Heme-mediated inhibition of Bach1 regulates the liver specificity and transience of the Nrf2-dependent induction of zebrafish heme oxygenase 1

Yuji Fuse[1,†], Hitomi Nakajima[1,†], Yaeko Nakajima-Takagi[1,a], Osamu Nakajima[2] and Makoto Kobayashi[1*]

[1]*Department of Molecular and Developmental Biology, Faculty of Medicine, University of Tsukuba, Tsukuba 305-8575, Japan*

[2]*Research Laboratory for Molecular Genetics, Yamagata University, Yamagata 990-9585, Japan*

[†]Equally contributed

[a]Present address: Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

[*]To whom correspondence should be addressed. E-mail: makobayash@md.tsukuba.ac.jp.

Short title: Regulation of zebrafish Hmox1 induction

Corresponding author:
Makoto Kobayashi
Address: Department of Molecular and Developmental Biology, Faculty of Medicine, University of Tsukuba, Tsukuba 305-8575, Japan
E-mail: makobayash@md.tsukuba.ac.jp
Phone: +81-29-853-8457
Fax: +81-29-853-5977

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Abstract

The induction of the gene encoding heme oxygenase 1 (Hmox1, HO-1) by Nrf2 is unique compared with other Nrf2 targets. We previously showed that the Nrf2a-mediated induction of zebrafish *hmox1a* was liver-specific and transient. We screened transcription factors that could repress the induction of *hmox1a* but not other Nrf2a targets and concluded that Bach1b was a prime candidate. In *bach1b* knocked-down larvae, the induction of *hmox1a* was observed ectopically in non-liver tissues and persisted longer than normal fish, suggesting that Bach1 is the only regulator for both the liver-specific and transient induction of *hmox1a*. Co-knockdown of *bach1b* with its paralog *bach1a* enhanced these effects. To determine why Bach1 could not repress the *hmox1a* induction in the liver, we analyzed the effects of a heme biosynthesis inhibitor, succinylacetone, and a heme precursor, hemin. Succinylacetone decreased the Nrf2a-mediated *hmox1a* induction, while pretreatment with hemin caused ectopic induction of *hmox1a* in non-liver tissues, implying that the high heme levels in the liver may release the repressive activity of Bach1. Our results suggested that Bach1 regulates the liver specificity and transience of the Nrf2a-dependent induction of *hmox1a*, and that heme mediates this regulation through Bach1 inhibition based on its level in each tissue.

Introduction

Nrf2 is a transcription factor that heterodimerizes with small Maf proteins and binds to the antioxidant responsive element (ARE) to induce the expression of genes encoding antioxidant proteins and drug metabolizing enzymes (Suzuki *et al.* 2013; Itoh *et al.* 2010; Kobayashi & Yamamoto 2005). Under basal conditions, Keap1, a Nrf2-specific adaptor protein for the Cul3 E3 ubiquitin ligase, facilitates the degradation of Nrf2. However, under oxidative or electrophilic stress, the Keap1-dependent degradation of Nrf2 is inhibited, and accumulated Nrf2 upregulates the induction of cytoprotective enzymes to remove stressors. Keap1 has also been demonstrated to be a sensor of electrophiles, and transduces their signal to Nrf2. The Keap1-Nrf2 system exists both mammals and other vertebrates, and its physiological roles and regulatory mechanism have been demonstrated to be conserved between mice and zebrafish (Mukaigasa *et al.* 2012; Kobayashi *et al.* 2002).

In zebrafish, two Nrf2 (*nrf2a*, Kobayashi *et al.* 2002; *nrf2b*, Timme-Laragy *et al.* 2012), two Keap1 (*keap1a* and *keap1b*, Li *et al.* 2008), and four small Maf proteins (*mafg1*, *mafg2*, *mafk* and *maft*, Takagi *et al.* 2004), have been reported. The finding of two Keap1 proteins was particularly interesting, since they have different sensor sites for different classes of electrophiles. This led to the finding that the Keap1-Nrf2 system enables multiple sensors to respond to a vast number of environmental stresses

(Tsujita *et al.* 2011; Kobayashi *et al.* 2009). Zebrafish embryos and larvae are good models to study the regulation of Nrf2 target genes, since it is easy to visualize their expression profiles by whole mount *in situ* hybridization (WISH) and/or green fluorescent protein analyses due to their transparency. We have been explored the Nrf2a target genes induced by a well-known Nrf2 activator, diethyl maleate (DEM), by RT-PCR and microarray analyses in zebrafish, and showed that the lineups of Nrf2 target genes were basically conserved among vertebrates (Nakajima *et al.* 2011).

Among the various target genes of zebrafish Nrf2a that we have examined, heme oxygenase 1a (*hmox1a*) showed an atypical induction profile compared with others, such as glutathione *S*-transferase pi 1 (*gstp1*) and peroxiredoxin 1 (*prdx1*) (Nakajima *et al.* 2011). The DEM-induced expression of *hmox1a* was liver-specific and transient, with a peak after three hours of the DEM treatment. Hmox1 (HO-1) is an enzyme which degrades heme into three molecules, biliverdin, carbon monoxide and ferric iron, generating a reactive oxygen species-scavenging cycle by producing antioxidants (biliverdin and bilirubin) and depleting the oxidant, heme (Gozzelino *et al.* 2010). In this study, we investigated the molecular basis of the liver-specific and transient induction of *hmox1a* by gain and loss of function analyses using zebrafish embryos/larvae. Our results indicated that the transcription repressors, Bach1a and Bach1b, inhibit the *hmox1a* induction in tissues other than the liver, and heme, which is considered to be predominantly present in the liver, removes this inhibition exclusively in the liver. In addition, the Bach1 inhibition may be potentiated by Nrf2a through its induction of the *bach1a* and *bach1b* genes, making the *hmox1a* induction transient.

Results

Identification of zebrafish Bach1b as a *hmox1a*-specific transcription repressor

The induction of *gstp1* and other zebrafish Nrf2a target genes was observed in the nose and gills, in addition to the liver, while that of *hmox1a* was restricted to the liver (Fig. 1, arrowheads) (Nakajima *et al.* 2011). This tissue-restricted induction of *hmox1a* seems to be associated with the intrinsic properties of the gene, not with the electrophilic sensor system of the Keap1-Nrf2 system, because its expression profiles were similar when Nrf2a-activating compounds other than DEM were used (Nakajima *et al.* 2011). We therefore presumed that there was/were *hmox1a*-specific transcriptional repressor(s) in non-liver tissues. To identify this hypothetical factor(s), we examined four candidates, Bach1, Nrf1, MafG and c-Fos, which had previously been reported to inhibit the activation of Nrf2 target genes (Sun *et al.* 2002; Wang *et al.* 2007; Dhakshinamoorthy & Jaiswal 2000; Venugopal & Jaiswal 1998). Full-length cDNA of zebrafish orthologs of these four genes (*bach1b*, *nrf1b*, *mafg1* and *cfos*) were cloned, and we then tested whether their products could inhibit the Nrf2a-dependent

induction of *hmox1a* by an overexpression analysis. One-cell stage embryos were co-injected with mRNA encoding Nrf2a and either Bach1b, Nrf1b, MafG1 or cFos, and we then analyzed the induction of five Nrf2a target genes [*hmox1a*, *gstp1*, *prdx1*, ferritin heavy chain like (*fthl*) and glutamate-cysteine ligase catalytic subunit (*gclc*)] eight hours later by RT-PCR. As shown in Figure 2, co-overexpression of Nrf1b, MafG1 and cFos basically suppressed the Nrf2a-dependent induction of all five genes, while overexpression of Bach1b only inhibited *hmox1a* induction. We hypothesized that the factor involved in the liver specificity of *hmox1a* induction must (1) inhibit the *hmox1a* induction and (2) not inhibit other Nrf2a targets. Our results suggest that zebrafish Bach1b fits these criteria, and we thus focused on Bach1b and its teleost-specific paralog, Bach1a, in further experiments.

