et al., 2012), which are derived from an ancient genome-wide duplication that occurred in teleost fish – including zebrafish (Aparicio et al., 2002; Jaillon et al., 2004; Kasahara et al., 2007). These two pairs of ancient duplicates appear to have evolved distinct functions through subfunctionalization (Postlethwait et al., 2004; Wang, 2008; Timme-Laragy et al., 2012). Here, our experiments, using overexpression and knockdown of *bach1b* and *nrf2a*, demonstrated the antagonism between Bach1b and Nrf2a in heme-mediated regulation of the zymogens Cpa5, Ctr1l, Ctrb1, Ela2l, Try and Tryl (Figs 3, 4; supplementary material Figs S2, S3). Previous mouse gene targeting studies have also shown the opposing functions of Bach1 and Nrf2 on the expression of mouse heme oxygenase 1 (*Ho-1*) – i.e. *Ho-1* is upregulated in Bach1 knockout mice and downregulated in *Nrf2*-deficient cells (Sun et al., 2002). Also, our *in vitro* experiments showed that higher doses of *nrf2a* outcompete *bach1b* and thus enhance the expression of the target zymogens, whereas higher doses of *bach1b* outcompete *nrf2a* and repress expression of the zymogens examined here (Fig. 5C; supplementary material Fig. S5C). Moreover, our *in situ* hybridization experiments showed that *bach1b* and *nrf2a*, as well as *mahk*, are indeed expressed in the zebrafish exocrine pancreas (Fig. 2). However, whether the other two duplicates *bach1a* and *nrf2b* play roles in heme-mediated regulation of the six zymogens requires further investigation. Importantly, the regulatory functions of Bach1 on exocrine zymogens appear to be highly conserved, as evidenced by the fact that overexpression of human BACH1 represses these six exocrine zymogens in zebrafish larvae (Fig. 7A).

The delay of the development of the exocrine pancreas in HEP fish does not play a substantial role in down-regulation of the zymogens that were investigated here. Although both qRT-PCR and *in situ* hybridization experiments with the exocrine cell determination gene exocrine differentiation and proliferation factor (*exdpf*) (Jiang et al., 2008) showed that there is, indeed, a degree of delay in the development of the exocrine pancreas in the HEP fish (Fig. 7B; supplementary material Fig. S7), the fact that treatment with hemin rapidly represses the expression of the exocrine zymogens...
indicates the presence of sufficient acinar cells (Wang et al., 2007). We believe that heme deficiency is the major cause of the downregulation of thezymogens Cp5, Ctr11, Ctrb1, Ela2l1, Try and Tryl in the HEP fish.

As zebrafish Bach1 contains HRMs with CP dipeptide (Fig. 1A), it mediates the regulatory role of heme in transcription of thezymogens investigated here (Wang et al., 2007). Heme binds to Bach1 through its CP-rich HRMs, leading to its Crn1-dependent nuclear export (Suzuki et al., 2004), and subsequently its ubiquitination and degradation (Zenke-Kawasaki et al., 2007). Consequently, Nrf2 heterodimerizes with MafK and activates the transcription of the downstream genes (Suzuki et al., 2004; Zenke-Kawasaki et al., 2007).

Our study reveals the roles of Bach1, Nrf2a and MafK in heme-mediated regulation of exocrine peptidase precursor genes. Similar to regulation of other genes by the MafK pathway (Igarashi et al., 1998; Ogawa et al., 2001; Kitamuro et al., 2003; Brand et al., 2004; Sun et al., 2004; Marro et al., 2010), we observed that the Bach1-MafK heterodimer represses the expression of the exocrinezymogens (Fig. 5C), whereas the Nrf2a-MafK heterodimer activates them (supplementary material Fig. S5C).

