## Invited Review

# Wearing Red for Signaling: The Heme-Bach Axis in Heme Metabolism, Oxidative Stress Response and Iron Immunology

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The connection between gene regulation and metabolism is an old issue that warrants revisiting in order to understand both normal as well as pathogenic processes in higher eukaryotes. Metabolites affect the gene expression by either binding to transcription factors or serving as donors for post-translational modification, such as that involving acetylation and methylation. The focus of this review is heme, a prosthetic group of proteins that includes hemoglobin and cytochromes. Heme has been shown to bind to several transcription factors, including Bach1 and Bach2, in higher eukaryotes. Heme inhibits the transcriptional repressor activity of Bach1, resulting in the derepression of its target genes, such as globin in erythroid cells and heme oxygenase-1 in diverse cell types. Since Bach2 is important for class switch recombination and somatic hypermutation of immunoglobulin genes as well as regulatory and effector T cell differentiation and the macrophage function, the heme-Bach2 axis may regulate the immune response as a signaling cascade. We discuss future issues regarding the topic of the iron/heme-gene regulation network based on current understanding of the heme-Bach axis, including the concept of "iron immunology" as the synthesis of the iron metabolism and the immune response.

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#### Introduction

Heme is a prosthetic group comprised of protoporphyrin IX and iron. Heme forms complexes with globin and myoglobin proteins to serve as the binding site for oxygen. Heme binds to diverse enzymes, subsequently conferring upon them the activities of oxygenase and peroxidase. In addition, heme plays an integral role in electron transfer reactions in mitochondria. As such, it is essential in most living organisms. However, lactic acid bacteria, for example, can depend on lactic acid fermentation and proliferate without the functions of cytochromes and heme. Nonetheless, these bacteria proliferate more vigorously when supplied with heme, a process exploited in the production of cheese (Lechardeur et al. 2011). The functions of heme are based on the principle that iron binds oxygen molecule and readily undergoes oxidation-reduction reactions under physiological conditions. Therefore, virtually every cell in the mammalian body must synthesize and utilize heme for survival. However, this background does not explain the whole picture of using (i.e., "wearing") heme.

In addition to the classic biochemical functions, the

regulatory role of heme as a signaling molecule is becoming clear in diverse biological systems (Padmanaban et al. 1989; Sassa and Nagai 1996). In this context, heme binds to heme-sensor proteins, changing their activities. The interactions between heme and sensor proteins occur at relatively lower affinities than those involving classical heme proteins, such as globin, making the system able to sense the level of heme within a cell. In addition, protein-bound heme further binds gas molecules, such as oxygen, carbon monoxide and nitric oxide, regulating the activities of the respective proteins in response to the concentrations of the gas molecules (Gilles-Gonzalez and Gonzalez 2005). In this review, we first summarize the metabolism of heme and provide recent examples of proteins that regulate the gene expression in response to heme, with the aim of providing a wider view of the regulatory roles of heme in the gene expression. Heme also regulates protein translation by binding to the heme-regulated kinase HRI (Chen 2007); however, in this review, we focus on nuclear heme sensors. Subsequently, we center on Bach1 and Bach2, two nuclear heme receptors/effectors that are related to each other in terms of structure and perform overlapping but distinct

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roles. We then discuss both the established and hypothetical roles of the heme-Bach axis in the context of heme metabolism as well as acquired and innate immunity.

Although it is beyond the scope of this review, iron is a critical target of innate immunity. The immune system attempts to sequester iron from colonizing microorganisms in order to suppress their proliferation by expressing ironbinding molecules (Correnti and Strong 2012; Zhao et al. 2012). In rivalry with the host, the microorganisms acquire iron and/or heme by producing siderophores (Correnti and Strong 2012). With these concepts in mind, the idea of "iron immunology" comes to the fore.

#### Metabolism of "Two-Face" heme

The synthesis of heme starts with two substrates, succinyl-CoA and glycine. Succinyl-CoA is a product of the tricarboxylic acid (TCA) cycle. The reactions leading to the formation of heme take place alternating between the cytoplasm and mitochondria (Fig. 1). In the last step of the reaction, iron is enzymatically incorporated into the protoporphyrin ring within mitochondria. Heme is then utilized within the mitochondria or the cytoplasmic region after being transported. Therefore, the amount of heme synthesized is affected by the activity of the TCA cycle and the level of available iron. The rate-limiting enzyme of the pathway is the first enzyme  $\delta$ -aminolevulinate synthase 1 (ALAS1), which is expressed ubiquitously. Under normal conditions, the level of heme is kept within a narrow range via the feedback regulation of ALAS1 by heme (Fig. 1) (Yamauchi et al. 1980; Kikuchi and hayashi 1982; Lathrop and Timko 1993; Munakata et al. 2004). The mitochondrial import of ALAS1 is inhibited by heme, which binds to the N-terminal mitochondrial import signal of ALAS1 depending on the presence of the CP motif (Fig. 2, see below). In erythroid cells, the erythroid-specific isozyme ALAS2 is not suppressed by heme, which allows for the synthesis of a large amount of heme in order to generate hemoglobin (Munakata et al. 2004).

Heme has another face: excess heme is toxic to cells due to the ability of heme to catalyze the so-called Fenton reaction, generating hydroxyl radicals, a highly reactive oxygen species (ROS). In order to avoid the phenomenon and complement the negative feedback regulation of heme synthesis, the expression of heme oxygenase-1 (HO-1) is induced in response to an increased level of heme (Shibahara 2003), which subsequently results in the degra-



#### Fig. 1. Metabolism of heme.

Heme is synthesized by a cascade of enzymes present in and outside of mitochondria (yellow). Iron is incorporated at the last step. Heme is bound to proteins in order to carry out essential functions. Excess heme is either degraded by HO-1 and/or HO-2 or excreted out of the cell by FLVCR. Iron is released upon heme degradation and excreted by ferroportin or maintained within the shell of ferritin as ferric iron. Genes regulated by the heme-Bach1 axis are shown in green. The induction of HO-1 and inhibition of ALAS1 by heme are depicted with dashed lines. ALA, aminolevulinic acid; PBG, porphobilinogen; HMB, hydroxymethylbilane; UPOgen III, uroporphyrinogen III; COPROgen III, coproporphyrinogen IX; PP IX, protoporphyrin IX.

dation of excess heme not bound to proteins (Fig. 1). In contrast to HO-1, HO-2 is constitutively expressed and participates in the basal level regulation of heme (Shibahara 2003). Therefore, evolution acquires mechanisms to handle the essential but toxic heme molecule, some of which are further described below.

#### Nuclear heme sensors

The list of heme-binding proteins now includes nuclear regulatory proteins of distinct protein families in addition to the classical hemoproteins, such as globin and cytochromes, which play significant roles in the development of biochemistry.

#### Hap1 in yeast

In yeast, oxygen availability is associated with the synthesis of cytochromes via the actions of heme. Under low concentrations of oxygen, yeast cells stop to depend on the electron transfer chain activity within mitochondria; therefore, the expression of cytochrome genes is kept at a low level. When oxygen is available, yeast cells express higher levels of cytochrome genes in order to activate the electron transfer chain. The level of oxygen determines the amount of heme synthesized (Mattoon et al. 1995), possibly because oxygen is a substrate for heme synthesis (see Fig. 1). Heme then directly interacts with the transcription factor heme activator protein 1 (Hap1), the first eukaryotic transcription factor shown to be regulated by heme (Zhang and Guarente 1995). Hap1 is activated by the binding of heme, resulting in the activation of genes required for respiration and oxidative damage control, such as catalase and cytochromes in the electron transfer chain. Therefore, the level of available oxygen is transduced to heme, activating the function of Hap1. Hap1 contains seven heme regulatory motifs (HRMs, Fig. 2) to which heme directly and independently binds (Zhang and Guarente 1995). The core motif of HRMs is the dipeptide sequence of cysteine and proline, termed the CP motif. The cysteine residue of the CP motif is the ligand of heme binding. Among the seven HRMs of Hap1, only the most C-terminal HRM7 is apparently required for heme-mediated regulation (Hach et al. 1999; Hon et al. 2000). The binding of heme to Hap1 activates its transactivation activity by dissociating it from an inhibitory protein complex containing heat shock proteins (Zhang et al. 1998; Lee et al. 2003). The functional significance of other CP motifs/HRMs remains unclear.

#### Circadian and metabolic regulators

In higher eukaryotes, the synthesis of heme is under the control of the circadian clock (Fig. 3). The expression of ALAS1 is regulated by the circadian transcription factors Per1, Per2 and NPAS2 (Zheng et al. 2001; Kaasik and Lee 2004). Heme binds to both NPAS2 and Per2. Heme binds to the two PAS domains of NPAS2 and is necessary for its DNA binding activity (Mukaiyama et al. 2006), whereas the binding of carbon monoxide (CO) to heme causes the decrease in the DNA binding activity of the Bmal1-NPAS2 dimer (Dioum et al. 2002). With respect to Per2, this molecule contains three heme binding regions: two PAS domains and a C-terminal HRM containing a CP motif. The binding of heme to the Per2 PAS domains appears to regulate DNA binding in response to carbon monoxide (Kitanishi et al. 2008; Hayasaka et al. 2011). Meanwhile, heme binding to the C-terminal HRM stimulates the degradation of Per2 (Yang et al. 2008). Therefore, heme functions to tune the circadian clock, forming a mutual regulatory loop.

