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Heme as a Differentiation-Regulatory Transcriptional Cofactor

Ruiqi Liao[1], **Emery H. Bresnick**[1]

[1]Wisconsin Blood Cancer Research Institute, Department of Cell and Regenerative Biology, Carbone Cancer Center, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

Abstract

The hematopoietic transcription factor GATA1 induces heme accumulation during erythropoiesis by directly activating genes mediating heme biosynthesis. In addition to its canonical functions as a hemoglobin prosthetic group and enzyme cofactor, heme regulates gene expression in erythroid cells both transcriptionally and post-transcriptionally. Heme binding to the transcriptional repressor BACH1 triggers its proteolytic degradation. In heme-deficient cells, BACH1 accumulates and represses transcription of target genes, including α- and β-like globin genes, preventing the accumulation of cytotoxic free globin chains. A recently described BACH1-independent mechanism of heme-dependent transcriptional regulation is associated with a DNA motif termed heme-regulated motif (HERM), which resides at the majority of loci harboring heme-regulated chromatin accessibility sites. Progress on these problems has led to a paradigm in which cell type-specific transcriptional mechanisms determine the expression of enzymes mediating the synthesis of small molecules, which generate feedback loops, converging upon the transcription factor itself and the genome. This marriage between transcription factors and the small molecules that they control is predicted to be a canonical attribute of regulatory networks governing cell state transitions such as differentiation in the hematopoietic system and more broadly.

Introduction

Heme, the iron-containing protoporphyrin IX, is essential for critical cellular processes, including oxygen transport, electron transfer and catalytic reactions (1). Mechanisms governing heme synthesis, transport and degradation establish and maintain intracellular heme homeostasis. As both excessive and inadequate accumulation of heme are deleterious, heme homeostasis must be tightly regulated as a vital physiological requirement (2, 3)

In mammalian cells, heme is synthesized by multi-step enzymatic reactions that occur in mitochondria and the cytoplasm (3). The first and rate-limiting step of heme biosynthesis is catalyzed by δ-aminolevulinic acid synthase (ALAS), encoded by the ubiquitously expressed *ALAS1* and erythroid-specific *ALAS2* genes, respectively (4). In erythroid cells, the transcription factor GATA1, which is required for erythropoiesis, activates *ALAS2* transcription and heme production via two intronic enhancers (5). Human *ALAS2* intron 1

Correspondence: Emery H. Bresnick, Department of Cell and Regenerative Biology, Carbone Cancer Center, University of Wisconsin School of Medicine and Public Health, 1111 Highland Avenue, 4009 WIMR, Madison, WI 53705; ehbresni@wisc.edu.

enhancer mutations disrupt heme synthesis and cause X-linked sideroblastic anemia (6, 7). Disruption of the murine intron 1 enhancer abrogates GATA1 occupancy, decreasing *Alas2* expression and heme synthesis, thus causing severe anemia and embryonic lethality (8).

In addition to its canonical function as a prosthetic group in metabolic enzymes in all cell types and hemoglobin in erythroid cells, heme regulates gene expression at both transcriptional and translational levels by engaging heme-sensing effector proteins. A variety of heme-sensing proteins have been identified in prokaryotes and eukaryotes that regulate heme homeostasis and fundamental cellular processes (9, 10). In erythroid cells, heme deficiency promotes autophosphorylation and activation of Heme Regulated Inhibitor (HRI), a kinase that phosphorylates and inactivates the translation initiation factor eIF2α (11, 12). Phosphorylation of eIF2α inhibits protein translation, with one important consequence being to restrict α- and β-globin mRNA translation, thus preventing proteotoxicity instigated by the excessive accumulation of heme-free globin chains during iron and heme deficiency (13). Excessive free globin chains form cytotoxic aggregates, analogous to protein aggregation characteristic of certain neurodegenerative disorders. Beyond HRI-mediated translational inhibition, the heme-regulated transcriptional repressor BTB and CNC homology1 (BACH1) provides an additional line of defense to prevent cytotoxic aggregates. In cells with low heme, BACH1 represses globin gene transcription (14, 15) to ensure an appropriate heme and globin stoichiometry. In this review, we discuss existing paradigms and recent progress that has extended and transformed paradigms for how heme utilizes BACH1-dependent and –independent mechanisms to regulate transcription during erythropoiesis and therefore to sustain a vigorous process of erythrocyte generation required for normal physiology and organismal survival.