Bach1 is the main reason for failing to induce *hmox1a* in non-liver tissues

Bach1 is one of two Bach proteins, Bach1 and Bach2, which are conserved in vertebrates and some urochordates (Fig. 3) (Igarashi & Watanabe-Matsui 2014; Oyake *et al.* 1996). It is a basic leucine zipper-type transcription repressor, and functions as a heterodimer with small Maf proteins, like Nrf2. Bach1 has previously been demonstrated to inhibit Nrf2-dependent induction of Hmox1 gene expression (Sun *et al.* 2002; 2004). In the zebrafish genome, there are two Bach1 homolog genes, *bach1a* and *bach1b*, which are located on chromosomes 15 and 10, respectively. To examine whether these orthologs of Bach1 are involved in the liver-specific induction of *hmox1a*, knockdown analyses were carried out using morpholino oligonucleotides (MO). Either or both of the *bach1a*MO and *bach1b*MO, which inhibit the translation of mRNA specifically (Fig. S1), were injected into one-cell stage zebrafish embryos, and the DEM-induced expression of *hmox1a* was analyzed at the five-days post fertilization (dpf) larval stage by a WISH analysis (Fig. 4). *hmox1a* induction was observed only in the liver in uninjected (3 h, 100%, n=103) and *bach1a*MO-injected (3h, 100%, n=32) larvae, while in *bach1b*MO-injected larvae, it was also detected in the nose and gills, in addition to the liver (3 h, non-liver, 50%, n=26). We did not take the induction in the intestine into account, because a considerable level of basal expression was detected in some uninjected larvae (Fig. 4, asterisks). We further confirmed the ectopic induction of *hmox1a* in *bach1b*-knocked down larvae using a splicing-type MO, *bach1b*MOS1, which inhibited normal splicing of mRNA encoding *bach1b* (Fig. S2, 3 h, non-liver, 64%, n=11). This ectopic induction of *hmox1a* in non-liver tissues was enhanced when *bach1a*MO and *bach1b*MO were co-injected (Fig. 4, 3h, non-liver, 87%, n=62). Collectively, Bach1b was the main reason why *hmox1a* was not induced in non-liver tissues and Bach1a had a supportive effect on the repression. We selected the *bach1a-bach1b* double-knockdown condition for further analysis, which showed the strongest effect on ectopic induction of *hmox1a* and no effect on the induction profile of *gstp1* (Fig. S3). Another zebrafish Hmox1 ortholog, *hmox1b*, was also induced by

DEM in the liver, but the *bach1a-bach1b* double-knockdown did not alter the induction profile, suggesting that the regulation of tissue specificity was not Bach1-dependent (Fig. S4).

Reduction of the heme levels diminished the induction of *hmox1a* in the liver

The next question raised was why the induction of *hmox1a* was not inhibited by Bach1 in the liver. We first thought that the expression of *bach1a* and *bach1b* might have been low in the liver. To explore this possibility, the expression profiles of the two Bach1 homologs were examined by a WISH analysis. As shown in Figure S5, *bach1a* and *bach1b* were expressed ubiquitously throughout the body, including the liver, ruling out the low expression and implicating liver-specific mechanism(s) in the *hmox1a* induction due to the Bach1 inhibition. Since the repressor functions of Bach1 were demonstrated to be inactivated by heme (Ogawa *et al.* 2001; Suzuki *et al.* 2004; Zenke-Kawasaki *et al.* 2007; Tan *et al.* 2013), and because the liver is known to be a heme-rich tissue (Meyer et al., 2002), we postulated that the high levels of heme in the liver inactivate Bach1 and eventually allow Nrf2a to induce the *hmox1a* expression.

To examine this second hypothesis, we reduced the endogenous heme levels by treating zebrafish larvae with succinylacetone (SA), an inhibitor of a rate-limiting enzyme in heme biosynthesis, δ-aminolevulinic acid dehydratase (Ebert *et al.* 1979), and evaluated its effects on the *hmox1a* induction by a WISH analysis. The effect of SA on the *hmox1a* induction was examined at 3 hours, since a single treatment of SA induced *hmox1a* after 6 hours and *gstp1* after 9 hours, probably due to indirect Nrf2 activation (Fig. S6). As shown in Figure 5, larvae treated simultaneously with SA and DEM (DEM + SA) displayed reduced *hmox1a* induction in the liver (strong 15%, weak 30%, negative 56%, n=54), compared with the larvae treated solely with DEM (strong 80%, weak 14%, negative 7%, n=44). This repressive effect of SA was not observed in *bach1a-bach1b* double knocked-down larvae in both liver (DEM, strong 74%, weak 26%, negative 0%, n=19; DEM + SA, strong 81%, weak 19%, negative 0%, n=21) and non-liver tissues (DEM, non-liver 74%, n=19; DEM + SA, non-liver 90%, n=21), suggesting that the effect of SA required the presence of Bach1 proteins. These results indicated that the high heme level in the liver is a critical factor for the Bach1 inhibitor and *hmox1a* induction.

An increase in the heme levels stimulated ectopic *hmox1a* induction in non-liver tissues

Next, we tried to increase the endogenous heme levels by treating zebrafish larvae with hemin (Fe^{3+}-bound heme), and examined its effects on the *hmox1a* induction. Since heme/hemin are Nrf2 activating compounds (Kim *et al.* 2001; Alam *et al.* 2003), we tested whether hemin could induce zebrafish *gstp1* (Fig. S7) and *hmox1a* (Fig. S8), and found that it could. Interestingly, weak ectopic

induction of *hmox1a* in the nose and gills was observed (Fig. S8, 6 h, uninjected, non-liver, 13%, n=47), suggesting that heme not only activated Nrf2 but derepressed the Bach1-dependent inhibition in these tissues. The *bach1a-bach1b* double-knockdown in the hemin-treated larvae showed no significant difference in the *hmox1a* induction profile (Fig. S8, 6 h, non-liver, 14%, n=35). These results suggested that the Bach1-dependent inhibition in these tissues was abrogated by the accumulated heme.

In order to evaluate the effects of hemin on the DEM-induced expression of *hmox1a*, hemin pretreatment was applied for 12 hours before DEM treatment (Fig. 6A). As expected, *hmox1a* was induced by DEM not only in the liver, but also in the nose and gills (Fig. 6B, Uninjected, Hemin > DEM, 3 h, non-liver, 90%, n=29). No significant difference was found when both Bach1 genes were knocked-down (Fig. 6B, *bach1a*MO + *bach1b*MO, Hemin > DEM, 3 h, non-liver, 96%, n=23). Pretreatment with DEM showed no effect on the *hmox1a* induction, thus excluding the possibility of unspecific effect of the drug pretreatment (Fig. S9, DEM > DEM, 3h, non-liver 0%, liver 0%, n=20). These results, together with the results using SA-treated larvae shown in Figure 5, led us to conclude that the heme levels and their mediator Bach1 are the fundamental regulators of the tissue-specific induction of Nrf2a-dependent *hmox1a* expression.

Bach1b is involved in the transient induction of *hmox1a*

Another notable difference in the induction profiles between *hmox1a* and other Nrf2a target genes is the time course of the induction. As shown in Figure 1, the induction of *hmox1a* was transient, with a peak after three hours of DEM treatment, while that of *gstp1* continued for more than 12 hours. Interestingly, as shown in Fig. 4, *bach1a-bach1b* double-knockdown not only induced ectopic *hmox1a* expression in non-liver tissues, but also extended the induction time in the liver (3 h, 98%, n=63; 6 h, 100%, n=69; 9 h, 98%, n=56; 12 h, 90%, n=59), compared to the uninjected control (3 h, 81% n=127; 6 h, 69%, n=124; 9 h, 17%, n= 115; 12 h, 11%, n=128), suggesting that Bach1 proteins were involved in the transient induction of *hmox1a*. Since the gene expression of Bach1 itself was shown to be induced by Nrf2 in human cultured cells (Jyrkkänen *et al.* 2011), similar regulation may occur in the zebrafish liver.

To test this possibility, the expression of *bach1a* and *bach1b* was examined after DEM treatment by WISH and RT-PCR analyses (Fig. 7). As expected, DEM induced both Bach1 genes, with more robust induction in *bach1b* than *bach1a*. Importantly, this expression was maintained for at least 12 hours after the treatment, suggesting that a significant amount of heme-free Bach1 proteins may be newly-synthesized subsequent to the Nrf2a accumulation in the liver nuclei. Intriguingly, the treatment with hemin, unlike treatment with DEM, resulted in a sustained *hmox1a* induction (Fig. S8,

Uninjected, liver: 3 h, 46%, n=52; 6 h, 68%, n=47; 12 h, 30%, n=46), implying that the excess amount of heme inhibited even the newly-synthesized Bach1 proteins. Similar results were obtained in the case of hemin pretreatment (Fig. 6B, Uninjected, Hemin > DEM, liver: 3 h, 97%, n=29; 6 h, 61%, n=31; 12 h, 23%, n=31). As in the case of the ectopic *hmox1a* induction, knockdown of Bach1 proteins showed no significant effects on the sustained *hmox1a* induction in either hemin pretreated larvae followed by DEM treatment (Fig. 6B) or hemin-alone treated larvae (Fig. S8). These results support our hypothesis that the accumulation of heme-free Bach1 is a key part of this negative feedback regulation of the *hmox1a* induction.

Discussion

The induction profile of the zebrafish *hmox1a* is unique compared with that of other Nrf2a target genes in that it is liver-specific and transient (Fig.1) (Nakajima *et al.* 2011). The present study revealed that the liver-specific induction of *hmox1a* was achieved by the ubiquitous transcriptional inhibition by Bach1a/Bach1b and their liver-specific inactivation by heme, and the transient induction was mediated also by Bach1 proteins, probably through the Nrf2a-dependent induction of their genes (Fig. 8).