Hap1	278	KCPINK	283	A	LAS1	7	RCPFLS	12
	297	KCPVDH	302			32	NCPKMM	37
	323	KCPVDH	328			109	KCPFLA	114
	347	KCPVDH	352					
	389	KCPVDH	394	H	0-2	263	KCPFYA	268
	414	KCPIDH	419			280	NCPFOT	285
	1192	KCPVYQ	1197				~	
				I	RP2	120	ACPTDL	125
Bach1	223	LCPKYR	228			201	LCPFHL	206
	300	QCPAEQ	305			375	MCPEYG	380
	435	ECPWLG	340			719	RCPSFF	724
	463	NCPFIS	368					
	494	PCPYAC	499	H	OIL-1	196	OCPGCT	201
	648	DCPLSF	653			279	- ECPVCY	284
						320	ACPFID	325
Bach2	368	ACPFNK	373			329	SCPGKL	334
	498	SCPVPI	503			388	TCPVCT	393
	505	VCPRSP	510			444	HCPQCR	449
	602	SCPVQD	607					
	728	YCPVLI	733	H	RI	405	ACPYVM	410
						547	RCPAOA	552
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Fig. 2. The CP motif within heme regulatory motifs.

The CP motifs of the indicated proteins are aligned with the numbers showing the positions of the amino acid residues.



Fig. 3. Cross-talk between the circadian clock and heme metabolism. Regulatory interactions (dashed lines: positive as arrowheads, negative as stop lines) between clock transcription factors (Bmal, Npas2, Per1, Per2, Cry1, Cry2) and metabolism are depicted. Putative heme-mediated feedback regulation is shown with the red dashed lines. The solid lines with arrowheads indicate the production of proteins from respective genes and the transactions of these proteins. CO, carbon monoxide.

However, one study has raised a concern regarding the specificity of the *in vitro* interaction between heme and Per2 or NPAS2 (Airola et al. 2010). According to their study, the PAS domain of Per2 binds heme in a non-specific manner. Therefore, the biochemical and biological significance for the heme-Per2 (and NPAS2) interaction awaits further studies.

Rev-Erb $\alpha$  and Rev-Erb $\beta$  are homologues of *Drosophila melanogaster* E75, all of which belong to the nuclear hormone receptor superfamily. These proteins bind to heme via their respective ligand binding domain (Reinking et al. 2005; Raghuram et al. 2007; Yin et al. 2007). Both Rev-Erb $\alpha$  and Rev-Erb $\beta$  are involved in the regulation of the circadian rhythm. Rev-Erb $\alpha$  represses the expression of Bmal1 by recruiting histone deacetylase 3 (HDAC3). Heme is essential for the repressor function of Rev-Erb $\alpha$ and promotes the interaction between Rev-Erb $\alpha$  and HDAC3 (Raghuram et al. 2007; Yin et al. 2007). Rev-Erb $\alpha$ also represses the expression of gluconeogenic genes (phosphoenolpyruvate carboxykinase and glucose 6-phosphatase) (Yin et al. 2007). Considering that heme is critical for the functions of diverse metabolic enzymes, these multiple regulatory interactions are expected to establish cross-talk between the circadian clock and various metabolic pathways (Fig. 3). The level of heme may thus provide feedback regulation of circadian clock transcription factors.

The regulation of circadian transcription factors by heme and the direct and indirect regulation of ALAS1 by these factors strongly suggest an interaction between the circadian rhythm and heme metabolism. While it is intuitively rational, the physiological significance of this connection remains elusive.

#### microRNA processor

Another interesting nuclear heme sensor is the RNAbinding protein DiGeorge critical region-8 (DGCR8) that participates, together with the RNase Drosha, in the first step of cleavage of pri-miRNA, the precursor of microRNA (miRNA). DGCR8 is a heme protein and requires heme for its efficient activity (Faller et al. 2007). Therefore, heme metabolism may be connected with the miRNA expression. However, the detailed mechanisms underlying the DGCR8 regulation by heme and/or the connection between DGCR8 and heme-related responses are unclear.

#### Role of Bach1 and Bach2 within the bZip network

A phylogenetic analysis recently revealed the presence of 19 families of basic leucine zipper (bZip) transcription factors in vertebrates (Amoutzias et al. 2007) (Fig. 4A). Bach1 and Bach2 constitute one of these 19 families and are most related in terms of evolution to the CNC family, which includes nuclear factor erythroid-2 (NF-E2) p45 and Nrf2 (see below) (Fig. 4B). The Bach family does not contain any representatives in arthropods or lower eukaryotes, although it is present in Ciona intestinalis, indicating that a common ancestor existed immediately prior to the vertebrate split (Yamada et al. 2003; Amoutzias et al. 2007). The Bach family is a relatively new invention in evolution. While there is only one Bach gene in ascidians, there are two distinct Bach1 and Bach2 orthologues in zebra fish. This evolutionary history suggests that Bach1 and Bach2 perform roles required for a sophisticated way of life in higher eukaryotes.

#### Bach repressors

Bach-small Maf heterodimers function as repressors of the gene expression by binding to Maf recognition elements (MAREs) and recruiting transcriptional co-repressors (Oyake et al. 1996; Muto et al. 1998; Dohi et al. 2008). The bZip domain mediates heterodimer formation with small Maf proteins and DNA binding (Fig. 5). Bach2 appears to be able to bind efficiently to DNA as a homodimer as well. In contrast, Bach1 must form heterodimers with small Maf proteins in order to bind to DNA (Oyake et al. 1996). Their binding sites are either AP-1-like sequences (Bach2 homodimer) or NF-E2 binding sites related to MARE (Bach-small Maf heterodimers). A comprehensive study of the dimer network of bZip proteins demonstrated that Bach1 and Bach2 are dedicated partners of small Maf proteins (Reinke et al. 2013).

The BTB/POZ domain (Albagli et al. 1995) located at the N-termini of Bach1 and Bach2 mediates dimer formation (Ito et al. 2009), resulting in the creation of multivalent DNA binding oligomers (Fig. 5) (Igarashi et al. 1998; Yoshida et al. 1999). Meanwhile, the Bach2 BTB domain mediates its targeting around PML nuclear bodies in order to repress transcription in this region (Tashiro et al. 2004). The basic region within the bZip domain functions as the nuclear localization signal (NLS) (our unpublished observations), whereas the C-terminal small cytoplasmic localization signal (CLS) promotes the nuclear export and cytoplasmic localization of Bach1 and Bach2 (Hoshino et al. 2000; Suzuki et al. 2003, 2004). CLS is dependent on the nuclear exporter Crm1 and is therefore a version of nuclear export signal (NES), although its primary sequence is not conserved with a typical NES (Hoshino et al. 2000). Another NES is located in front of the basic region of Bach1, spanning two of the CP motifs (Suzuki et al. 2004) (Fig. 5, see below). A portion of Bach1 molecule mediates cytoplasmic anchoring by interacting with microtubules via IHABP А



В





(A) Among the 19 families of bZip transcription factors in vertebrates (Amoutzias et al. 2007), representatives of 17 families are compared in their bZip domains. For the Bach family, both Bach1 and Bach2 are included for comparison. Sequences of bZip regions, defined in the Uniprot protein knowledge database (http://www.uniprot. org/) were aligned using the CLUSTALW program (http://www.genome.jp/tools/clustalw/). The tree is depicted as an unrooted phylogenetic tree with a specific branch length. (B) The members of the NF-E2 p45 and Bach families are compared as in (A).



Fig. 5. Schematic structures of Bach factors. The domains and motifs of Bach1 and Bach2 are depicted to scale.

binding (Yamasaki et al. 2005) (see below). Therefore, the subcellular localization of Bach1 and Bach2 is regulated by multiple mechanisms.

#### NF-E2-related activators

NF-E2 is critical for the expression of globin genes in erythroid cells. It binds to the enhancer elements within the locus control region (LCR) in order to stimulate the expression of  $\beta$ -globin genes (Ney et al. 1990a, b, 1993). NF-E2 is a heterodimer of p45 and the small Maf protein MafK (Andrews et al. 1993a, b; Chan et al. 1993b; Ney et al. 1993; Igarashi et al. 1994, 1995). Other small Maf proteins, such as MafG and MafF, may be redundant to MafK (Igarashi et al. 1994; Blank et al. 1997). Small Maf proteins do not possess a transcription activation domain, whereas p45 does (Bean and Ney 1997; Nagai et al. 1998), thus contributing to the transactivation activity of the heterodimer NF-E2. The expression of p45 is relatively specific to hematopoietic cells, including erythroid cells, whereas small Maf proteins are expressed rather ubiquitously (Igarashi et al. 1995). NF-E2 also activates the expression of the porphobilinogen deaminase gene encoding an enzyme in the heme synthesis pathway (Mignotte et al. 1989a, b; Romeo et al. 1990).