BACH1-Dependent Transcriptional Regulation by Heme

BACH1 contains an N-terminal BTB domain that mediates protein-protein interactions and a C-terminal basic leucine zipper (bZip) domain mediating sequence-specific DNA binding (Figure 1a). Like other bZip transcription factors, e.g., NF-E2 and NRF2, BACH1 heterodimerizes with small Maf proteins such as MafF, MafG, and MafK. BACH1/Maf heterodimers bind a subset of the Maf recognition elements (MARE) in the genome to repress transcription (16). BACH1 contains 6 cysteine-proline (CP) dipeptide motifs (17) that mediate heme binding within heme regulatory motifs (HRM) of other heme-regulated proteins including ALAS (18, 19) and HRI (20). Biochemical analyses revealed that heme directly binds BACH1 via 4 of the 6 CP motifs (21). Heme binding inhibits BACH1-MafK heterodimer DNA binding and transcriptional regulatory activity without disrupting the BACH1 – MafK protein-protein interaction (17, 22). Heme inhibits BACH1-dependent repression of *Hmox1* transcription by preventing BACH1 binding to the MARE motifs in *Hmox1* enhancers. In *Bach1* knockout mice, *Hmox1* expression is elevated in various tissues, providing evidence that BACH1 represses *Hmox1* transcription *in vivo* (23). Similarly, BACH1 inhibits α- and β-like globin gene transcription by binding to MARE motifs within DNaseI hypersensitive sites within the α- and β-like globin gene clusters (24). Treating cells with hemin reduces BACH1 protein levels, elevating α- and β-like globin transcription (14, 15). The BACH1-dependent repression of α- and β-like globin gene transcription ensures a physiological stoichiometry of heme relative to globin chains, thus

preventing the cytotoxic accumulation of heme-free globin chains in heme-deficient cells (13).

Beyond inhibiting DNA binding, heme regulates BACH1 activity by facilitating BACH1 nuclear export and promoting its proteasomal degradation. Hemin induces the cytoplasmic translocation of BACH1 (25), which is independent of the BTB domain that mediates protein-protein interactions or the cytoplasmic localization signal that mediates cadmium-dependent nuclear export of BACH1 (26). Two of the six CP motifs that mediate heme binding are critical for heme-dependent nuclear export of BACH1. This process requires the broadly utilized nuclear exporter protein XPO1, also known as CRM1 (25).

Zenke-Kawasaki et al. (27) demonstrated that hemin treatment of cells reduces the BACH1 protein level in a dose-dependent manner, and proteasomal inhibition with MG-132 counteracts the downregulation. Using heme-deficient proerythroblast-like G1E-ER-GATA1 cells, Tanimura et al. (5) demonstrated that *Bach1* RNA expression is unaffected by heme, while BACH1 protein only accumulates in heme-deficient cells. The E3 ubiquitin ligase HOIL1 (encoded by *Rbck1*) induces BACH1 polyubiquitination *in vitro*. HOIL1 is a component of the linear ubiquitin chain assembly complex (LUBAC) (28), and human *RBCK1* mutations cause autoimmunity and inflammatory disease (29, 30). Upon co-expression of BACH1 and HOIL1 in NIH 3T3 cells, BACH1 interacts with HOIL1. A dominant-negative HOIL1 mutant delays BACH1 degradation in MEL cells, suggesting that HOIL1 might mediate BACH1 polyubiquitination and degradation (27). Subsequent studies identified FBXL17 (31) or FBXO22 (32) as an E3 ubiquitin ligase that mediates BACH1 degradation in the cervical cancer cell line HeLa or the lung cancer cell lines KP or A549, respectively. Thus, heme-dependent BACH1 degradation might involve tissue-specific E3 ubiquitin ligases that operate in a context-dependent manner (Figure 1b).