We showed that the liver specificity of the *hmox1a* induction is based on the liver-specific heme-dependent inactivation of Bach1proteins. Bach1a and Bach1b are expressed ubiquitously in zebrafish larvae and repress their target gene, *hmox1a*, in almost all tissues except liver, which has a high heme content compared to other tissues (Meyer *et al.* 2002). A similar heme- and Bach1-dependent and tissue-specific expression of the Hmox1 gene has been observed in mice, although the expression in that study was under uninduced conditions (Sun *et al.* 2002). The expression of the mouse *Hmox1* gene is high in heme-rich tissues, such as the liver and spleen, and low in others, such as the brain and heart, while in *Bach1* knockout mice, the expression in the latter tissues was elevated to levels similar to that in the former tissues, suggesting the involvement of the heme-Bach1 axis in the tissue-specific expression of the *Hmox1* gene in mice.

The critical resemblance between zebrafish and mice in the regulation of Hmox1 gene expression is its heme dependence. This implies that there is strong conservation at the molecular level with regard to the tissue specificity of the Hmox1 gene among vertebrates. We showed that alterations in the heme levels induced by hemin and SA modulated the tissue specificity of the *hmox1a* induction, suggesting that the Hmox1 expression profiles can be altered during development or by pathophysiological states, depending on the changes in the heme concentration.

Independent from our findings, an interesting study related to heme and zebrafish Bach1 was

recently published (Zhang *et al.* 2014) showing that the pancreas-specific gene expression of zebrafish exocrine peptidases, such as trypsin and chymotrypsin, was upregulated in *bach1b* knocked-down embryos, and was downregulated in mutant embryos that had defects in heme biosynthesis. Their results also implicated a gene expression system which is regulated by heme-mediated inactivation of Bach1-dependent transcriptional inhibition. The major point of difference between their study and our present study is that they did not observe any ectopic expression of the exocrine peptidase genes in non-pancreas tissues of *bach1b* knocked-down larvae, suggesting that the regulation of tissue specificity by the heme-Bach1 axis is specific for *hmox1a*.

The transient induction of the Hmox1 expression is another unique trait among Nrf2 target genes, and was observed not only in fish, but also in mammals (Zhang *et al.* 2006; Motterlini *et al.* 2000; Ewing & Maines 1993). The molecular basis of this Hmox1-specific regulation is not well understood. In the present study, we found that *bach1a-bach1b* double-knockdown prolonged the *hmox1a* induction, which suggested that Bach1 plays a role to terminate the Nrf2-dependent Hmox1 induction. Since Bach1 has been shown to compete with Nrf2 proteins for binding to the gene regulatory region of Hmox1 (Sun *et al.* 2002), the possible mechanism underlying this Bach1a/Bach1b-dependent termination of *hmox1a* induction may be a result of increased heme-free Bach1 proteins produced by the Nrf2a-mediated induction. Nrf2-dependent induction of Bach1 expression was previously demonstrated in mammals (Jyrkkänen *et al.* 2011), and we demonstrated that this regulation was also conserved in fish (see Fig. 7). Based on this result, together with the results from the *bach1a-bach1b* double-knockdown analysis, we hypothesize that the transient induction of *hmox1a* is caused by its initial transactivation by Nrf2a, followed by transcriptional repression by Nrf2a-induced Bach1a/Bach1b.

As Sun *et al.* (2002) suggested in their paper, the dual regulation of the Hmox1 gene induction by the oxidative stress-Nrf2 and heme-Bach1 axes is analogous to that of the bacterial *lac* operon by the cAMP receptor protein and *lac* repressor, the textbook concept provided by Jacob, Monod, and others. A physiological role of the latter regulation in bacteria is considered to be the selective utilization of lactose as a carbon and energy source only when glucose is absent in the growth environment, known as "carbon catabolite repression". We think that the tissue-specific and transient induction of the vertebrate Hmox1 gene by Nrf2 and Bach1 may have a similar physiological role, wherein there is a selective utilization of the heme oxygenase products, bilirubin and biliverdin, as endogenous antioxidants only in heme-rich tissues. Compared with other Nrf2-regulated antioxidant proteins, such as peroxiredoxins and catalases, which can directly reduce oxidative stress, Hmox1 is unique, since its antioxidant activity is indirectly exerted by its products (Wegiel *et al.* 2014), and this unique characteristic would be a useful cellular antioxidant strategy. However, this process requires heme as a

substrate, and heme also plays important roles as a prosthetic group for critical hemoproteins in a variety of tissues. Therefore, the utilization of Hmox1 may be restricted to heme-rich tissues, and even in such tissues, overuse may be strictly prevented, leading to only transient Hmox1 induction.

The Keap1-Nrf2 system is present not only in vertebrates, but also invertebrates, such as fruit flies (Kobayashi *et al.* 2002; Sykiotis & Bohmann 2008). In contrast, the Bach protein family does not contain any representatives in arthropods or lower eukaryotes (Igarashi & Watanabe-Matsui 2014). This fact suggests that the modified regulation of the Nrf2-mediated Hmox1 gene induction by the heme-Bach1 axis is a "new instruction" for the Keap1-Nrf2 system which may be important for vertebrate life. The only non-vertebrate animals that have the Bach protein are ascidians. It is interesting to note that ascidians have no acquired immunity nor hemoglobin production, both processes in which Bach proteins play critical roles (Igarashi & Watanabe-Matsui; Igarashi *et al.* 2007). It is possible that the modification of the Hmox1 induction mechanism by Bach1 may be a key event that occurred during the appearance of vertebrates.

Experimental procedures

Fish and chemical treatments

Zebrafish embryos and larvae were obtained by natural mating. All experiments were carried out using a wild-type AB strain. For induction studies, fish were placed in culture dishes containing 100 μM DEM (Wako, Osaka, Japan), 0.5 mM SA (Sigma-Aldrich, St. Louis, MO) and/or 100 μM hemin (Wako). All animal experiments were carried out with the approval of the Animal Research Committee of the University of Tsukuba.

Construction of plasmids

For *bach1b*, a full-length cDNA clone was isolated by screening a λgt10 phage cDNA library of one-month-old zebrafish (Takara Bio Inc, Otsu, Japan) with a *bach1b* probe prepared by PCR using primers designed based on the corresponding expressed sequence tag sequence. The *bach1b* probe was labeled using an AlkPhos Direct DNA labeling kit and positive plaques on the membrane filters were detected with CDP-Star as a substrate, according to the manufacturer's instructions (GE Healthcare Japan, Hino, Japan). The open reading frame (ORF) region of *bach1b* was made by PCR using this isolated cDNA clone and specific primers, and was subcloned into $pCS2^{+}$ to construct pCS2bach1b. It was also subcloned into pBluescript II KS^{+} and named pKSbach1b. To make pCS2nrf1b, pCS2cfos, pKSbach1a and pKShmox1b, the ORF regions of *nrf1b, cfos, bach1a and hmox1b* were prepared by RT-PCR using total RNA of 5-7 dpf zebrafish larvae and specific primers. pCS2bach1aMeGFP was

generated by inserting the target region of *bach1a*MO prepared by annealing two oligonucleotides (5’-GATCAGCTAAAGCCCTGCGTCATGTCTGTGGATGGCCCCCG and 5’-CATGCGGGGGCCATCCACAGACATGACGCAGGGCTTTAGCT) into pCS2eGFP (Kobayashi *et al.* 2001b). To construct pCS2bach1bMeGFP, the target region of *bach1b*MO was prepared by RT-PCR using specific primers and inserted into pCS2eGFP. The sequences of the oligonucleotide primers used for plasmid construction are shown in Table S1. The nucleotide sequence of each construct was verified by DNA sequencing. The pSKhmox1a, pSKprdx1, pKSfthl, pKSgclc (Nakajima *et al.* 2011), pKSgstp1N (Suzuki *et al.* 2005), pCS2nrf2a (Kobayashi *et al.* 2002) and pCS2mafG1 (Takagi *et al.* 2004) plasmids have been described previously.

Overexpression and knockdown analyses

pCSnrf2a, pCSbach1b, pCS2nrf1b, pCS2mafG1, pCS2cfos, pCS2bach1aMeGFP and pCS2bach1bMeGFP were digested with *Bsp*120I (Thermo Fisher Scientific, Waltham, MA) and the subsequent mRNA synthesis was conducted using a SP6 mMESSAGE mMACHINE in vitro transcription kit (Ambion, Austin, TX). The MOs were purchased from Gene Tools LLC (Philomath, OR) (*bach1a*MO: 5’-CATCCACAGACATGACGCAGGGCTT, *bach1b*MO: 5'-AGCTTTCCACCGACATCTTGAGTTA and *bach1b*MOS1: 5'-CCTTTGATTGTGTCTTTACCTCATC). mRNA or MOs were injected by a IM300 microinjector (Narishige, Tokyo, Japan) into the yolk of one-cell stage zebrafish embryos. GFP expression was analyzed under a GFP-BP filter (470 nm excitation, 525 nm emission) of a M205 FA microscope (Leica, Wetzler, Germany) equipped with a DFC310 FX digital camera (Leica).