In non-erythroid cells, small Maf proteins form heterodimers with other p45-related factors, including Nrf1/ LCR-F1 (Chan et al. 1993a; Caterina et al. 1994), Nrf2/ ECH (Moi et al. 1994; Itoh et al. 1995) and Nrf3 (Kobayashi et al. 1999), all of which are related to each other in both structure and evolution (Fig. 4B). These factors activate transcription by forming heterodimers with small Maf proteins in order to bind to MARE. The heterodimers of Nrf2-small Maf are important for the activation of genes in response to oxidative stress (Taguchi et al. 2011). The physiological and/or pathological roles of Nrf1 and Nrf3 remain enigmatic. Nrf1 has been shown to activate proteasome-related genes (Tsuchiya et al. 2013), whereas Nrf3 has been shown to be targeted to the endoplasmic reticulum (Zhang et al. 2009). These observations suggest that Nrf1 and Nrf3 regulate protein homeostasis.

# Competitive repression and activation involving Bach and Nrf factors

MARE is an extended version of the AP-1 binding site containing a central AP-1 motif (Kataoka et al. 1994) (Fig. 6A), which allows for the binding of the heterodimers of Bach-small Maf, NF-E2 and Nrf-small Maf as well as Maf homodimers in addition to AP-1 (Mignotte 1989a; Ney et



Fig. 6. Regulation of Bach by heme.

(A) Bach1/2-small Maf and p45/Nrf heterodimers compete for MARE binding, resulting in either the repression or activation of the target genes. Heme inhibits the activities of Bach1 by directly binding to them, inducing its nuclear export, polyubiquitination and degradation. Hoil-1 and Fbxl17 are the E3 ligases for Bach1. Heme also induces the degradation of Bach2, although the mechanism remains unclear. The AP-1 site within the MARE is shown in red. (B) Recombinant glutathione *S*-transferase (GST)-Bach2 fusion protein expressed in *E. coli* and purified exhibits a brown color. (C) Heme binds to Bach1 and Bach2 in two modes. The five-coordinate mode involves the CP motifs.

al. 1990b; Kataoka et al. 1994; Oyake et al. 1996; Motohashi et al. 2000; Kyo et al. 2004). Within the heterodimers of small Maf proteins, small Maf recognizes the longer half of the sequence. In various experimental systems, these heterodimers and Maf homodimers appear to compete with each other for MARE binding sites and subsequent gene regulation (Fig. 6A). This competitive regulation by Maf heterodimers appears to allow for the dynamic gene expression under a chromatin environment, as the binding of Bach-Maf heterodimers keeps MAREs accessible for activators even when the target genes are repressed. Otherwise, the target genes may be completely inactivated by heterochromatin formation when the activators are not active. One important fundamental question, the answer to which remains unclear, is whether these MARE-binding activators and repressors are functionally similar to each other in their respective classes. Do Nrf1 and Nrf2 heterodimers regulate the same battery of genes? Do Bach1 and Bach2 heterodimers regulate the same battery of genes? If not, what is the molecular basis for the specificity beyond the apparently shared MAREs?

# Regulation of Bach1 and Bach2 by heme pathways and other mechanisms

Purified recombinant proteins of Bach1 and Bach2 expressed in *E. coli* become brown in color (Fig. 6B). These observations led to the discovery that these proteins are heme-binding proteins (Ogawa et al. 2001; Watanabe-Matsui et al. 2011).

#### Heme-mediated regulation of Bach1

Bach1 is the first mammalian transcription factor shown to be regulated by heme (Ogawa et al. 2001). Mouse Bach1 possesses six CP motifs resembling those of HAP1 (Fig. 2). Each of the four C-terminal CP motifs (CP3-CP6) is involved in one molecule of heme binding (Hira et al. 2007). When heme binds to Bach1, heme decreases the DNA binding activity of Bach1 in vitro (Ogawa et al. 2001; Sun et al. 2002), reducing its half-life by promoting polyubiquitination and subsequent proteasome-mediated degradation in vivo (Zenke-Kawasaki et al. 2007) (Fig. 6A). Regarding the polyubiquitination of Bach1 in response to heme, Hoil-1 is the candidate ubiquitin E3 ligase (Zenke-Kawasaki et al. 2007). A recent study demonstrated that Fbxl17 participates in both steady state and heme-induced polyubiquitination and in the quantitative regulation of Bach1 (Tan et al. 2013). Heme also induces the Crm1dependent nuclear export of Bach1 (Suzuki et al. 2004). The four CP motifs appear to divide the labor of hememediated regulation, as only two of the CP motifs (CP3 and CP4) are responsible for the induction of nuclear export upon heme binding (Suzuki et al. 2004) (see Fig. 5). The critical motifs for the regulation of DNA binding and/or polyubiquitination are unclear at present.

Spectroscopic studies have been performed to elucidate the interactions between Bach1 and heme (Hira et al. 2007). A recombinant fragment of Bach1 containing all of the CP motifs (176-739 a.a.) has been expressed in E. coli and purified. The complex of Bach1 (176-739 a.a.) and heme exhibits an optical absorption spectrum with two Soret peaks at 423 and 371 nm. The determination of the spin state and coordination structure of these species using resonance Raman spectroscopy has revealed that the Soret peak at 423 nm reflects Cys coordination as one of the six axial ligands (Fig. 6C). Another axial ligand is likely to be a histidine residue. Therefore, this binding reflects a sixcoordinate heme complex. The 371-nm Soret peak becomes dominant at higher heme concentrations, which indicates the presence of another heme binding mode with affinity lower than that of the 423-nm six-coordinate mode. This binding appears to reflect a five-coordinate heme complex and involves the CP motifs (Hira et al. 2007) (Fig. 6C). These results raise the possibility that the two modes of heme binding (the six- and five-coordinate heme complexes) individually regulate the activity of Bach1 with distinct effects. The relationships of these modes to the inhibition of DNA binding and polyubiquitination await further studies.

#### Heme-mediated regulation of Bach2

Bach2 possesses five CP motifs (Fig. 2). Recently, we found that heme binds to both Bach2 and Bach1 (Watanabe-Matsui et al. 2011). Heme binds to Bach2 in order to inhibit its DNA binding activity *in vitro*. When added to culture media, heme reduces the half-life of Bach2 in B cells (Watanabe-Matsui et al. 2011) (Fig. 6A). In an analogy to that observed for Bach1, heme may induce the poly-ubiquitination of Bach2. An optical absorption spectrum analysis of recombinant Bach2 demonstrated that Bach2 possesses two heme-binding modes (five- and six-coordinates), as does Bach1 (Watanabe-Matsui M., Murayama K. and Igarashi K., unpublished observation) (Fig. 6C). These findings suggest that heme is a physiological regulator of Bach2.

#### Regulation of Bach by redox and phosphorylation

Considering their diverse functions (see below), one may expect that Bach1 and Bach2 proteins are regulated by other post-translational mechanisms in addition to heme. Both Bach1 and Bach2 are modulated in response to oxidative stress. Cadmium ions induce the nuclear export of Bach1, which facilitates the transactivation of oxidative stress response genes by competing transcriptional activators, such as Nrf2. Cadmium ions activate the function of the C-terminal CLS of Bach1, which mediates Crm1dependent nuclear export (Suzuki et al. 2003), resulting in the derepression of its target genes. Diethyl maleate (DEM), which depletes the reduced form of glutathione, induces the nuclear accumulation of Bach2 in part by repressing CLS-mediated nuclear export (Hoshino et al. 2000). Because CLS contains essential cysteine residues (Hoshino et al. 2000), cadmium ions and DEM may each affect their redox status, although in opposite directions. However, the precise mechanisms underlying the regulation of CLS of Bach1 and Bach2 remain unclear. In human cells, Bach1 is inactivated under low concentrations of arsenite ions, resulting in the transcriptional induction of HMOX1 (Reichard et al. 2008). Because Bach1 and Bach2 are rich in cysteine residues, some of the cysteine residues, either within or outside of CLS, may function under redox regulation. Consistent with this idea, diamide, a sulfhydryloxidizing agent, induces the cytoplasmic localization of Bach1 reporter fragment, which is dependent on the presence of cysteine 574 (Ishikawa et al. 2005). Therefore, it is clear that Bach1 acts under redox regulation, which may also apply to Bach2.