While studies in diverse cell types have analyzed BACH1-linked biochemical and genetic mechanisms, only recently have insights emerged on how BACH1 negotiates the numerous MAREs in the genome to control genome function. In addition to the BACH1 ChIP-seq analysis in human K562 erythroleukemia cells and H1 human ES cells from the ENCODE project (33), Warnatz et al. (34) combined BACH1 ChIP-seq and microarray in HEK293 cells with BACH1 knockdown and identified BACH1 target genes linked to cell cycle regulation and apoptosis. Matsumoto et al. (35) analyzed BACH1 function in immortalized mouse embryonic fibroblasts (iMEF) and discovered multiple BACH1 target genes involved in lipid metabolism, including *Pparg*, encoding the master regulator of adipocyte differentiation PPARγ. *Bach1* deficiency promotes the differentiation of iMEFs to adipocytes in the presence of PPARγ ligands, suggesting that BACH1 regulates adipocyte differentiation through PPARγ. Ebina-Shibuya et al. (36) analyzed BACH1 and BACH2 targeting in the M1 murine myeloid leukemia cell line and demonstrated that BACH1 and BACH2 regulate genes involved in the inflammatory response. Unlike BACH1, which is expressed in erythroid and myeloid cells, BACH2 is expressed primarily in lymphoid cells and regulates B- and T-lymphocyte differentiation and function (37). BACH2 has 5 CP motifs and binds heme via an intrinsically disordered region (38). Heme binding inhibits BACH2 DNA binding *in vitro* and promotes its degradation in B cells (39). Analyses of *Bach1* and *Bach2* knockout mice revealed that both transcription factors promote

erythropoiesis, while suppressing myelopoiesis by reducing expression of C/EBPβ and its target genes in myeloid progenitor cells (40). Although the studies highlighted above identified BACH1 target genes in non-erythroid cells, how heme regulates the expression of these genes remains unclear.

BACH1-Independent Transcriptional Regulation by Heme

To systematically analyze heme-dependent transcriptional mechanisms during erythropoiesis, Tanimura et al. (5) utilized the murine G1E-ER-GATA1 cell genetic complementation system (41, 42). These GATA1-null proerythroblast-like cells stably express a conditionally active allele of GATA1 fused to the ligand binding domain of the estrogen receptor. In the absence of β-estradiol, ER-GATA1 resides in an inactive state, and β-estradiol activates ER-GATA1, instigating an erythroid gene expression program and erythroid maturation (43, 44). CRISPR-Cas9 was used to engineer a mutant G1E-ER-GATA1 cell line, in which the WGATAR motifs within two intronic enhancers of *Alas2* were mutated (5). The mutant cells contain 30-fold lower heme in comparison to wild-type cells after 48 h of β-estradiol treatment. Supplementing the mutant cells with 5-aminolevulinic acid (5-ALA), the small molecule product of ALAS enzymatic activity, restored heme biosynthesis. RNA-seq analyses of β-estradiol-induced wild-type, mutant, and 5-ALA-treated mutant cells revealed heme-regulated genes during erythroid maturation. While 1340 genes are heme-regulated (differentially expressed between wild-type and mutant cells), 1033 of which are also GATA1-regulated; 94 are rescued by 5-ALA. Of the 94 GATA1/heme/5-ALA-regulated genes, 66 are up-regulated by both GATA1 and heme, suggesting that heme facilitates GATA1 activity in these contexts (5).