Gene expression analyses

The WISH analysis was carried out as described previously with some modifications (Kobayashi *et al.* 2001a). Briefly, 5-dpf larvae were fixed in 4% paraformaldehyde (PFA) in PBS at 4ºC. After fixation, the larvae were washed twice with PBS, once in 50% methanol and once in 100% methanol and were stored at -20ºC overnight in 100% methanol. After two washes with PBT (0.1% Tween 20 in PBS), the larvae were treated with 9% H_2O_2 in PBT for two hours to decolorize them. After two washes with PBTw (0.2% bovine serum albumin in PBT), the larvae were treated with 50 μg/ml Proteinase K (Sigma-Aldrich) in PBTw for 20 min and fixed in 4% PFA in PBS. To make RNA probes for the WISH analysis, pKSgstp1N, pKSbach1a, pKSbach1b and pKShmox1b were digested with *Bam*HI (Takara Bio Inc) and pSKhmox1a was digested with *Xho*I (Takara Bio Inc), mixed with DIG RNA labeling mix (Roche, Mannheim, Germany) and transcribed with T3 RNA polymerase (Roche). All

pictures were taken using a Leica MZ16 microscope equipped with an Olympus DP73 digital camera (Tokyo, Japan).

For the RT-PCR analyses, total RNA was extracted from zebrafish embryos or larvae using the QIAzol Lysis Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized with SuperScript II (Life Technologies, Carlsbad, CA) and used for the subsequent PCR analysis with specific oligonucleotide primers (Table S2). The expression level of each gene was normalized to the *ef1α* transcripts.

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References

Alam, J., Killeen, E., Gong, P., Naquin, R., Hu, B., Stewart, D., Ingelfinger, J.R. & Nath, K.A. (2003) Heme activates the heme oxygenase-1 gene in renal epithelial cells by stabilizing Nrf2. *Am. J. Physiol. Renal Physiol.* **284**, F743-F752.

Dhakshinamoorthy, S. & Jaiswal, A.K. (2000) Small maf (MafG and MafK) proteins negatively regulate antioxidant response element-mediated expression and antioxidant induction of the NAD(P)H:Quinone oxidoreductase1 gene. *J. Biol. Chem.* **275**, 40134-40141.

Ebert, P.S., Hess, R.A., Frykholm, B.C. & Tschudy, D.P. (1979) Succinylacetone, a potent inhibitor of heme biosynthesis: effect on cell growth, heme content and delta-aminolevulinic acid dehydratase activity of malignant murine erythroleukemia cells. *Biochem. Biophys. Res. Commun.* **88**, 1382-1390.

Ewing, J.F. & Maines, M.D. (1993) Glutathione depletion induces heme oxygenase-1 (HSP32) mRNA and protein in rat brain. *J. Neurochem.* **60**, 1512-1519.

Gozzelino, R., Jeney, V. & Soares, M.P. (2010) Mechanisms of cell protection by heme oxygenase-1. *Annu. Rev. Pharmacol. Toxicol.* **50**, 323-354.

Igarashi, K., Ochiai, K. & Muto, A. (2007) Architecture and dynamics of the transcription factor network that regulates B-to-plasma cell differentiation. *J. Biochem.* **141**, 783-789.

Igarashi, K. & Watanabe-Matsui, M. (2014) Wearing red for signaling: the heme-Bach axis in heme metabolism, oxidative stress response and iron immunology. *Tohoku J. Exp. Med.* **232**, 229-253.

Itoh, K., Mimura, J. & Yamamoto, M. (2010) Discovery of the negative regulator of Nrf2, Keap1: a historical overview. *Antioxid. Redox Signal.* **13**, 1665-1678.

Jyrkkänen, H.K., Kuosmanen, S., Heinäniemi, M., Laitinen, H., Kansanen, E., Mella-Aho, E., Leinonen, H., Ylä-Herttuala, S. & Levonen, A.L. (2011) Novel insights into the regulation of antioxidant-response-element-mediated gene expression by electrophiles: induction of the transcriptional repressor BACH1 by Nrf2. *Biochem. J.* **440**, 167-174.

Kim, Y.C., Masutani, H., Yamaguchi, Y., Itoh, K., Yamamoto, M. & Yodoi, J. (2001) Hemin-induced activation of the thioredoxin gene by Nrf2: a differential regulation of the antioxidant responsive element by a switch of its binding factors. *J. Biol. Chem.* **276**, 18399-18406.

Kobayashi, M., Itoh, K., Suzuki, T., Osanai, H., Nishikawa, K., Katoh, Y., Takagi, Y. & Yamamoto, M. (2002) Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. *Genes Cells* **7**, 807-820.

Kobayashi, M., Li, L., Iwamoto, N., Nakajima-Takagi, Y., Kaneko, H., Nakayama, Y., Eguchi, M., Wada, Y., Kumagai, Y. & Yamamoto, M. (2009) The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Mol. Cell. Biol.* **29**, 493-502.

Kobayashi, M., Nishikawa, K., Suzuki, T. & Yamamoto, M. (2001a) The homeobox protein Six3 interacts with the Groucho corepressor and acts as a transcriptional repressor in eye and forebrain formation. *Dev. Biol.* **232**, 315-326.

Kobayashi, M., Nishikawa, K. & Yamamoto, M. (2001b) Hematopoietic regulatory domain of *gata1* gene is positively regulated by GATA1 protein in zebrafish embryos. *Development* **128**, 2341-2350.

Kobayashi, M. & Yamamoto, M. (2005) Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid. Redox Signal.* **7**, 385-394.

Li, L., Kobayashi, M., Kaneko, H., Nakajima-Takagi, Y., Nakayama, Y. & Yamamoto, M. (2008) Molecular Evolution of Keap1: Two Keap1 molecules with distinctive intervening region structures are conserved among fish. *J. Biol. Chem.* **283**, 3248-3255.

Meyer, R.P., Podvinec, M. & Meyer, U.A. (2002) Cytochrome P450 CYP1A1 accumulates in the cytosol of kidney and brain and is activated by heme. *Mol. Pharmacol.* **62**, 1061-1067.

Motterlini, R., Foresti, R., Bassi, R. & Green, C.J. (2000) Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. *Free Radic. Biol. Med.* **28**, 1303-1312.

399 Mukaigasa, K., Nguyen, L.T., Li, L., Nakajima, H., Yamamoto, M. & Kobayashi, M. (2012) Genetic
400 evidence of an evolutionarily conserved role for Nrf2 in the protection against oxidative stress.
401 *Mol. Cell. Biol.* **32**, 4455-4461.
402 Nakajima, H., Nakajima-Takagi, Y., Tsujita, T., Akiyama, S., Wakasa, T., Mukaigasa, K., Kaneko, H.,
403 Tamaru, Y., Yamamoto, M. & Kobayashi, M. (2011) Tissue-restricted expression of Nrf2 and its
404 target genes in zebrafish with gene-specific variations in the induction profiles. *PLoS One* **6**,
405 e26884. (Erratum in: PLoS One 7)
406 Ogawa, K., Sun, J., Taketani, S., Nakajima, O., Nishitani, C., Sassa, S., Hayashi, N., Yamamoto, M.,
407 Shibahara, S., Fujita, H. & Igarashi, K. (2001) Heme mediates derepression of Maf recognition
408 element through direct binding to transcription repressor Bach1. *EMBO J.* **20**, 2835-2843.
409 Oyake, T., Itoh, K., Motohashi, H., Hayashi, N., Hoshino, H., Nishizawa, M., Yamamoto, M. &
410 Igarashi, K. (1996) Bach proteins belong to a novel family of BTB-basic leucine zipper
411 transcription factors that interact with MafK and regulate transcription through the NF-E2 site.
412 *Mol. Cell. Biol.* **16**, 6083-6095.
413 Sun, J., Brand, M., Zenke, Y., Tashiro, S., Groudine, M. & Igarashi, K. (2004) Heme regulates the
414 dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network.
415 *Proc. Natl. Acad. Sci. USA* **101**, 1461-1466.
416 Sun, J., Hoshino, H., Takaku, K., Nakajima, O., Muto, A., Suzuki, H., Tashiro, S., Takahashi, S.,
417 Shibahara, S., Alam, J., Taketo, M.M., Yamamoto, M. & Igarashi, K. (2002) Hemoprotein Bach1
418 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J.* **21**, 5216-5224.
419 Suzuki, H., Tashiro, S., Hira, S., Sun, J., Yamazaki, C., Zenke, Y., Ikeda-Saito, M., Yoshida, M. &
420 Igarashi, K. (2004) Heme regulates gene expression by triggering Crm1-dependent nuclear export
421 of Bach1. *EMBO J.* **23**, 2544-2553.
422 Suzuki, T., Motohashi, H. & Yamamoto, M. (2013) Toward clinical application of the Keap1-Nrf2
423 pathway. *Trends Pharmacol. Sci.* **34**, 340-346.
424 Suzuki, T., Takagi, Y., Osanai, H., Li, L., Takeuchi, M., Katoh, Y., Kobayashi, M. & Yamamoto, M.
425 (2005) Pi-class glutathione S-transferase genes are regulated by Nrf2 through an evolutionarily
426 conserved regulatory element in zebrafish. *Biochem. J.* **388**, 65-73.
427 Sykiotis, G.P. & Bohmann, D. (2008) Keap1/Nrf2 signaling regulates oxidative stress tolerance and
428 lifespan in *Drosophila*. *Dev. Cell* **14**, 76-85.
429 Takagi, Y., Kobayashi, M., Li, L., Suzuki, T., Nishikawa, K. & Yamamoto, M. (2004) MafT, a new
430 member of the small Maf protein family in zebrafish. *Biochem. Biophys. Res. Commun.* **320**,
431 62-69.