The phosphorylation of Bach1 also regulates its nuclear export. Tyrosine 486 of Bach1 is phosphorylated in response to the antioxidant tert-butylhydroquinone and is essential for the nuclear export of Bach1 (Kaspar and Jaiswal 2010). It should be noted that tyrosine 486 juxtaposes to the heme-regulated nuclear export signal containing Cys435 of CP3 and Cys463 of CP4 (Figs. 2 and 5). Another line of research has shown Bach1 to likely be inactivated by the phosphatidylinositol-3 kinase (PI-3K) pathway (Sakamoto et al. 2009). Ferritin H transcription is robustly induced in tert-butylhydroquinone (t-BHQ)-treated Jurkat cells via an antioxidant responsive element (ARE), a version of MARE, due to a deficiency of PTEN. The PI3K inhibitor LY294002 blocked recruitment of Nrf2 to the ARE and the subsequent Bach1 release (Sakamoto et al. 2009). Regarding human Bach2, the phosphorylation of serine 521 is required for the cytoplasmic accumulation of Bach2. This modification is dependent on the PI-3K/S6 kinase pathway, thus raising the possibility that Bach2 is regulated via phosphorylation in response to extracellular cytokines and growth factors (Yoshida et al. 2007). In addition to this site, we recently found that additional multiple sites of Bach2 are modified by phosphorylation in response to the PI-3K pathway (Ando R., Shima H., Sato Y., Muto A. and Igarashi K., unpublished observation).

#### Regulation of Bach1 and Bach2 expression

The expression of Bach1 is rather widespread while that of Bach2 is restricted in tissues and cell types. The expression of Bach1 gene is activated by the ubiquitous transcription factor Sp1, which may explain its diverse expression (Sun et al. 2001). In addition, Bach1 activates its own gene expression in transfection assays by binding to the MARE-like element present downstream of its promoter (Sun et al. 2001). This positive-feedback may also contribute to the wide expression of Bach1. However, considering its activity as principally a repressor, the mechanism for this self-activation is unclear yet. Other MARE-binding factors may also transactivate Bach1. Indeed, Nrf2 induces the expression of Bach1, thus generating a negative feedback regulation of the Nrf2 response (Jyrkkanen et al. 2011).

Bach2 is more specific to hematopoietic cells and developing neurons (Muto et al. 1998; Hoshino et al. 2002). Its expression is high in B lymphoid cells and effector T cells (Muto et al. 1998; Roychoudhuri et al. 2013; Tsukumo et al. 2013). It appears to be also expressed in subtypes of tissue macrophages such as alveolar macrophages (Nakamura et al. 2013). Bach2 expression in B cells is activated by the B cell-specific transcription factor Pax5 (Casolari et al. 2013). Considering the developmental stage-specific expression of Bach2 in B cells (Muto et al. 1998), other transcription factors seem to participate in the expression of Bach2 in B cells as well. Also, little is known for its regulation outside of B cells including T cells.

One of the important aspects of the regulation of Bach1 gene expression is the participation of multiple microRNAs (miRNAs). Kaposi's sarcoma-associated herpesvirus (KSHV) encodes miR-K12-11 that shares significant sequence homology with human miR-155. Ectopic expression of either miR-155 or miR-K12-11 repressed a

Bach1 3'UTR-containing reporter (Gottwein et al. 2007; Skalsky et al. 2007). miR-155 has an important role in the mammalian immune system, specifically in regulating T helper cell differentiation and the germinal center reaction to produce an optimal T cell-dependent antibody response (Thai et al. 2007). Considering the involvement of miR-155 and miR-K12-11 in the regulation of B cell function and transformation, it will be an interesting possibility that these miRNAs regulate the function and/or transformation of B cells by targeting Bach1 mRNA. miRNA let-7 represses the expression of Bach1 to promote metastatic responses of breast cancer cells (Yun et al. 2011). In addition, let-7 has been shown to induce the expression of HO-1 by reducing Bach1 mRNA (Hou et al. 2012). The occurrence of multiple miRNAs targeting Bach1 suggests that, while it is widely expressed, the level of Bach1 mRNA is finely tuned. In contrast to Bach1, little is known regarding miRNA-mediated regulation of the Bach2 mRNA.

#### Systemic roles of Bach1 and Bach2

The expression of Bach1 is widespread, including hematopoietic cells, fibroblastic cells and neurons (Oyake et al. 1996; Kanezaki et al. 2001). The well-established target genes for Bach1 regulation are the heme oxygenase-1 gene (*Hmox1*) and the  $\beta$ -globin gene. While the expression of Bach2 is considered to be rather specific to B cells and neurons (Muto et al. 1998; Hoshino and Igarashi 2002), recent studies have established that Bach2 is also expressed in T cells (Roychoudhuri et al. 2013; Tsukumo et al. 2013). The physiological and pathological roles of Bach1 and Bach2 in diverse cell types have been investigated using genetically engineered mice. Some of the inferences regarding the Bach2 function stem from the results of genome-wide association studies (GWAS) of patients with diseases of the immune system.

# Bach1 in the regulation of iron homeostasis and oxidative stress response

The human body contains roughly 3-4 g of iron, of which two-thirds is present in red blood cells (RBCs) as hemoglobin (Andrews 1999). Iron is vital for life, although it can be highly toxic due to its reactivity. Free iron, like free heme, catalyzes the Fenton reaction, generating ROS (Sheftel et al. 2012). Therefore, almost every cell is equipped with systems to handle iron overload and utilize its reactivity in safe ways. The principle of iron homeostasis is to maintain a sufficient level of iron within the cell and body while avoiding excess iron accumulation. Therefore, the human body takes up only a small amount of iron (1-2 mg/day), which is balanced by a basal obligatory loss of iron (1-2 mg/day) due to the exfoliation of cells from the skin and gastrointestinal tract. In contrast to the small amount of iron taken in and excreted by the body, erythropoiesis requires roughly 20-30 mg/day of iron. In order to meet these demands, vertebrates, including humans, recycle most of the iron in iron- or heme-containing molecules (primarily hemoglobin) following their degradation (reviewed in Andrews 1999; Immenschuh et al. 2010; Sheftel et al. 2012).

Macrophages play critical roles in maintaining the iron homeostasis. Iron is taken up by macrophages via diverse mechanisms in the form of iron, heme and hemoglobin. Senescent red blood cells are phagocytized by macrophages. Hemoglobin molecules released into the blood due to hemolysis are bound to haptoglobin and taken up by macrophages. Free heme released from hemoproteins, including hemoglobin, is bound to hemopexin and taken up by macrophages via receptor-mediated endocytosis (Hamza and Dailey 2012; Sheftel et al. 2012). At the end of the day, heme-protein complexes are degraded in the endosome-lysosome system, and heme is transferred to the cytoplasm where it is degraded by HO-1 and HO-2, which are bound to the cytoplasmic surface of the endoplasmic reticulum (Fig. 1). The transport of heme between the cytoplasmic compartment and other membrane compartments is dependent on specific heme transporters, including Hrg1 (Hamza and Dailey 2012). Following the degradation of heme, iron is either released to the outside of the cell by ferroportin or caged within ferritin in an inactive, storage form (Fig. 1).

Considering that Bach1 is a heme-regulated factor, it is not surprising that Bach1 participates in the regulation of iron metabolism at multiple steps. Among the molecules involved in these pathways, Bach1 represses the genes encoding HO-1 (Ogawa et al. 2001; Sun et al. 2002, 2004), ferritin (Hintze et al. 2007; Katoh et al. 2011) and ferroportin (Marro et al. 2010) (Figs. 1 and 7A). The two critical enhancers of Hmox1 are bound and repressed by Bach1small Maf heterodimers in diverse types of cells under normal conditions (Sun et al. 2002). When the intracellular level of heme is increased, Bach1 is inhibited, allowing for the activation of these iron-related genes, likely by transcriptional activators, such as Nrf2 (Fig. 7A). This system co-regulating genes for iron handling achieves a homeostatic link between input (heme) and output (iron). Therefore, it would be useful to compare iron homeostasis in wild-type and Bach1-deficient mice. In addition to the regulation of iron-related genes, Bach1 represses the expression of Spi-c, which encodes a transcription factor essential for the differentiation of macrophages dedicated for the iron recycling (e.g., red pulp macrophages) (Haldar et al. 2014). It appears that the expression of iron-related genes and the differentiation of macrophages are highly coordinated.

Recently, it has been suggested that human Bach1 does not regulate the same battery of genes as Nrf2 in keratinocytes when the cells are treated with electrophiles and redox-cycling agents (MacLeod et al. 2009). The clear target of Bach1 in this system is HO-1. A chromatin immunoprecipitation-sequence (ChIP-seq) analysis of Bach1 in HEK 293 human kidney cells has been reported (Warnatz et al. 2011), the results of which indicate that, in addition to known Bach1 targets involved in heme degradation (HMOX1, FTL, FTH1, ME1 and SLC48A1) and redox regulation (GCLC, GCLM and SLC7A11), Bach1 target genes include those affecting cell cycle and apoptosis pathways (ITPR2, CALM1, SQSTM1, TFE3, EWSR1, CDK6, BCL2L11 and MAFG) as well as subcellular transport processes (CLSTN1, PSAP, MAPT and vault RNA). It remains unclear how these new target genes of Bach1 are activated by Nrf2 or other related activators in response to heme and/or oxidative stress.