Our data indicate that heme does not only activatezymogens in a concentration-dependent manner (Fig. 5A; supplementary material Fig. S5A), but also that Bach1 dissociation from the MafK-occupiedMARE sites is heme-dependent. In wild-type fish, more Nrf2a than Bach1 protein was associated with the MafK-occupiedMARE sites in the regulatory regions of thezymogens. By contrast, in hemedeficient HEP fish, more Bach1 than Nrf2a protein was associated with the MafK-occupiedMARE sites, which was reversed by treatment with hemin (Fig. 6B,C; supplementary material Fig. S6A-F). It appears that, through binding to Bach1, heme induces the replacement of Bach1 with Nrf2a on the MafK-occupiedMARE sites, switching from the repressive Bach1-MafK heterodimer to the activating Nrf2a-MafK heterodimer (Fig. 6B,C; supplementary material Fig. S6A-F). Thus, through mediating the dynamic exchange of Bach1 and Nrf2a in the MafK-occupiedMARE sites, heme plays a crucial role in the regulation ofzymogen production.

Taken together, these data provide a molecular explanation of the frequently reported acute episodic abdominal pains with co-occurrences of nausea and vomiting in someporphyria individuals (Bickers, 1981; Pierach, 1982; Bonkovsky, 2005; Puy et al., 2010; Balwani and Desnick, 2012). In fact, treatment with hemin can effectively cure these symptoms, although the underlying mechanism for this kind of therapy has been elusive (Bickers, 1981; Pierach, 1982; Ryter and Tyrrell, 2000; Anderson et al., 2005; Bonkovsky, 2005; Solinas and Vajda, 2008; Cappellini et al., 2010; Puy et al., 2010; Siegesmund et al., 2010; Balwani and Desnick, 2012). Our study reveals why the HEP fish produce lower levels ofzymogens and how hemin restores production of these proteins (Fig. 6B,C; supplementary material Fig. S6A-F). Because the roles of Bach1, Nrf2a and MafK in heme-mediated regulation ofexocrinezymogens appear to be highly conserved betweenzebrafish and human (Fig. 7A), we believe that the acute episodic abdominal pains in theseporphyria individuals are likely to stem from decreased production ofpancreaticzymogens. It is tempting to speculate that our study also provides a new therapeutic target for theseporphyria individuals – for instance, the possibility of knocking downBACH1 specifically in the exocrine pancreas to enhancezymogen levels (Fig. 7A), thereby, alleviating acute episodic abdominal pains.

In summary, we have revealed the antagonistic roles of the Nrf2a-MafK and Bach1b-MafK heterodimers in heme-mediated regulation of the exocrine pancreaticzymogens Cp5, Ctr11, Ctrb1, Ela2l1, Try and Tryl. In conditions of heme deficiency, such as porphyria, the repressive Bach1b-MafK heterodimer prevails; and through its binding to Bach1b, hemin facilitates the formation of the activating Nrf2a-MafK heterodimer, restoring the expression of thesezymogens in porphyria (Fig. 7C). These findings should provide novel targets for diagnosing and treating porphyria.

**MATERIALS AND METHODS**

**Fish husbandry and embryo production**

The Soochow University Animal Use and Care Committee approved all animal protocols. Zebrafish (Danio rerio) wild-type AB strain and the mutant line yquem (tyetm2168) were raised at the Soochow University Zebrafish Facility according to standard protocols (Westfield, 1995). Wild-type and mutant embryos were produced by pair mating, the embryos were then collected for RNA isolation and fixed for in situhybridization experiments at the specified stages. Homozygous mutants (yquem/urod, /−) were obtained by mating heterozygous fish (yquem/urod, /−/−) and then identified by using an epifluorescent stereomicroscope (Leica M165 FC).

**RNA extraction and qRT-PCR**

Total RNA was extracted using the TRizol® Reagent, according to the manufacturer’s instructions (Invitrogen). cDNA was synthesized by using reverse transcription with the M-MLV reverse transcription kit (Invitrogen), which was then used as the template for qRT-PCR analysis. qRT-PCR reactions were performed with the ABI StepOnePlus™ system, using SYBR® Premix Ex Taq™ (Takara) and the following thermal profile: 95°C for 3 minutes; 95°C for 10 seconds; 58°C for 30 seconds for 40 cycles. The primers that were used to amplify cpa, ctri1, ctri1b, ela2l, try, tryl and actb1 (as an internal control) are listed in supplementary material Table S1. Relative mRNA expression levels were quantified using the comparative CΔCt method and expressed as 2−CΔΔCt. The calculations and statistical analyses were performed by using Microsoft Office Excel or SPSS10.0.1. Each PCR assay was performed on three biological samples.