Depleting Bach1 with shRNA in *Alas2* enhancer mutant, heme-deficient cells restores the expression of select heme-regulated genes, raising the possibility that additional mechanisms, potentially not involving Bach1, contribute to dysregulated gene expression in this system (5). The BACH1-sensitive genes include established BACH1 targets, such as *Hba-a1* (14), *Hbb-b1* (15), and *Slc7a11* (34), and genes not known to be BACH1 targets, including *Tbcel* and *Fbxo30*. By contrast, downregulating BACH1 did not affect expression of the heme-regulated genes *Sqstm1* and *Slc30a1*. Since *SQSTM1* is upregulated upon BACH1 knockdown in HEK293 cells (34), a given gene might be BACH1-regulated in certain, but not all, cellular contexts. The GATA1- and heme-activated gene *Slc30a1* encodes a zinc exporter that regulates intracellular zinc in erythroblasts. Zinc chelation, which reduces intracellular zinc, induces cell death in immature erythroblasts, while *Slc30a1* knockdown, which increases intracellular zinc, promotes terminal differentiation (45). Thus, the heme-dependent, BACH1-independent mechanism regulates the expression of genes critical for erythropoiesis.

To elucidate the mechanism of heme-dependent genomic regulation, Liao et al. utilized the assay for transposase-accessible chromatin sequencing (ATAC-seq) (46) to establish a genome-wide atlas of heme-regulated chromatin with wild-type and mutant (heme-deficient) G1E-ER-GATA1 cells. This study identified 11,340 GATA1-regulated ATAC-seq peaks and 1,122 heme-regulated peaks; 526 peaks are co-regulated by GATA1 and heme. The majority of the 526 GATA1/heme-co-regulated peaks are co-activated or co-repressed by GATA1

and heme, suggesting that heme amplification of GATA1 activity to regulate chromatin accessibility is the most common mechanism (47).

De novo motif finding in heme-regulated ATAC-seq peaks identified a DNA motif that resembles the BACH1 binding motif (35). Surprisingly, the BACH1 motif resides at less than 30% of the heme-regulated peaks. The application of *de novo* motif finding with peaks lacking BACH1 motifs revealed an AG-rich motif, which is deemed Heme-Regulated Motif (HERM). HERM resides at greater than 70% of heme-regulated peaks. Statistical analyses demonstrated that HERM is enriched in heme-regulated ATAC-seq peaks relative to random genomic regions or non-heme-regulated peaks (47).

Amalgamating ATAC-seq-detected accessible chromatin sites with RNA-seq data (5) revealed 169 genes regulated by heme at the RNA and chromatin levels (47). These genes conform to six regulatory modes based on the upregulation or downregulation of their expression by heme and GATA1, and whether a given gene contains BACH1 motif or HERM. Each regulatory mode contains genes with BACH1 motif or HERM. Thus, the BACH1 motif and HERM do not appear to predict the regulatory mode. Depleting BACH1 with shRNA in heme-deficient cells rescued the expression of BACH1-motif-containing, but not HERM-containing genes, in the group of GATA1- and heme-co-activated genes. These results are consistent with a model in which HERM and BACH1 function in parallel to facilitate GATA1-mediated transcriptional activation (Fig. 2).

The study described above provided evidence that HERM can mediate heme-dependent transcriptional repression that counteracts GATA1-mediated transcriptional activation (47). *Pklr* encodes the liver- and erythroid-specific pyruvate kinase essential for glycolysis (48), and *PKLR* mutations cause pyruvate kinase deficiency and nonspherocytic hemolytic anemia (49). *Pklr* expression is upregulated by GATA1, but further upregulated (by ~100 fold) in heme-deficient cells. shRNA-mediated BACH1 depletion does not affect *Pklr* expression, suggesting that *Pklr* is regulated by a BACH1-independent mechanism. *Pklr* contains an intronic heme-regulated ATAC-seq peak, which harbors two HERMs that are 29 bp apart. Disrupting the HERMs by CRISPR-Cas9 reduced *Pklr* expression in heme-deficient cells, but did not elevate *Pklr* expression in cells with normal heme, suggesting that HERM mediates the upregulated *Pklr* expression in a heme-deficient cell (Fig. 3a). Comparison of HERM to known motifs revealed similarity to a motif implicated in ZNF263 binding, which was identified from a ChIP-seq analysis in K562 cells (50). However, whether heme regulates binding of ZNF263 or another transcription factor to HERM requires future studies.