### Bach1 in the regulation of erythropoiesis and megakaryopoiesis

In higher eukaryotes, most iron is utilized to form hemoglobin. Approximately 30 mg of iron is used to form 200 billion red blood cells every day. Each RBC contains roughly a billion iron atoms in the form of heme (Knutson and Wessling-Resnick 2003). Therefore, developing erythroid cells synthesize a large amount of heme during differentiation. Considering the intrinsic toxicities of free (i.e., non-protein binding) iron and heme, erythroid cells must balance the amounts of synthesized heme and globin.

We previously suggested that Bach1 participates in the transcriptional regulation of the coordination of the synthesis of heme and globin in erythroid cells (Sun et al. 2004; Igarashi and Sun 2006). In this case, Bach1 binds to the locus control regions of both the  $\alpha$ - and  $\beta$ -globin genes (Brand et al. 2004; Sun et al. 2004; Tahara et al. 2004a, b). This repression appears to take place in the early stages of erythroid cell development in which the globin expression has yet to be induced. When the synthesis of heme increases due to the expression of the rate-limiting enzyme ALAS2, an increase in the heme level is believed to result in the inhibition of the activity of Bach1, thereby inducing the expression of the  $\alpha$ - and  $\beta$ -globin genes by the tissuespecific activator NF-E2 (Fig. 7B). These observations were made using the in vitro differentiation of leukemic erythroid cells. Therefore, it remains unclear how the system contributes to erythroid differentiation in vivo. Indeed, Bach1-deficient mice do not exhibit anemia or any other defect in erythropoiesis under normal conditions (Sun et al. 2002), leaving the model unproven. This conundrum may be explained by the presence of additional distinct mechanisms for regulating the heme levels in developing erythroid cells.

One well-studied system involves the heme-regulated eIF2 $\alpha$  kinase (HRI) (Chen 2007). HRI phosphorylates eIF2 $\alpha$  in order to suppress the translation of globin and other mRNAs. HRI is a heme receptor itself that is inhibited upon heme binding (Rafie-Kolpin et al. 2000). Therefore, when heme is sufficiently synthesized, the translation of globin is also stimulated in order to allow the rapid incorporation of heme into hemoglobin. Another interesting system involves the heme exporter FLVCR. FLVCR was recently cloned as a receptor for feline leukemia virus subgroup C, which induces red cell aplasia (Quigley et al. 2004). Subsequent studies established that FLVCR plays



Fig. 7. Regulation of heme oxygenase-1 and globin genes by the heme-Bach1 axis.
(A) The Bach1-small Maf heterodimer represses the expression of HO-1 and ferritin. An increased level of heme inactivates the repressor, resulting in their expression. This mechanism results in the coordination of degradation of heme and storage of potentially dangerous iron. (B) The Bach1-small Maf heterodimer represses the expression of globin prior to the terminal differentiation of erythroid cells. The induction of heme synthesis upon terminal differentiation results in the inactivation of Bach1 via direct heme binding, leaving the locus control region (LCR) available for the activator NF-E2 (heterodimer of small Maf and NF-E2 p45). This mechanism results in the coordination of the synthesis of globin and heme, both of which are potentially dangerous on their own.

an essential protective role in erythropoiesis by exporting excess heme, counteracting the toxicity of free heme (Keel et al. 2008). Hemopexin, an abundant heme carrier protein in the serum, accepts heme by directly interacting with FLVCR (Fig. 1) (Yang et al. 2010). FLVCR achieves a balance between heme and globin by exporting excess heme, preventing the toxicity of free heme. Therefore, HRI and/ or FLVCR1 may confer sufficient protection against free heme in *Bach1*-deficient mice. In addition to this prediction, Bach2 may be redundant to Bach1.

The transgenic overexpression of Bach1 in mice under the control of the GATA-1 locus hematopoietic regulatory domain results in megakaryocytic impairment (Toki et al. 2005). These mice display thrombocytopenia associated with impaired maturation of megakaryocytes, subsequently developing myelofibrosis. Considering that NF-E2 is critical for thrombopoiesis (Motohashi et al. 2010), these results suggest that Bach1 acts as a transcriptional repressor in the regulation of MARE-dependent genes in megakaryocytes as well.

### Bach1 as a potential therapeutic target in oxidative stressrelated diseases

While Bach1 is expressed in diverse types of cells, Bach1-deficient mice do not show any overt defects under normal conditions (Sun et al. 2002). However, heme oxygenase-1 (HO-1) is highly expressed in diverse tissues and cells in the absence of Bach1 (Sun et al. 2002). In normal mice, the expression of HO-1 is highly induced in response to diverse stimuli, such as oxidative stress, heat shock, heavy metals, inflammatory cytokines, lipopolysaccharide and its substrate heme (Tenhunen et al. 1970; Shibahara et al. 1978; Alam et al. 1989; Terry et al. 1998; Wijayanti et al. 2004). The induction of HO-1 is critical for the survival of cells and tissues under the harsh conditions. The mechanistic base underlying the protective role of HO-1 is twofold. First, HO-1 reduces the level of the free form of heme (i.e., non-protein binding), which mediates the Fenton reaction generating ROS. Second, two of the reaction products are endowed with cytoprotective activities. Biliverdin and bilirubin generate a cycle in which ROS are scavenged (Baranano et al. 2002; Gozzelino et al. 2010). CO mediates vasodilation and the regulation of cell proliferation as a gaseous second messenger (Morita et al. 1997; Brouard et al. 2000; Peyton et al. 2002; Gozzelino et al. 2010). The third reaction product is ferrous ion, which can catalyze the Fenton reaction, although ferric ions are usually managed by ferritin (Fig. 1).

As summarized above, the inducible expression of HO-1 is primarily regulated by the balance of the repressor Bach1-small Maf and the activator Nrf2-small Maf heterodimers. Nrf2 activates the expression of Bach1, which appears to constitute a negative feedback loop intended to prevent the overexpression of HO-1 following the resolution of oxidative stress (Jyrkkanen et al. 2011). Other transcription factors, such as NF-kB also play roles in the basal and inducible expression of HO-1, depending on the cell

type and inducing signal/stressor (Paine et al. 2010).

Since the induction of HO-1 is cytoprotective in multiple disease models (Gozzelino et al. 2010), *Bach1*-deficient mice, which are apparently healthy and overexpress HO-1 in diverse tissues and cells, are expected to exhibit an increase in tolerance in disease models. Over the past few years, this possibility has been investigated using diverse disease models considered to involve oxidative stress (Table 1).

Cardiovascular system: Bach1 appears to modulate the disease processes in the cardiovascular system. For example, the deletion of Bach1 in mice results in the marked upregulation of HO-1 proteins in the cardiovascular system, including cardiac and vascular smooth muscle cells (Omura et al. 2005). Bach1-deficient mice demonstrate the suppression of neointimal formation after vascular injury in vivo (Omura et al. 2005). Accordingly, the proliferative activity of Bach1-deficient vascular smooth muscle cells in vitro is suppressed (Omura et al. 2005). This phenomenon may explain the suppression of neointimal formation in Bach1-deficient mice. In another model of atherosclerosis, mice lacking the apolipoprotein E (Apo E) gene are fed a high fat diet. In this model, the presence of Bach1deficiency suppresses the development of atherosclerosis (Watari et al. 2008). The effects of Bach1 ablation on the area of plaque and other parameters are almost completely abolished by treating mice lacking both Bach1 and Apo E with Sn protoporphyrin, an inhibitor of the HO activity (Watari et al. 2008). Taken together, these observations suggest that Bach1 normally represses the expression of HO-1 in the arteries and that therapeutic interventions involving Bach1 may activate HO-1, thus resulting in the suppression of atherosclerosis.

Myocardial ischemia/reperfusion (I/R) injury offers a model of cardiac infarction. *Bach1*-deficient mice exhibit a higher expression of HO-1 before and after I/R than wild-

system/organ	effects of Bach1-deficiency	references
blood vessel	reduction of cuff injyury with reduced smooth muscle proliferation	(Omura et al, 2005)
	reduction in atherosclerosis in Apo E kockout mice	(Watari et al, 2008)
heart	reduction in I/R injury	(Yano et al, 2006)
	reduced hypertrophy after pressure overload	(Mito et al, 2008)
lung	reduced damages after high oxygen exposure	(Tanimoto et al, 2009)
intestine	reduced injury in small intestine after indomethacin administration	(Harusato et al, 2011; Harusato et al, 2009)
	reduction in trinitrobenzene sulfonic acid (TNBS)-induced colitis	(Harusato et al, 2013)
liver	reduction in steatohepatitis induced by methionine-choline deficient diet	(Inoue et al, 2011)
	reduced damages induced by D-galactosamine and lipopolysaccharide	(Iida et al, 2009)
pancreas	reduction of apoptosis of beta cells and less increase of blood glucose upon alloxan treatment	(Kondo et al, 2013)
intervertebral disc	reduced damages in annulus puncture model	(Ochiai et al, 2008)
spinal cord	reduced damages after mechanical injury and better locomoter activity	(Kanno et al, 2009; Yamada et al, 2008)

Table 1. Evaluation of Bach1-deficient mice in disease models.

type mice and reduced areas of infarction after I/R (Yano et al. 2006). Cardiac hypertrophy is a compensatory mechanism of the heart that functions to maintain cardiac output in the presence of sustained increases in the hemodynamic load, although it can lead to the development of cardiac failure. In a pressure overload model of cardiac hypertrophy, *Bach1*-deficient mice display lower cardiac weights as well as reduced fibrosis and wall thickening than wild-type mice; these alterations are cancelled by treating the mice with Sn-protoporphyrin (Mito et al. 2008).