**Generation of RNA probes**

RNA probes for the sixzymogens (cpa5, ctri11, ctri1b, ela2l, try and tryl) and evadep (Jiang et al., 2008) were generated, as previously described (Wang et al., 2007). The cDNA fragments forbach1b and mafk were amplified by using PCR andzebrafish larvae [96 hours post-fertilization (hpf)] cDNAs. These fragments were then cloned into the pEASY-T3 vector using the pEASY-T3 Cloning Kit (TransGen). A cDNA fragment ofnrf2a was amplified, by using PCR, from pCS2nrf2a, a kind gift from Makodo Kobayashi (Takagi et al., 2004), and was then cloned into the pEASY-T3 vector. DIG-labeled RNA probes forbach1b, nrf2a and mafk were produced using the DIG RNA Labeling Kit, and a fluorescein-labeled tryl probe was generated using the Fluorescein RNA Labeling Mix, according to the manufacturer’s instructions (Roche). Primers forbach1b, nrf2a and mafk are listed in supplementary material Table S1.

**Whole-mount in situ hybridization analysis of gene expression**

Whole-mount in situ hybridization experiments were conducted as previously described (Wang et al., 2007). Briefly, 10-15 larvae were used for each in situ hybridization experiment. At least three independent experiments were conducted for each gene by using an antisense probe. Double in situ hybridization assays were performed by using digoxigenin and fluorescein-labeled probes, and developed with the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium, and Fast Red. Following in situ hybridization staining, selected larvae were rehydrated and embedded in 30% sucrose at 4°C overnight for cryostat sectioning. Sectioning was performed with a Leica CM 1850 cryostat. Stained larvae and sections were photographed using a stereomicroscope (Leica M165 FC) with a digital camera, and the images were analyzed by using ImageJ (National Institutes of Health) and Adobe Photoshop.
Construction of the expression vectors

The full-length bach1b cDNA sequence was isolated by using the SMARTer™ RACE cDNA Amplification Kit, according to the manufacturer’s instructions (Clontech) and deposited in NCBI (accession number KJ420533). The largest bach1b open reading frame (ORF) of 1899 bp encodes a peptide of 632 amino acids (Fig. 1A), which was amplified by using PCR of zebrafish larval cDNAs (120 bp) as template. This ORF was then cloned into the pEASY-T3 vector, and the final construct was named bach1b-T3. The plasmids pcDNA3.1mafK-FLAG, pcDNA3.1nrf2a-FLAG and pcDNAbach1b-HA were constructed by inserting the cDNA sequences corresponding to the FLAG-tag (DYKDDDDK) or HA-tag (YPYDVPDYA) before the stop codon TAA sequence at the 3′ ends of the mafK, nrf2a and bach1b ORF cDNAs. The primers used for bach1b ORF cloning are listed in supplementary material Table S1.

Overexpression and knockdown of bach1b and nrf2a

The plasmids bach1b-T3 and pcS2nrf2a were linearized and used as templates for generating capped mRNAs with the mMESSAGE mMACHINE® Kit according to the manufacturer’s instructions (Ambion). The SPL MO (5′-CCTTGTAGTGTGTCTTCTCATC-3′) and ATG MO (5′-TGACTTTGAGCTTTCCACCGACATC-3′) against bach1b, and the nrf2a ATG MO (5′-CATTCACXCTCCAATGTCTCAG-3′) (Kobayashi et al., 2002; Kobayashi et al., 2009; Wang and Gallagher, 2013) were purchased from Gene Tools. A mixture of bach1b (or nrf2a) capped mRNA and Tris-HCl (0.01 M, pH 7.0), or MO against bach1b (or nrf2a) dissolved in Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0 mM HEPES (pH 7.6)] was microinjected into one-cell stage zebrafish embryos. Microinjection controls with the vehicle solution, lacking mRNA or MO, were also performed. Reverse transcription PCR showed that the bach1b SPL MO effectively blocked the splicing of intron 2 (supplementary material Fig. S1A,B). The ATG MO knocked down bach1b with equal efficacy to that of the SPL MO (supplementary material Fig. S1C). The efficacy of the nrf2a ATG MO has been demonstrated previously (Kobayashi et al., 2002; Kobayashi et al., 2009; Wang and Gallagher, 2013). Total RNA was isolated from 50-50 larvae at 84 hpf and reverse transcribed to cDNAs for qRT-PCR analysis. The corresponding larvae were also fixed in situ hybridization experiments. At least three independent replicates were performed for each experiment. The primers that were used for the bach1b intron 1 splicing analysis are listed in supplementary material Table S1.