Tan, M.K.M., Lim, H.J., Bennett, E.J., Shi, Y. & Harper, J.W. (2013) Parallel SCF adaptor capture proteomics reveals a role for SCFFBXL17 in NRF2 activation via BACH1 repressor turnover. *Mol. Cell* **52**, 9-24.

Timme-Laragy, A.R., Karchner, S.I., Franks, D.G., Jenny, M.J., Harbeitner, R.C., Goldstone, J.V., McArthur, A.G. & Hahn, M.E. (2012) Nrf2b, novel zebrafish paralog of oxidant-responsive transcription factor NF-E2-related factor 2 (NRF2). *J. Biol. Chem.* **287**, 4609-4627.

Tsujita, T., Li, L., Nakajima, H., Iwamoto, N., Nakajima-Takagi, Y., Ohashi, K., Kawakami, K., Kumagai, Y., Freeman, B.A., Yamamoto, M. & Kobayashi, M. (2011) Nitro-fatty acids and cyclopentenone prostaglandins share strategies to activate the Keap1-Nrf2 system: a study using green fluorescent protein transgenic zebrafish. *Genes Cells* **16**, 46-57.

Venugopal, R. & Jaiswal, A.K. (1998) Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene* **17**, 3145-3156.

Wang, W., Kwok, A.M. & Chan, J.Y. (2007) The p65 Isoform of Nrf1 Is a Dominant Negative Inhibitor of ARE-mediated Transcription. *J. Biol. Chem.* **282**, 24670-24678.

Wegiel, B., Nemeth, Z., Correa-Costa, M., Bulmer, A.C. & Otterbein, L.E. (2014) Heme oxygenase-1: a metabolic nike. *Antioxid. Redox Signal.* **20**, 1709-1722.

Zenke-Kawasaki, Y., Dohi, Y., Katoh, Y., Ikura, T., Ikura, M., Asahara, T., Tokunaga, F., Iwai, K. & Igarashi, K. (2007) Heme induces ubiquitination and degradation of the transcription factor Bach1. *Mol. Cell. Biol.* **27**, 6962-6971.

Zhang, J., Ohta, T., Maruyama, A., Hosoya, T., Nishikawa, K., Maher, J.M., Shibahara, S., Itoh, K. & Yamamoto, M. (2006) BRG1 interacts with Nrf2 to selectively mediate *HO-1* induction in response to oxidative stress. *Mol. Cell. Biol.* **26**, 7942-7952.

Zhang, S., Xu, M., Huang, J., Tang, L., Zhang, Y., Wu, J., Lin, S. & Wang, H. (2014) Heme acts through the Bach1b/Nrf2a-MafK pathway to regulate exocrine peptidase precursor genes in porphyric zebrafish. *Dis. Models Mech.* **7**, 837-845.

Figure legends

Figure 1 Liver-specific induction of *hmox1a*. The expression levels of *hmox1a* and *gstp1* were analyzed by a WISH analysis in 5-dpf larvae treated with 100 μM DEM for the indicated times. The upper and lower panels are lateral and ventral views, respectively. It should be noted that the induction of *hmox1a* was liver-specific and transient compared with that of *gstp1*. The arrowheads indicate the positive expression of each gene in the nose, gills and liver.

Figure 2 The screen for a candidate *hmox1a*-specific repressor. Candidate *hmox1a*-specific repressors were screened by analyzing their inhibition of the Nrf2a-mediated induction of *hmox1a* and other Nrf2a targets. mRNA for Nrf2a (60 pg) and the *hmox1a*-specific repressor candidates (Bach1b, Nrf1b, MafG1 or cFos) (120 pg) was co-injected into one-cell stage embryos. After eight hours, the expression of five Nrf2a target genes, *hmox1a*, *gstp1*, *prdx1*, *fthl* and *gclc*, was analyzed by RT-PCR. The amount of cDNA used for RT-PCR was standardized by the *ef1α* expression. B1: Bach1b, N1: Nrf1b, Mg: MafG1, cF: cFos.

Figure 3 A phylogenetic tree of the Bach family proteins. The amino acid sequences of the cap'n'collar (CNC) domains in each protein were analyzed. The tree was constructed by the neighbor-joining method using the Clustal W program and the NJplot software program (version 2.2). The scale bar indicates the genetic distance. c: chicken, ci: *Ciona intestinalis*, cs: *Ciona savignyi*, h: human, m: mouse, ol: *Oryzias latipes*, tn: *Tetraodon nigroviridis*, tr: *Takifugu rubripes*, xt: *Xenopus tropicalis*, z: zebrafish.

Figure 4 Ectopic and prolonged *hmox1a* induction in Bach1 knocked-down larvae. The expression of *hmox1a* was analyzed in 5-dpf larvae injected with either or both of *bach1a*MO and *bach1b*MO (1 pmol each) compared with uninjected control, and treated with 100 μM DEM for the indicated times. It should be noted that the ectopic induction of *hmox1a* in non-liver tissues, such as the nose and gills, was observed in *bach1b*MO-injected and *bach1a*MO-*bach1b*MO co-injected larvae. In addition, prolonged *hmox1a* induction in the liver was also detected in these morphants. The arrowheads indicate the *hmox1a* induction in the nose, gills and liver. The asterisks denote the basal expression in the intestine.

Figure 5 The effects of reducing the endogenous heme levels on the *hmox1a* induction. The expression of *hmox1a* was analyzed in 5-dpf larvae treated with 100 μM DEM and 0.5 mM SA for 3 hours. It should be noted that the DEM-induced expression of *hmox1a* in the liver was significantly decreased when SA co-treatment was performed, while *bach1a-bach1b* double-knockdown blocked this effect of SA. The arrowheads indicate the *hmox1a* induction in the nose, gills and liver. The asterisks denote the basal expression in the intestine.

Figure 6 The effects of increasing the heme levels on the *hmox1a* induction. (A) A schematic diagram of the experiment. (B) The expression of *hmox1a* was analyzed in 5-dpf larvae treated with 100 μM

DEM for the indicated times, after either a 12-hour pretreatment with or without 100 μM hemin. Ectopic and sustained *hmox1a* induction was observed when hemin pretreatment was performed in both *bach1a*MO-*bach1b*MO co-injected and uninjected larvae. The arrowheads indicate the *hmox1a* induction in the nose, gills and liver. The asterisks denote the basal expression in the intestine.

Figure 7 The induction of *bach1a* and *bach1b* expression in DEM-treated larvae. The results of the expression analyses of *bach1a* and *bach1b* in 5-dpf larvae treated with 100 μM DEM for the indicated times. (A) The results of the WISH analysis. (B) The results of the RT-PCR analysis. The amount of cDNA used for RT-PCR was standardized by the *ef1α* expression.

Figure 8 Hypothetical models of the tissue-specific and transient *hmox1a* induction.