Respiratory system: Hyperoxic lung injury is caused by the prolonged administration of fractional concentrations of oxygen greater than 50-60%. Toxic concentrations of  $O_2$ generate oxygen-derived free radicals that damage lung epithelial and endothelial cells. The HO-1 levels in the lungs of Bach1-deficient mice are significantly higher than those observed in WT mice before and after hyperoxic exposure (Tanimoto et al. 2009). Bach1-deficient mice survive for a longer period of time under hyperoxic conditions compared with wild-type mice. Furthermore, congestion and interstitial edema of the lungs are significantly reduced in Bach1deficient mice. Interestingly, these alterations are not dependent on the catalytic activity of HO-1, and an increased level of IL-6 has been suggested to provide protection against hyperoxia in Bach1-deficient mice (Tanimoto et al. 2009). Therefore, not all of the protective effects of Bach1 ablation can be accounted for by the actions of HO-1. Nonetheless, Bach1 represses the expression of HO-1 in neonatal mouse lungs (Kassovska-Bratinova et al. 2009), which may underlie the vulnerability of newborns to hyperoxia. It should be noted that Bach1 is transcriptionally induced under conditions of hypoxia to inhibit the expression of HO-1 in human cultured lung cancer cells (Kitamuro et al. 2003).

Digestive tract and glucose metabolism: In a model of small-intestinal mucosal injury induced by indomethacin, the degree of injury is improved in Bach1-deficient mice. A reduced level of apoptotic cells and a higher expression of HO-1 are observed in the intestines of these mice compared to wild-type mice (Harusato et al. 2009, 2011). In addition, the development of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis is suppressed in Bach1-deficient mice (Harusato et al. 2013), and the HO-1 expression is highly induced in the mucosa of these mice. Very interestingly, the alterations in the colitis model are further accompanied by changes in macrophages (see below). Steatohepatitis induced by the administration of a methionine-choline deficient (MCD) diet constitutes a model of nonalcoholic steatohepatitis (NASH). In this model, Bach1-deficient mice exhibit negligible amounts of hepatic steatosis compared to the pronounced steatohepatitis observed in wild-type mice (Inoue et al. 2011). Liver injury induced by the combination of D-galactosamine (GalN) and lipopolysaccharide (LPS) has also been reported to be reduced in Bach1-deficient mice (Iida et al. 2009).

Oxidative stress plays a key role in the progressive

deterioration of pancreatic  $\beta$ -cells during the development of diabetes. In a model of alloxan-induced pancreatic  $\beta$ -cell loss, *Bach1*-deficient mice show less apoptosis of  $\beta$ -cells than control wild-type mice (Kondo et al. 2013). Accordingly, their levels of blood glucose are lower than those of wild-type mice after alloxan treatment. In contrast, *Bach1*-deficiency does not demonstrate any protective effects in mice with high-fat diet-induced insulin resistance (Kondo et al. 2013). Therefore, *Bach1*-deficiency protects pancreatic  $\beta$ -cells from oxidative stress-induced apoptosis.

*Locomotor system:* Oxidative stress has been shown to be an important factor for intervertebral disc degeneration. In an age-related model of intervertebral disc degeneration, there are no significant differences between *Bach1*-deficient and wild-type mice (Ochiai et al. 2008b). However, in an annulus puncture model, histological scoring has revealed significantly reduced damage and increased numbers of HO-1-positive cells in *Bach1*-deficient mice.

Similar observations have been reported using a spinal cord injury model. After incurring mechanical injury, *Bach1*-deficient mice exhibit better locomotor activity, a higher expression of HO-1 in both the gray and white matter and reduced loss of neural cells compared with wild-type mice (Yamada et al. 2008; Kanno et al. 2009). Therefore, Bach1 may also be a target of therapy in the field of orthopedics. However, Bach1 ablation does not necessarily protect neural tissue from damage. In a cold injury model of the mouse cortex, *Bach1*-deficient mice demonstrate a higher and wider expression of HO-1 around the injured area, although they do not show any reductions in the degree of tissue damage (Sakoda et al. 2008).

*Skin:* Several observations suggest that the Bach1-HO-1 axis is involved in disease processes in human skin. The upregulation of HO-1 by ultraviolet A (UVA; 320-380 nm) irradiation of human skin cells protects the cells against oxidative stress. In addition, UVA irradiation of cultured human skin fibroblasts enhances the accumulation of Bach1 mRNA and proteins, which may function as a compensatory mechanism for controlling HO-1 overexpression (Zhong et al. 2010). While HO-1 is induced during keratinocyte differentiation, the HO-1 induction does not involve any changes in the expression of Bach1, suggesting that HO-1 induction upon keratinocyte differentiation is independent of the actions of Bach1 (Okada et al. 2010).

*Malaria:* Vulnerability to malaria is determined in part by HO-1 induction in response to heme released from infected red blood cells (Ferreira et al. 2011a). The presence of the microsatellite polymorphism in the HO-1 promoter has been shown to be correlated with the disease severity and outcomes of cerebral malaria (Takeda et al. 2005). Heme may play a role in the induction of HO-1 in response to malaria infection via the inactivation of Bach1.

*Pros and cons of Bach1 as a therapeutic target:* The results of the above experiments comparing the effects of Bach1 ablation in various disease models strongly suggest that the absence Bach1 or a reduction in its activity result in

less damage in the models examined. In many of the models, the involvement of HO-1 has been verified using inhibitors of its catalytic activity. Considering the fact that the induction of HO-1 is protective in diverse disease models (Gozzelino et al. 2010), these results appear to point to Bach1 as a promising therapeutic target. The absence of any particular defects in Bach1-deficient mice under normal conditions warrants Bach1-directed drug development. However, several precautions should be taken along this line. First, it remains possible that *Bach1*-deficient mice may suffer from disorders that have eluded conventional laboratory health checks. For example, because a subclinical dose of CO modulates the development of the neural system and affects memory (Cheng et al. 2012), detailed analyses of the higher function of the brain in Bach1deficient mice are needed. Second, although Bach1 binds heme, heme and/or related compounds may not be used as drugs, as they catalyze the production of ROS. Therefore, the heme-Bach1 interaction may not be exploited when designing leading compounds. Third, such drugs must be specific to Bach1, without affecting Bach2, since a drug targeting both Bach1 and Bach2 may induce severe side effects due to modification of the immune system (see below).

#### Bach2 and Bach1 orchestrate immunity

Several observations suggest that iron and heme are related to the function of the immune system. In the innate immune system, heme is essential for the production of highly reactive hydroxyl radicals, a bactericidal effector. Examples include NADPH oxidases, which are heme enzymes (Sumimoto 2008). The induction and absence of HO-1 have been shown to modify the immune system (Woo et al. 1998; Kapturczak et al. 2004; Takeda et al. 2004; Chauveau et al. 2005; McDaid et al. 2005; Matsushima et al. 2009; Tzima et al. 2009; Schumacher et al. 2012; So et al. 2012). However, the mechanisms underlying the HO-1mediated modulation of the immune system remain elusive (Soares et al. 2009). Recent findings showing that Bach2 and Bach1 play critical roles in the regulation of the immune system raise the interesting possibility that HO-1 affects Bach2 and/or Bach1 by reducing the intracellular heme level.

#### Regulation of acquired immunity by Bach2

"Delay-driven diversity" model for class switch recombination in B cells: The Bach2 expression is relatively specific to hematopoietic cells. Initially, the Bach2 expression was considered to be specific to B lymphoid cells (Muto et al. 1998). However, recent studies have shown that Bach2 is also expressed in T cells (Roychoudhuri et al. 2013; Tsukumo et al. 2013) and macrophages (Nakamura et al. 2013) to varying degrees, depending on the developmental stage of the cells. In B cells, Bach2 is required for the efficient diversification of the antibody function via class switch recombination (CSR) and the improvement of antigen-binding affinity via the somatic hypermutation (SHM) of antibody genes (Muto et al. 2004). Bach2-deficient mice produce higher levels of immunoglobulin M (IgM) but much less isotype antibodies, such as IgG, compared with wild-type mice under normal conditions as well as when challenged with synthetic antigens. The formation of a germinal center, in which both CSR and SHM take place in antigen-activated follicular B cells with help from helper T cells, is diminished in the absence of Bach2 (Muto et al. 2004). Activation-induced cytidine deaminase (AID) initiates the double strand breakage of DNA, thereby stimulating both CSR and SHM (Muramatsu et al. 2000). The expression of AID is abolished in Bach2-deficient B cells (Muto et al. 2004), suggesting that the Bach2-dependent expression of AID in activated B cells is critical for both CSR and SHM.