Construction of the luciferase reporter vectors

The 5′ promoter regions of these sixzymogens all contain multiple MARE (Maf recognition element) or MARE like sites (supplementary material Fig. S1). Among them, the ctrll and tryl promoter fragments containing MARE sites were PCR amplified from the genomic DNA, first cloned into pcMID19-T simple vector (TaKaRa), and then subcloned into luciferase reporter vector pGL3-Basic. The ctrll-luc construct contained the 5′ upstream 1183-bp region that harbored two MAREs (starting at position −6638 bp through to position −5456 bp) and a 1558-bp basic promoter region (starting at position −1439 bp through to position 119 bp), whereas the tryl-luc construct contains 1604 bp harboring one MARE (−6371 bp to −4768 bp) and a 1077-bp basic promoter region (−886 bp to 191 bp). The MARE binding sites also were mutated using the site-directed mutagenesis kit according to the manufacturer’s instructions (TaKaRa). The corresponding primers are listed in supplementary material Table S1.

Cell culture, cell transfection and luciferase assay

The NIH3T3 and 293T cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM; high glucose, Invitrogen). The medium was supplemented with 10% heat-inactivated low-endotoxin fetal bovine serum (FBS; Invitrogen), 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C under 5% CO2 inside an incubator. NIH3T3 or 293T cells were plated at 70% confluence in 24-well plates and incubated overnight. Transfections were performed using Lipofectamine 2000, according to the manufacturer’s instructions (Invitrogen). Five hours after transfection, the cells were treated with the indicated concentrations of hemin. Cells were lysed in passive lysis buffer (Promega) at the indicated timepoints, and cellular extracts were analyzed for luciferase activity using the Dual-Luciferase-Reporter Assay System (Promega) and a Luminoskan Ascent Microplate Luminometer (Thermo Scientific). The pcS2nrf2a and pcDNA3.bach1b-HA constructs were co-transfected with luciferase reporter constructs. Control cells were transfected with the pGL3-Basic vector, as well as a control plasmid containing the Renilla gene under the control of the HSV-TK promoter. Transfection efficiencies were normalized to the activity of a Renilla luciferase expression plasmid pRL-TK. Each experiment was performed three times.

ChIP assays

The ChIP analyses were performed using the ChIP Assay Kit, according to the manufacturer’s instructions (Millipore). Briefly, the embryos that had been microinjected with capped mRNAs of mafK-FLAG, nrf2a-FLAG or bach1b-HA were fixed at 96 hpf by using formaldehyde (final concentration at 1% for 20 minutes at room temperature. The larvae were then lysed using SDS lysis buffer. The chromatin lysates were sonicated to 200-1000 bp DNA fragments. Monoclonal antibodies against the FLAG and HA tags (EarthOx) were used for immunoprecipitation. After extensive washing, the DNA fragments were eluted using elution buffer (1% SDS, 0.5 M NaHCO3) and the eluted DNA was analyzed by using PCR. The PCR products were quantified by using qRT-PCR (Invitrogen). Relative enrichment was calculated as the difference between the specific antibody and normal IgG signals that had been normalized to the respective input signals. The primers flanking the MARE sites in the upstream regions of the six exocrinezymogens are listed in supplementary material Table S1.

Treatment with hemin

Hemin (Sigma) was dissolved in 0.2 ml of 1 N NaOH and then 1 ml of 0.2 M Tris-HCl (pH 8) was added and the solution was stored at −80°C. The pH was adjusted to 7.8 by using 1 N HCl. Treatment with hemin was performed as described previously (Wang et al., 2007).

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Competing interests

The authors declare no competing financial interests.

Author contributions

H.W., S.Z. and M.X. conceived and designed the experiments; S.Z., M.X., J.H., L.T., Y.Z. and J.W. performed the experiments; H.W., S.Z. and M.X. analyzed the data; and H.W., S.Z., M.X. and S.L. wrote the paper.

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Supplementary material

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References