Heme exporters can contribute to intracellular heme content. Feline leukemia virus subgroup C receptor (FLVCR1), a member of the major facilitator superfamily of transporter proteins, is a human heme exporter (51). Depleting *Flvcr1* in mice elevates intracellular heme and is embryonic lethal, while neonatal depletion of *Flvcr1* causes macrocytic anemia (52, 53). Single-cell RNA-seq analysis in wild-type and *Flvcr1* knockout mice revealed that ribosomal protein gene expression is elevated in *Flvcr1*-deleted early erythroid cells. *Gata1* and its target genes are repressed in late erythroid cells lacking *Flvcr1* (54). Treating primary human bone marrow cells or K562 cells with 5-ALA, which elevates intracellular

heme, reduces GATA1 RNA and protein expression (54). Thus, GATA1 and heme operate in a feedback loop in which GATA1 elevates heme synthesis, and as heme accumulates, it initially facilitates GATA1 activity during early erythropoiesis (5, 45, 47). As heme levels increase further, this is associated with reduced GATA1 expression during terminal differentiation (54) (Figure 3b).

The heme-dependent eIF2α kinase, HRI, which inhibits the translation of globin mRNAs via eIF2α phosphorylation, induces ATF4 (55, 56), a basic leucine zipper (bZip) protein that mediates stress responses (57). RNA-seq analysis in wild-type and *Eif2ak1* (encoding HRI) knockout mice during iron deficiency revealed that the majority of genes elevated in wild-type mice with iron deficiency appear to be ATF4 targets involved in amino acid metabolism and synthesis, and these genes are not elevated in *Eif2ak1* mutant mice (58). Thus, the HRI-dependent increase of ATF4 in heme-deficient cells may mediate heme-dependent transcriptional repression (Figure 3c). ATF4 shares similar DNA binding motifs with other bZip transcription factors (59, 60). Interestingly, MafK, NFE2, BACH2, and AP-1 motifs, which involve related sequences, also reside within sequences encompassing ATF4 ChIP-seq peaks (60). In principle, ATF4 may function through Bach motifs in chromatin. Thus, since BACH1 is a transcriptional repressor that mediates heme-dependent gene activation, the heme-repressed genes might be regulated by ATF4, or perhaps a related bZip factor through BACH1 motifs in heme-deficient cells.

Conclusion

Heme exerts critical functions during erythropoiesis beyond its canonical role as a hemoglobin component. These functions include facilitating or antagonizing GATA1 function to establish and/or maintain the erythroblast transcriptome via BACH1-dependent and independent mechanisms (5, 45, 47). GATA1 upregulates heme synthesis and expression of the transcriptional repressor BACH1. Heme binding inhibits DNA binding, promotes nuclear export, and induces proteasomal degradation of BACH1, thus enhancing GATA1-dependent activation of genes, including globins and *Hmox1* (17, 23, 25, 27). Furthermore, heme can antagonize GATA1 activity by inhibiting GATA1 expression (54) or, in principle, by targeting the genome via the DNA motif HERM (47). HERM mediates heme-dependent activation or repression of genes that are essential for erythropoiesis, including the zinc exporter, *Slc30a1* and the metabolic enzyme, *Pklr* (45, 47). Major unsolved problems include whether HERM-dependent genome targeting involves sequence-specific transcription factor binding, how HERM-dependent and BACH1-dependent mechanisms interface, and how these transcriptional mechanisms are interlinked with heme-dependent post-transcriptional and post-translational mechanisms. As GATA1 activates *Alas2* and other genes encoding heme biosynthetic enzymes that generate heme, and heme functions in feedback loops to control GATA1 and the genome, we envision that diverse small molecules, including metals and metabolites, instigate such loops, which are likely to be vital components of GATA factor mechanisms that control cellular functional states, including differentiation (Figure 4).