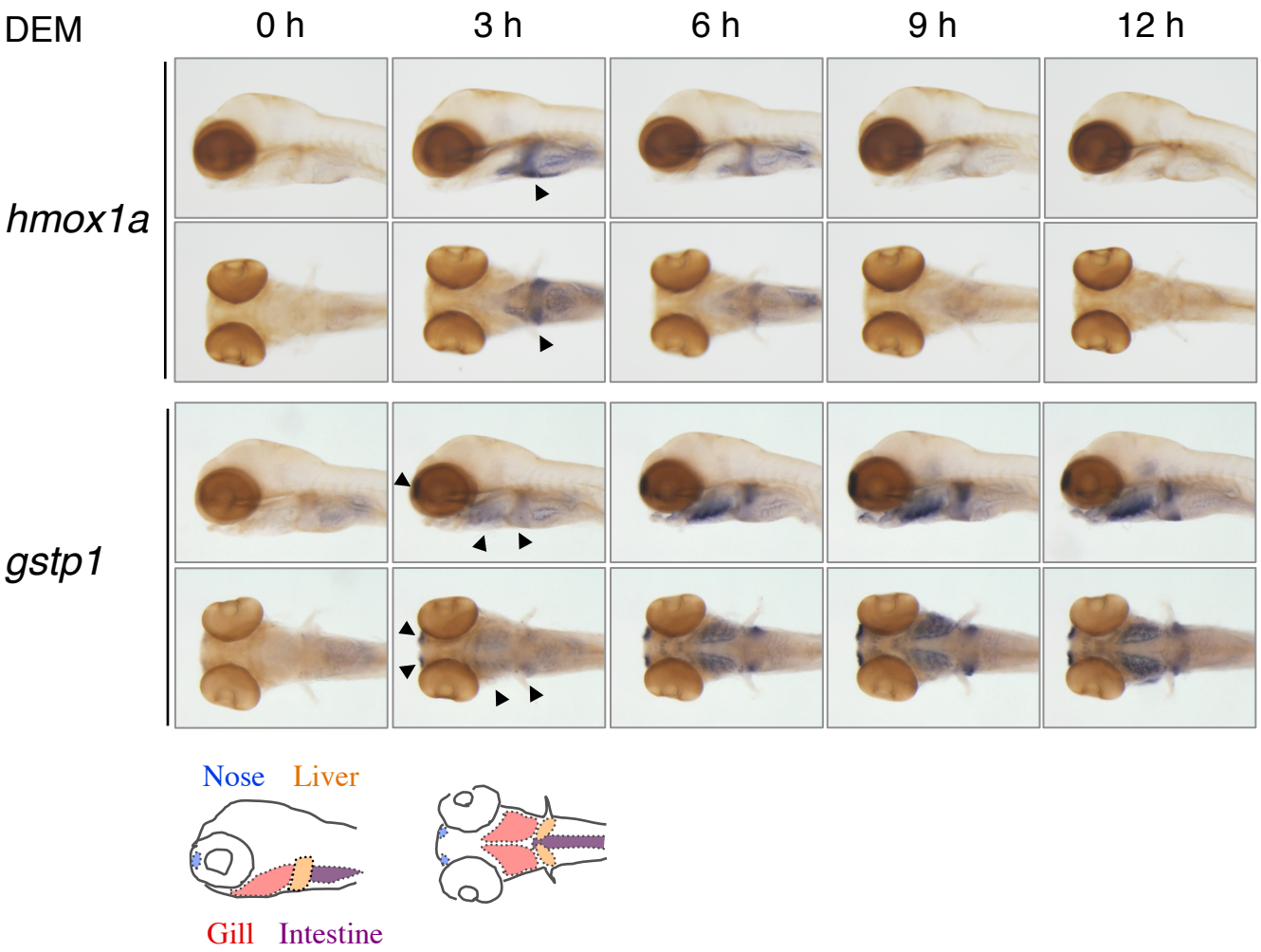
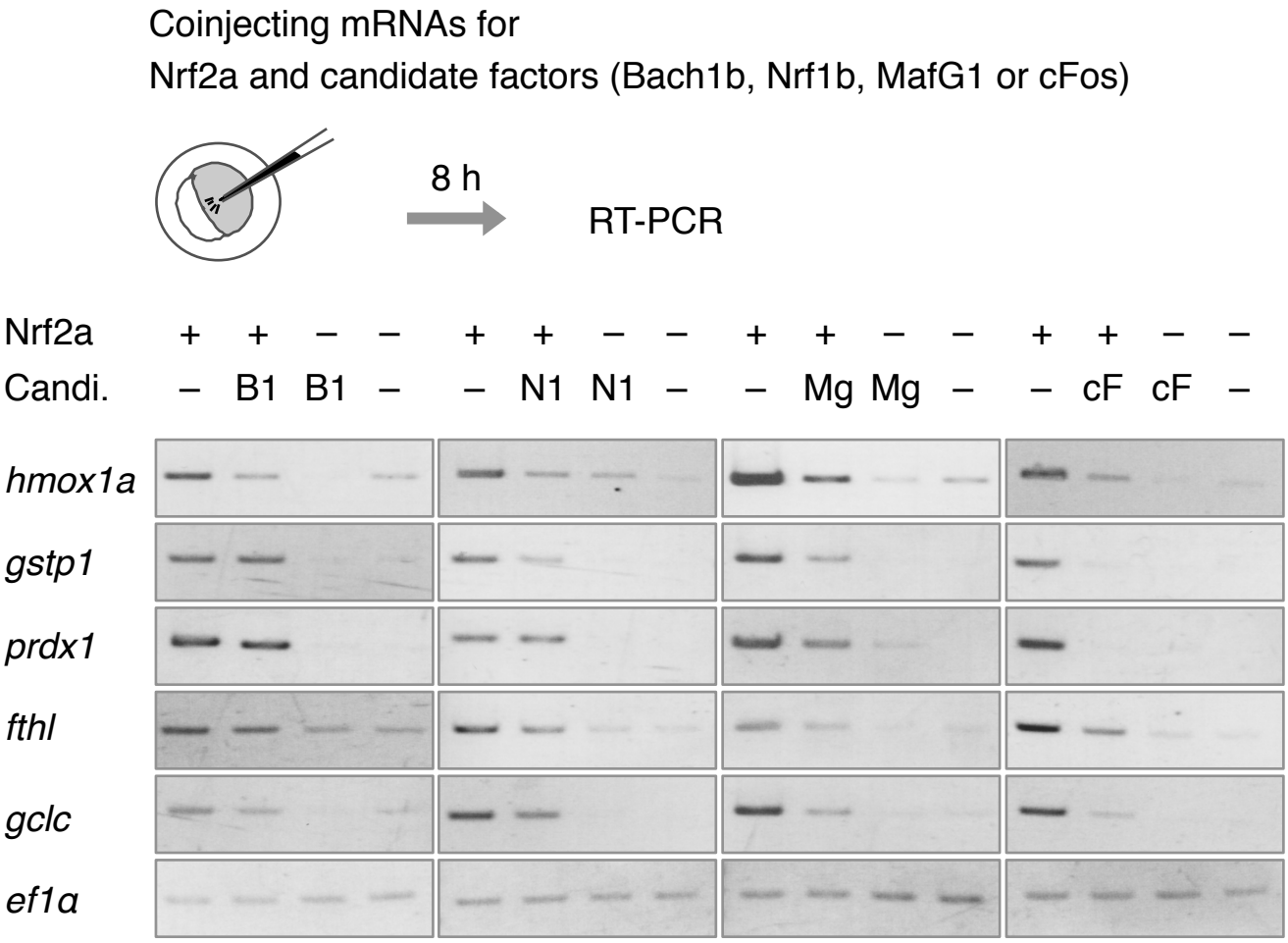


Fig. 1 Fuse et al.



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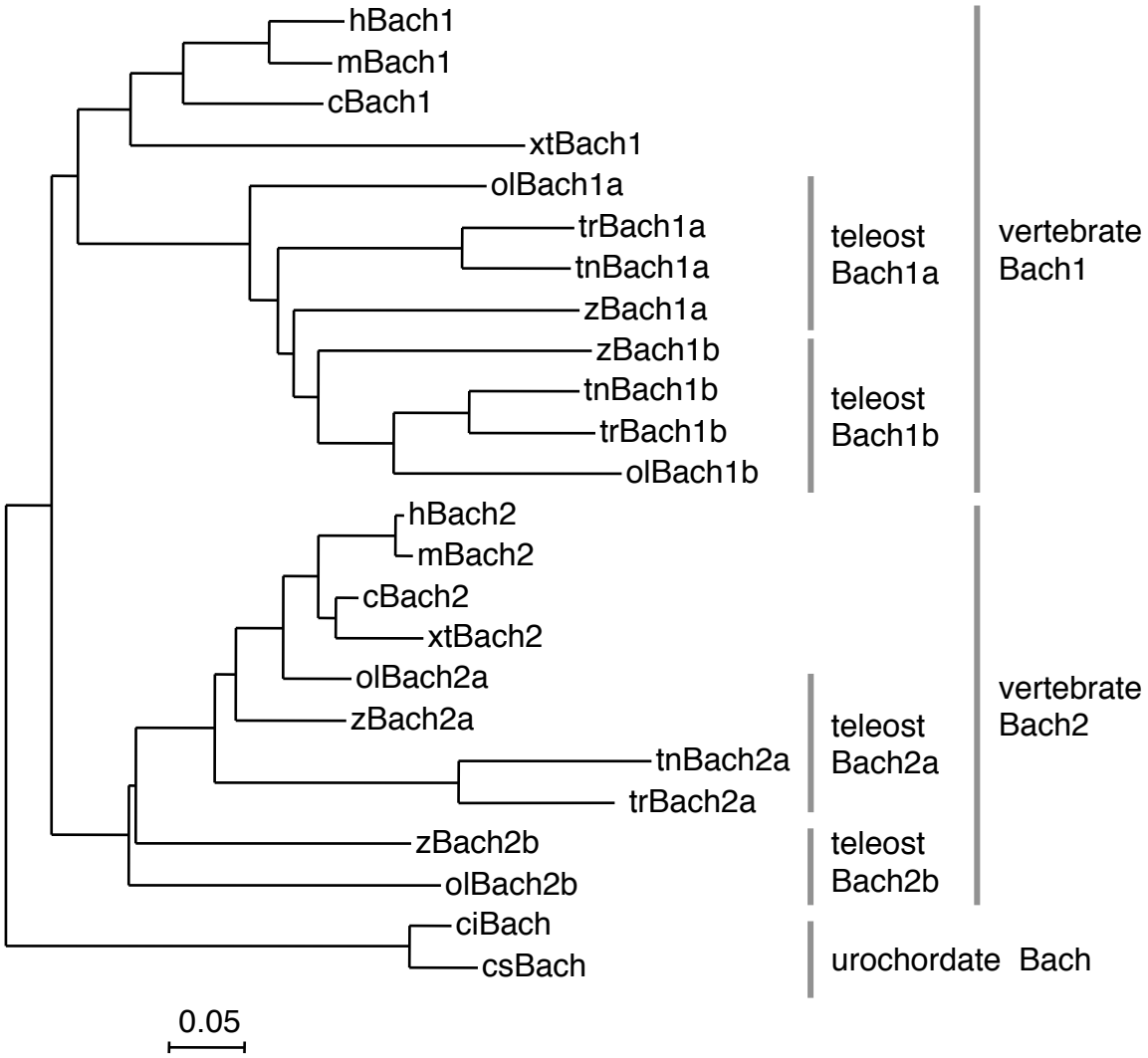