Bach2 participates in a gene regulatory network (GRN) of B cells and plasma cells in which two self-reinforcing modules are connected in order to counteract each other (Muto et al. 2010) (Fig. 8A). The balance of the two modules determines the identity of the B and plasma cells as well as the isotype of the antibodies secreted by the plasma cells (Fig. 8B). This model proposes that the kinetic regulation of the Blimp-1 expression by Bach2 determines whether activated B cells undergo CSR and is thus called the "delay-driven diversity model" (Muto et al. 2010). In one of the modules active in B cells, Pax5 dictates the identity of B cells, including the expression of Bach2. Bach2 directly represses the expression of Blimp-1, a master regulator of plasma cell differentiation, thereby suppressing the deployment of the plasma cell program (Ochiai et al. 2006, 2008a; Muto et al. 2010). Blimp-1 represses the expression of many B cell-associated genes, including Pax5 (Kallies et al. 2004; Sciammas and Davis 2004; Calame 2008). Therefore, Pax5 generates a self-reinforcing module by repressing Blimp-1 via Bach2. On the other hand, Blimp-1 activates IRF4, which then activates Blimp1, thus generating another reinforcing module (Sciammas et al. 2006). This architecture of GRN provides a critical function for CSR. Usually, a mutually repressing GRN behaves like a toggle switch with a swift on and off dynamic. In contrast, mathematical modeling predicts that the presence of Bach2 between Pax5 and Blimp-1 allows for a longer duration before the induction of Blimp-1, at which time the expression of Pax5 begins to decrease (Muto et al. 2010). This transient suspension of the Blimp-1 expression is critical for the expression of AID in activated B cells, as Blimp-1 represses the expression of AID, possibly by repressing the Pax5 expression (Lin et al. 2002) (Fig. 8B). Following the successful completion of SHM and CSR and a reduction in the Bach2 activity, activated B cells begin to express Blimp-1, terminally differentiating toward plasma cells (Fig. 8B).

When added to primary cultures of murine B cells, heme enhances the transcription of Blimp-1 and skews plasma cell differentiation toward the immunoglobulin M



Fig. 8. Regulation of antibody class switching and plasma cell differentiation via the Bach2 gene regulatory network.
(A) A gene regulatory network (GRN) consisting of the transcription factors Pax5, Bach2, Blimp-1 and Irf4 determine the stages of differentiation of B cells (Pax5<sup>hi</sup>, Bach2<sup>hi</sup>, Blimp-1<sup>low</sup>, Irf4<sup>low</sup>) and plasma cells (Pax5<sup>low</sup>, Bach2<sup>low</sup>, Blimp-1<sup>high</sup>, Irf4<sup>high</sup>). In addition to their mutual regulation, these transcription factors regulate other genes that are directly or indirectly related to the functions of B cells and plasma cells.

(B) The level of Bach2 activity dictates whether activated B cells undergo class switch recombination (CSR) prior to terminal differentiation into plasma cells. A higher level of Bach2 protein suspends the expression of Blimp-1, thereby allowing for the expression of AID. Based on the fact that the suspension of Blimp-1 induction is critical for CSR, this mechanism is called the "delay-driven diversity model."

(IgM) isotype, decreasing the IgG levels *in vitro* (Watanabe-Matsui et al. 2011). These observations raise the possibility that heme regulates humoral immunity by changing the activity of Bach2.

*Bach2 function in the pre-B cell stage:* In addition to SHM and CSR, Bach2 has been shown to regulate the early stage of B cell development (Swaminathan et al. 2013). Upon the recombination of the V-D-J region of the immu-

noglobulin heavy chain gene, pre-B cells with successful rearrangement become quiescent, while those with nonproductive rearrangement are eliminated. This phenomenon is termed the pre-B cell receptor (pre-BCR) checkpoint (Martensson et al. 2010). *Bach2*-deficient pre-B cells fail to activate p53, allowing those with non-productive rearrangement of the V-D-J genes to remain viable. Bach2 appears to directly activate the expression of p21 and other tumor suppressor genes (Swaminathan et al. 2013). In addition, Bach2 may modulate cell death in B cells, considering the findings of two previous reports showing that Bach2 promotes B cell death when overexpressed in several B cell lines, particularly in the presence of oxidative stress (Muto et al. 2002; Chen et al. 2013). Such activity may be related to the pre-BCR checkpoint.

Bach2 function in T cells: Bach2 is also emerging as an important regulator of T cells. CD4<sup>+</sup> T cells undergo functional diversification on the periphery in order to provide both effector and regulatory functions in the immune response. While the Bach2 expression is low in developing T cells, it is expressed at substantial levels in naïve CD4<sup>+</sup>, CD8<sup>+</sup> and regulatory T cells on the periphery (Roychoudhuri et al. 2013; Tsukumo et al. 2013). The development of T cells in the thymus remains largely unaffected by Bach2-deficiency in mice (Roychoudhuri et al. 2013; Tsukumo et al. 2013). Bach2 is required for the efficient generation of FoxP3<sup>+</sup> regulatory T cells (Treg) and thus the suppression of inflammation (Roychoudhuri et al. 2013). Bach2 appears to suppress a diverse set of genes that are important for the effector T cell function (Roychoudhuri et al. 2013; Tsukumo et al. 2013). Therefore, Bach2 suppresses the differentiation of effector T cells to support Treg differentiation. Consistent with this role, Bach2 maintains T cells in a naïve state by repressing the genes involved in the effector function (Tsukumo et al. 2013). The number of naïve T cells on the periphery is reduced in Bach2-deficient mice. Interestingly, one of the target genes of Bach2 in CD4+ T cells is Blimp-1 (Roychoudhuri et al. 2013; Tsukumo et al. 2013), which plays a role in the generation of effector T cells (Kallies et al. 2006; Martins et al. 2006). The Bach2-mediated repression of Blimp-1 is expected to suppress the terminal differentiation of CD4<sup>+</sup> T cells. Other target genes in T cells include Gata3, Irf4, Il12rb1, HO-1, S100a2 and Phd3, which are also important for the regulation of the CD4<sup>+</sup> T cell function and differentiation (Tsukumo et al. 2013).

#### Regulation of innate immunity by Bach2 and Bach1

*Bach2 in alveolar macrophages:* Bach2 regulates the activation of tissue macrophages (Nakamura et al. 2013). The lungs of *Bach2*-deficient mice gradually develop conspicuous pathological changes very similar to those observed in human pulmonary alveolar proteinosis (PAP) (Nakamura et al. 2013). PAP is characterized by the massive accumulation of lung surfactant proteins and lipids in the alveolar spaces, resulting in abnormal gas exchange.

PAP results from the dysfunction of alveolar macrophages (AMs), chiefly due to the disruption of signaling of granulocyte macrophage colony-stimulating factor (GM-CSF) (Trapnell et al. 2009). AMs in *Bach2*-deficient mice fail to handle surfactants and become foamy as the mice grow. While *Bach2*-deficient AMs exhibit a normal expression of the genes involved in GM-CSF signaling, they show an altered expression of the genes involved in chemotaxis, lipid metabolism and alternative M2 macrophage activation, with an increased expression of Ym1 and arginase-1, as well as the M2 regulator Irf4 (Nakamura et al. 2013). Therefore, Bach2 regulates the immune system at multiple steps, not only in B and T cells, but also in macrophages. Bach2 may therefore be a coordinator of innate and acquired immunity.

Role of Bach1 in innate immunity: Bach1 is also emerging as an important regulator of the immune system, more specifically in the development of macrophages and dendritic cells (DCs). Macrophages, DCs and B cells function as antigen-presenting cells (APCs), which are critical for mediating adaptive immunity to foreign antigens as well as inducing tolerance to self-antigens. Bach1-deficient mice demonstrate partial protection from the development of autoimmune diseases in an experimental model of autoimmune encephalomyelitis (EAE) (So et al. 2012), the murine model of human multiple sclerosis (MS). This phenotype is accompanied by defective peripheral T-cell responses in mice. However, unlike Bach2, the absence of Bach1 does not appear to affect the intrinsic T-cell function, but rather impairs the development of macrophages and DCs. One critical target gene in this regulatory pathway is HO-1, as mice deficient for both Bach1 and HO-1 exhibit defects in the development of the mono-macrophage system as early as the stage of macrophage-DC precursors (MDPs) (So et al. 2012). Bach1-deficient murine bone marrowderived macrophages are partially resistant to RANKLdependent HO-1 reduction and show impaired osteoclastogenesis in vitro (Hama et al. 2012). Consistent with these results, the expression profiles of tissue macrophages identify Bach1 as a candidate regulator (Gautier et al. 2012). Taken together, Bach1 is required for the efficient and proper development of APCs and osteoclasts in addition to the iron-recycling macrophages.