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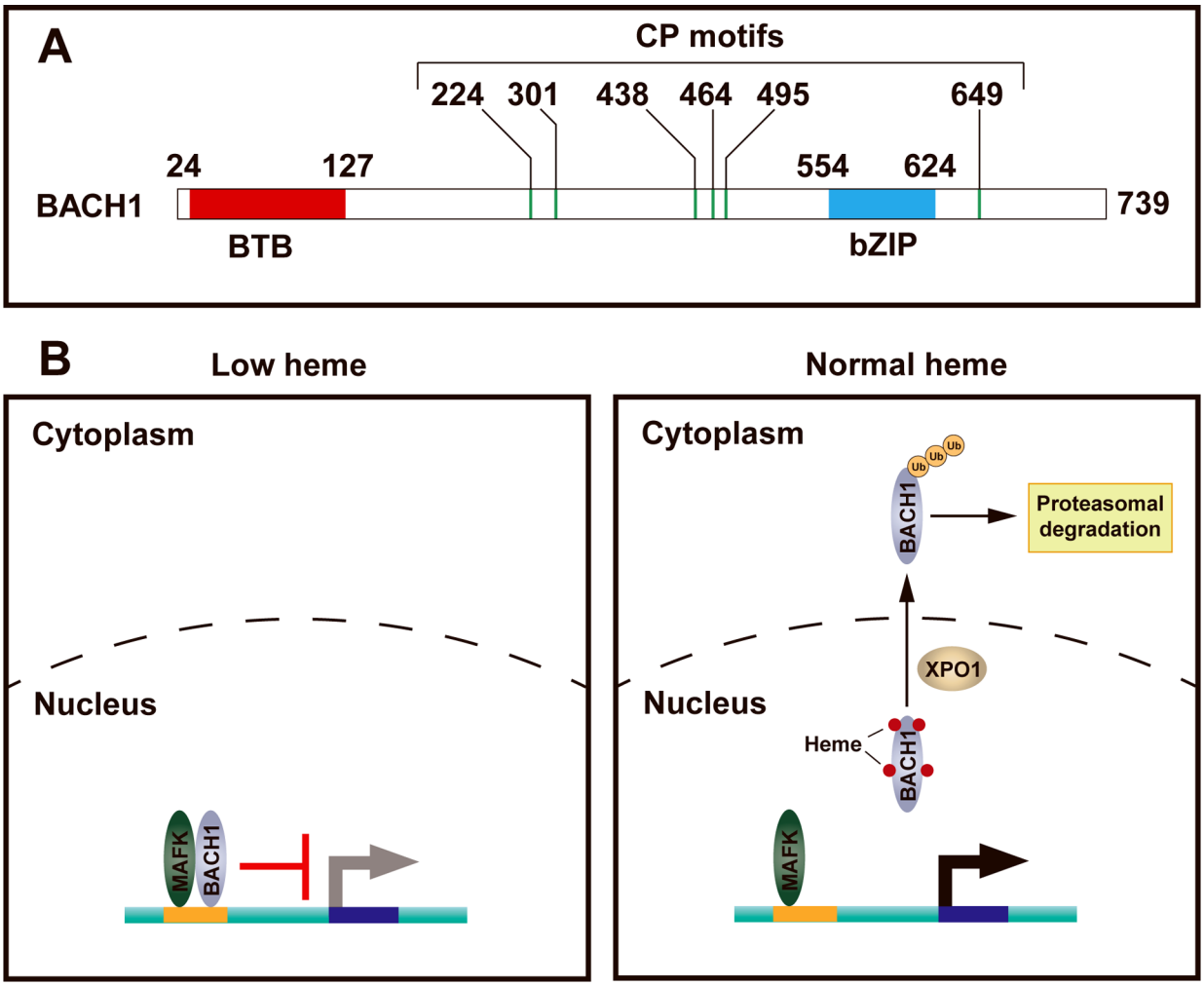

Figure 1. Heme post-translationally regulates BACH1 function.