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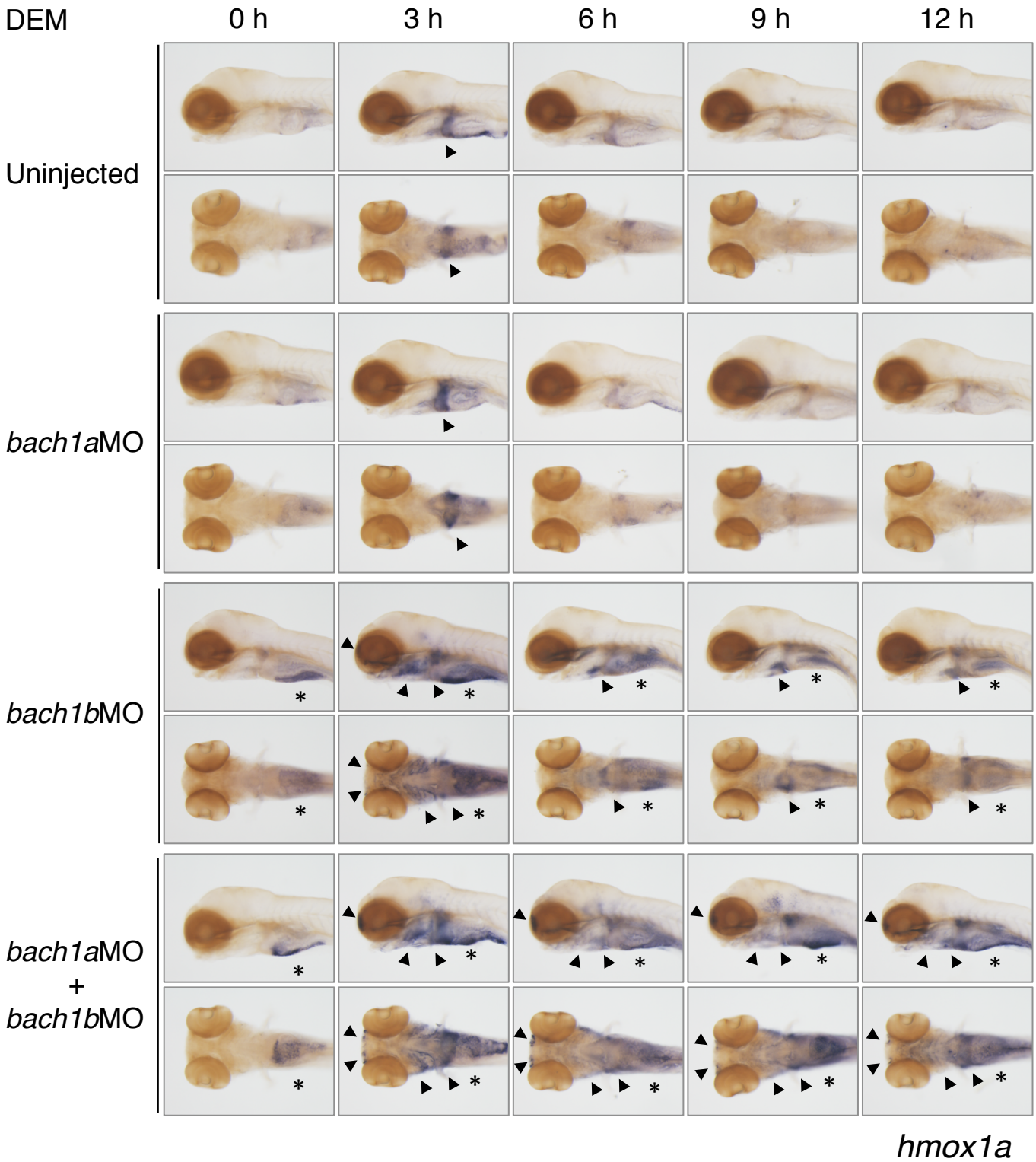

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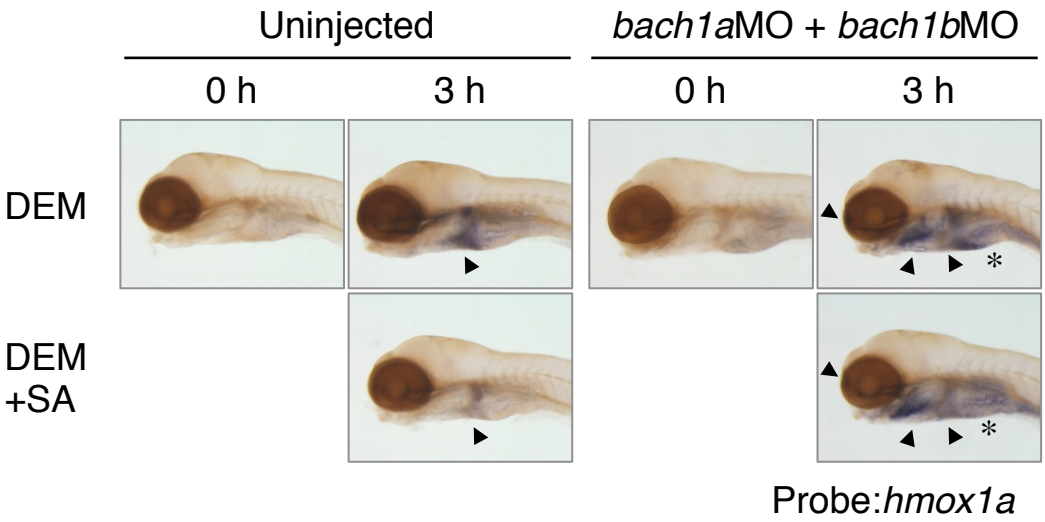

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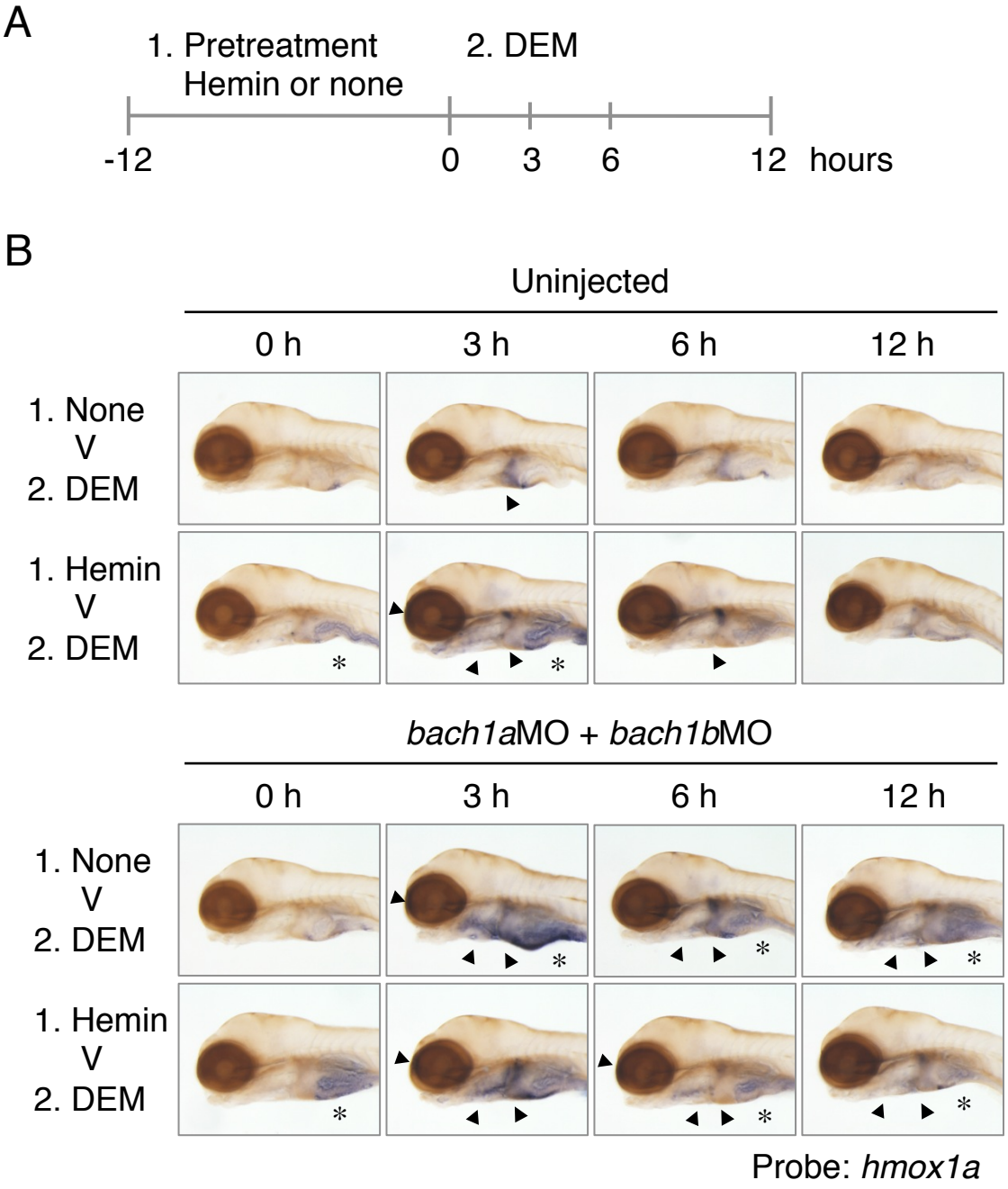