# Association between Bach2 and immune-related human diseases

Consistent with the critical functions of Bach2 in the immune cells, genome-wide association studies (GWAS) suggest the involvement of Bach2 in the development of diverse immune system-related diseases, such as generalized vitiligo (Jin et al. 2012), asthma (Ferreira et al. 2011b), type 1 diabetes (Cooper et al. 2008; Plagnol et al. 2011), type 2 diabetes (Lee et al. 2011), Crohn's disease (Franke et al. 2010; Christodoulou et al. 2013) and celiac disease (Dubois et al. 2010). The utilization of *Bach2*-deficient mice in disease models related to these human diseases is

expected to provide insight into the causal links and mechanistic basis of these conditions. An altered function of Bach2 may predispose affected individuals to developing immune-related diseases, possibly due to an altered regulatory T cell function. In contrast to that observed for Bach2, no GWAS reports have described an association between Bach1 and human diseases.

### Senescence and the senescence-associated secretory phenotype (SASP) as possible modulators of the immune function and tumorigenesis

One interesting possibility regarding the function of Bach1 in the immune system is that Bach1 also regulates the immune response outside of the immune system via its role in cellular senescence. Cellular senescence is a form of permanent cell cycle arrest which is dependent on the tumor suppressors p53 and/or pRb (Campisi et al. 2011; Larsson 2011; Liu and Xu 2011). Because cellular senescence is induced in response to DNA damage and oncogene activation, it is considered to be a barrier precluding the proliferation of cells with genetic damage and therefore an important mechanism of tumor suppression (Campisi et al. 2011; Larsson 2011; Liu and Xu 2011). Recent research has identified the senescence-associated secretory phenotype (SASP) as another functional factor of cellular senescence (Campisi et al. 2011). SASP is a phenotype in which senescent human fibroblastic cells secrete a diverse array of cytokines and chemokines, including Il-6, -7, MCP-2, GRO and HGF (Coppe et al. 2008). Senescent cells are believed to induce local inflammation and immune responses (Hoenicke and Zender 2012).

Bach1 suppresses the onset of cellular senescence in murine embryonic fibroblastic cells (MEFs) by forming a complex with p53 that includes histone deacetylase 1 (HDAC1), thereby promoting the histone deacetylation and repression of a subset of p53 target genes involved in cellular senescence (Dohi et al. 2008; Ota et al. 2011). The Bach1-p53 interaction is decreased by the tumor suppressor p19Arf, raising the possibility that the tumor suppressor activity of p19Arf involves the dissociation of p53 and Bach1 (Nishizawa et al. 2012). In addition to genes directly involved in cell cycle arrest, several cytokine genes, including that of IL-6, are more highly expressed in Bachldeficient senescent MEFs than in wild-type senescent MEFs (our unpublished observations). Therefore, it will be an interesting line of research to investigate the interactions between immune cells and non-immune cells in Bach1deficient mice in the context of SASP.

These observations suggest that Bach1 facilitates cell transformation by suppressing senescence. Consistent with this idea, *Bach1*-deficient MEFs are resistant to transformation by the H-Ras<sup>V12</sup> oncogene and less susceptible to 4-nitroquinoline-1-oxidide (4-NQO)-induced tongue carcinoma than wild-type mice (Nakanome et al. 2013). These results suggest that the development of some cancers depends on Bach1 to modulate the gene expression pattern

in a favorable way for transformation. Bach1 has been characterized as a key metastatic gene located downstream of the RKIP-Let-7 pathway (Yun et al. 2011; Lee et al. 2014). When the tumor suppressor RKIP is inactivated, the processing of Let-7 microRNA is decreased, resulting in a higher expression of its target gene, Bach1 (Yun et al. 2011). This observation also suggests that Bach1 plays a role as a factor facilitating tumorigenesis. Therefore, Bach1 appears to affect tumorigenesis in multiple ways, and suppression of the SASP phenotype by Bach1 may be relevant to this process.

### **Mitotic Bach1 function**

Bach1 is endowed with another interesting, enigmatic capacity in mitotic chromosome alignment during metaphase that is mechanistically independent of its role in gene regulation. Knockdown of Bach1 in HeLa cells results in disordered mitotic chromosome alignment, including a thicker mitotic chromosome plate, collapsed ring-like shape in the presence of monastrol and oscillating, unstable chromosome axis (Li et al. 2012). These phenotypes are rescued not only by murine Bach1, but also by its derivative lacking the bZip DNA binding domain. Therefore, this particular Bach1 function is separable from its role in gene regulation (Li et al. 2012). CLS, the motif for Crm1dependent nuclear export, is critical for the mitotic Bach1 function. Because Crm1 is involved in mitotic chromosome alignment (Arnaoutov et al. 2005), Bach1 may function in mitosis by interacting with Crm1. Therefore, Crm1 appears to regulate the functions of Bach1 in both the interphase (i.e., gene regulation) and M phase (chromosome alignment). Considering that the nuclear export of Bach1 by Crm1 is sensitive to heme and redox (Suzuki et al. 2003, 2004), one may wonder whether the mitotic Bach1 function is tuned in response to such signals. A Bach1-interactor IHABP (a.k.a, HMMR) has been reported to participate in the regulation of mitotic spindle orientation (Yamasaki et al. 2005; Dunsch et al. 2012). Therefore, Bach1 may participate in determining the mitotic spindle orientation together with IHABP/HMMR. The physiological and pathological significance of the mitotic Bach1 function awaits further study. It will also be important to examine whether Bach2 is also involved in mitotic regulation.

#### "Iron immunology," a perspective on heme

Heme metabolism and immune cell responses appear to constitute distinct features of higher eukaryotes. Why are these events regulated by Bach1 and Bach2? One interesting possibility is that these two events are actually connected in our system. Taking into the account the fact that iron and heme are critical resources for colonizing microorganisms (Hammer and Skaar 2011; Correnti and Strong 2012; Hamza and Dailey 2012), the connection between heme metabolism and immune cell responses may be summarized as "iron immunology."

Every cell contains heme, meaning that, when a cell



plays a new role as a signaling molecule. Necrotic cells release heme, which is then taken up by immune cells via specific heme trafficking systems, such as that involving hemopexin and its receptor. Cell debris is phagocytized by macrophages in which heme inhibits Bach1 or Bach2 in order to alter the macrophage function. Alternatively, heme may be exported by the exporter FLVCR to affect surrounding immune cells.

dies, it leaves behind heme. In innate immunity, some of the components of cells are sensed by other molecules, emitting signals for danger (danger signals or damage-associated molecular patterns (DAMPs)) in order to activate the immune cell function (Gallo and Gallucci 2013). Considering that Bach1 and Bach2 regulate the function of macrophages, heme may tune immunity by affecting these transcription factors (Fig. 9). In the germinal center, in which Bach2 is important (Muto et al. 2004), many proliferating cells eventually undergo apoptosis (Peperzak et al. 2012). What happens to the heme they contain? Of course, macrophages handle the dead cells; however, is it also possible that they secrete heme after degrading heme proteins? This idea may well be supported by the expression of the heme exporter FLVCR in macrophages (Keel et al. 2008). Once extracellular, heme may be bound by heme chaperons, such as hemopexin, and taken up by immune cells. Even if macrophages do not excrete heme, heme derived from apoptotic cells surely affect the function of macrophages by altering heme receptors, such as Bach1 and Bach2. Therefore, an analysis heme trafficking at the site of infection and in lymphoid tissue may reveal a novel communication network between macrophages and B and T cells mediated by heme.

Small Maf-Bach1 heterodimers repress the gene expression by recruiting methionine adenosyltransferase II (MATII) to their target genes (Katoh et al. 2011; Kera et al. 2013). MATII synthesizes *s*-adenosylmethionine (SAM), which serves as a methyl donor for the methylation of diverse molecules, including DNA and histone. The recruitment of MATII by small Maf-Bach1 is expected to facilitate the localized, efficient methylation of the epigenome (the "local synthesis for local consumption" model) (Igarashi and Katoh 2012). This model suggests the presence of cross-talk among the pathways of heme and SAM at the level of the gene expression.

Therefore, there may lie an interesting and fruitful field at the intersection of heme, immunity and metabolism. Heme may be a signaling molecule regulating diverse aspects of higher eukaryotes, including humans, ranging from the oxidative stress response and erythroid differentiation to both innate and acquired immunity.

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#### **Conflict of Interest**

The authors declare no conflict of interest.

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