A. Domain structure of murine BACH1 protein. BTB, bric-a-brac–tramtrack–broad complex domain; bZIP, basic leucine zipper domain; CP motif, cysteine-proline dipeptide that mediates heme binding.

B. Heme regulates BACH1 DNA binding, nuclear export and proteasomal degradation. In heme-deficient cells, BACH1 dimerizes with a small Maf protein and inhibits target gene transcription via BACH1 DNA motifs. Upon heme binding to BACH1, BACH1 dissociates from DNA, exits the nucleus into the cytoplasm, and following ubiquitination, it is degraded by the proteasome.

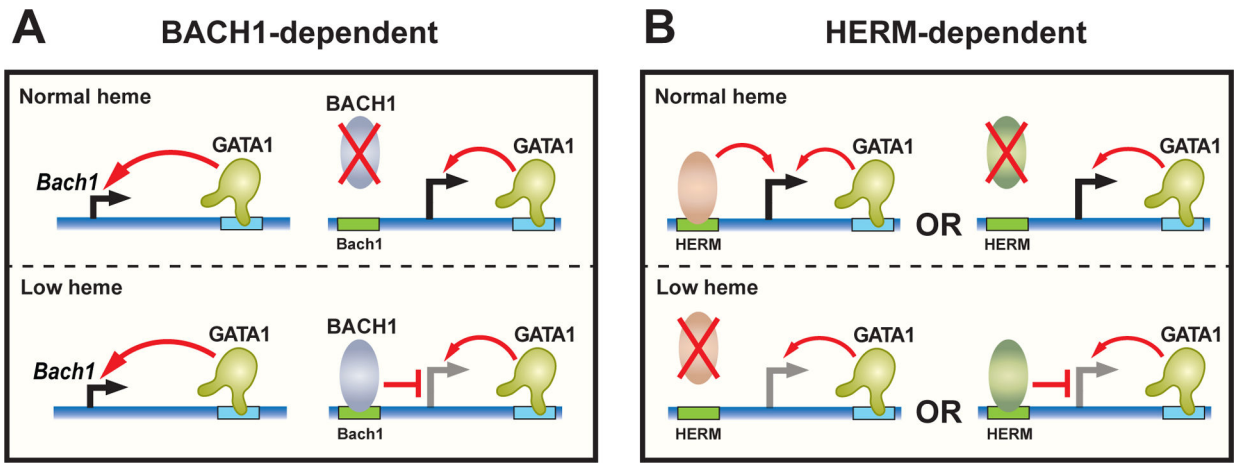

Figure 2. Context-dependent heme amplification of GATA1 activity: parallel BACH1-dependent and independent mechanisms.

A. Heme facilitates GATA1 activity via BACH1. GATA1 activates *Bach1* transcription. In heme-deficient cells, BACH1 protein accumulates, binds BACH1 DNA motifs, and opposes GATA1 activity to activate target gene transcription. In a normal heme environment, BACH1 protein proteolysis dominates over mechanisms that promote BACH1 synthesis, thus negating the mechanism that antagonizes GATA1.

B. Heme facilitates GATA1 activity via HERM. The presence of a HERM-binding transcriptional activator or the absence of a HERM-binding repressor in a physiological heme environment facilitates GATA1-mediated activation of target gene transcription.