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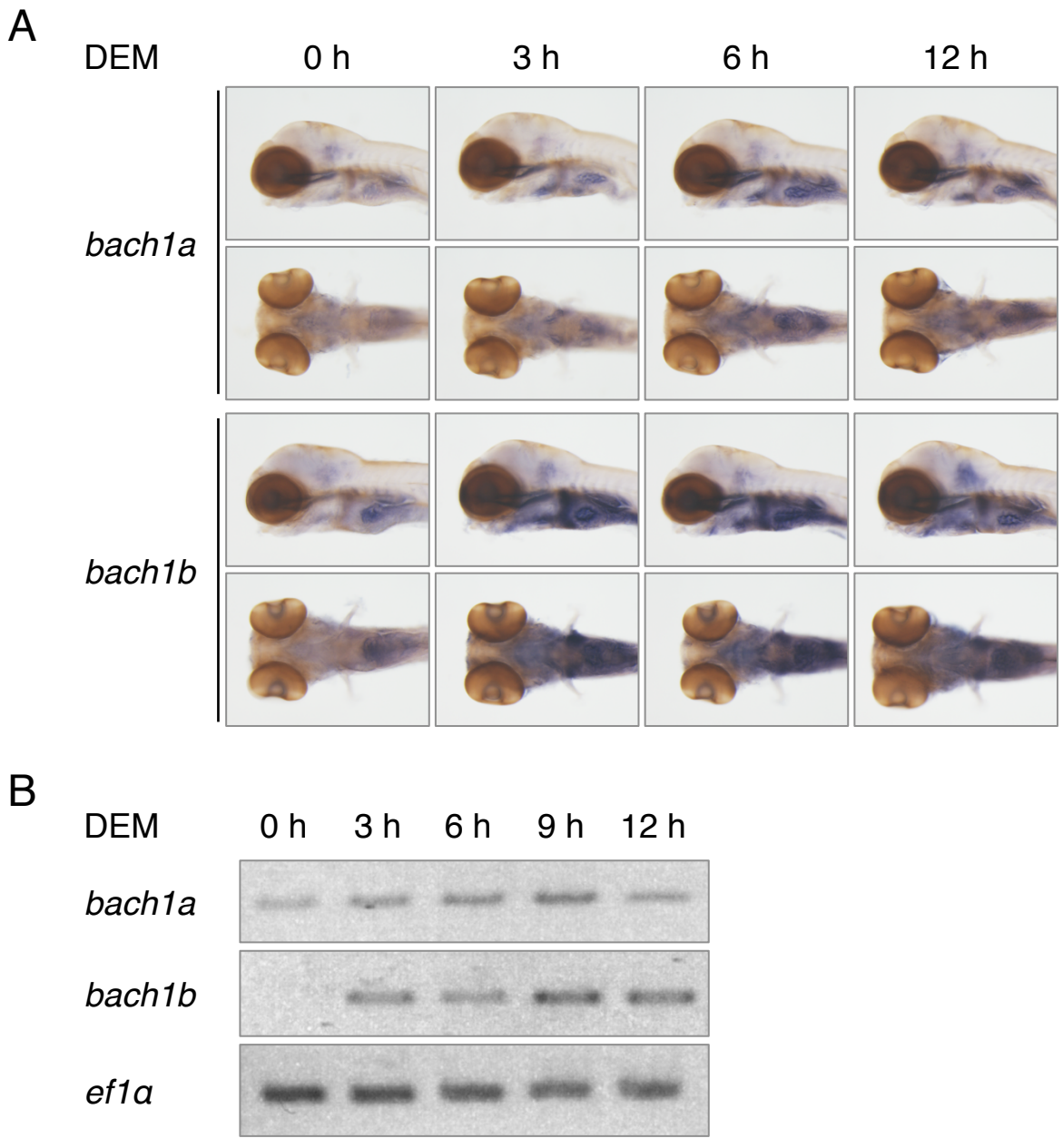

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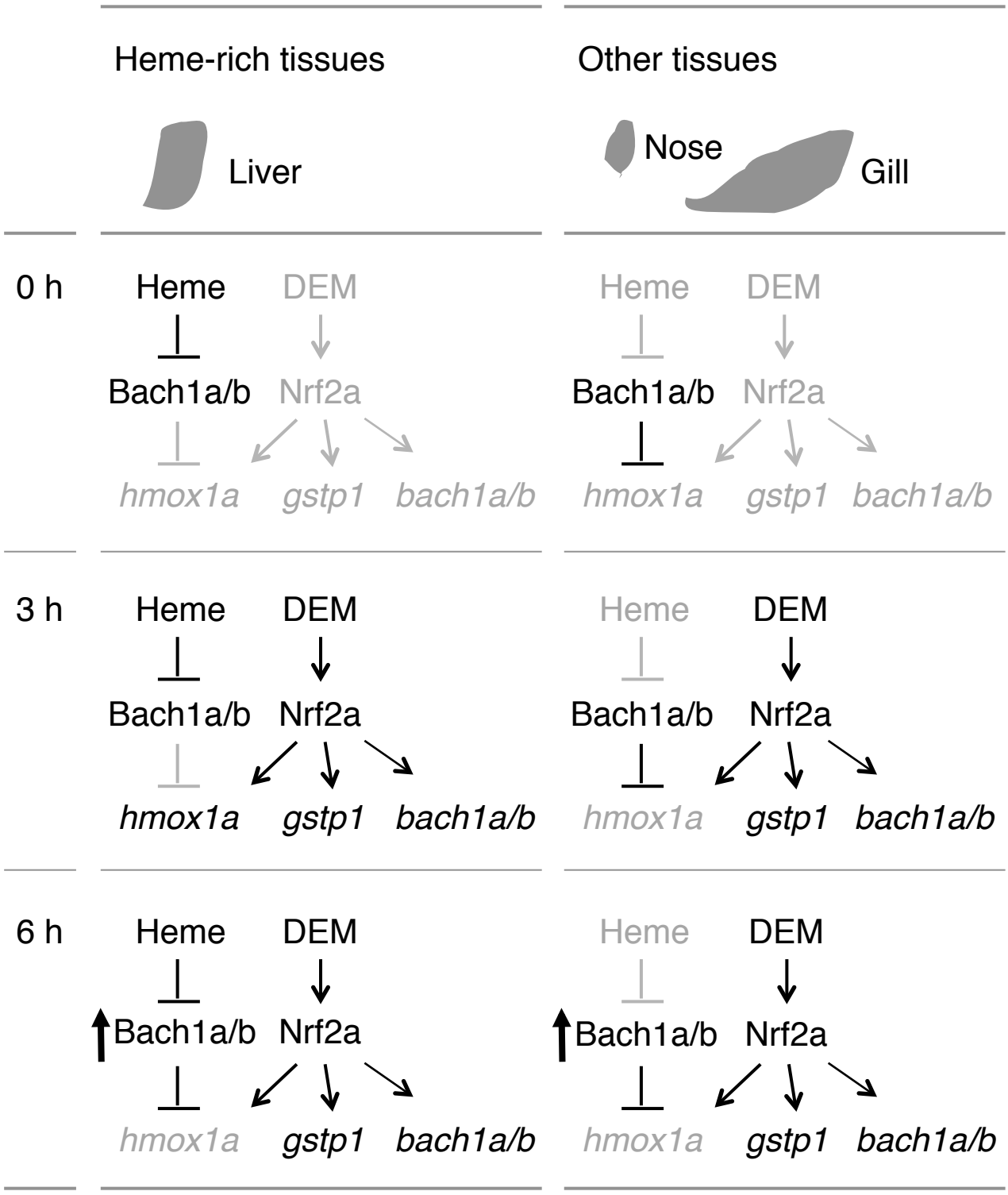

Fig. 8 Fuse et al.