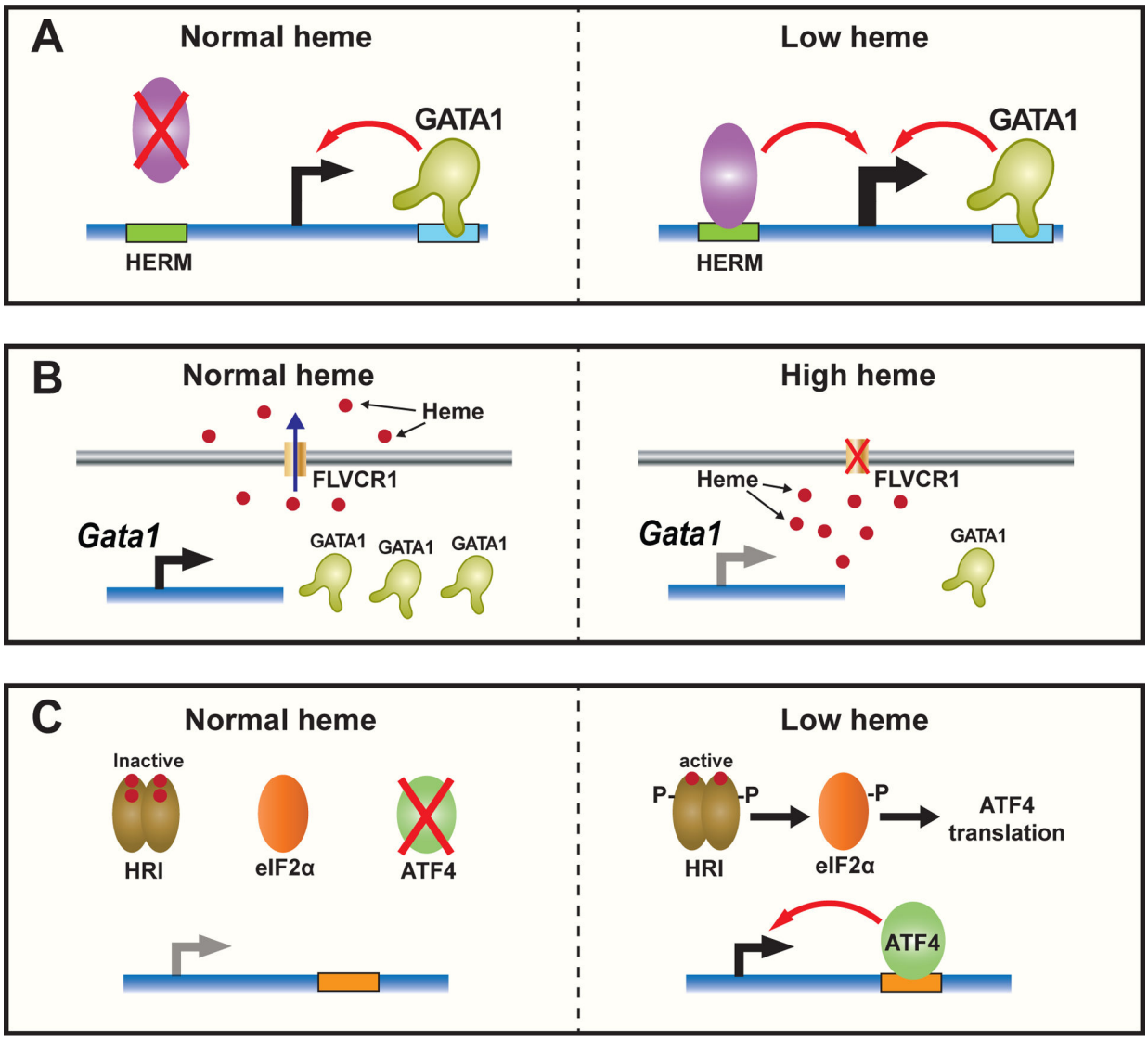

Figure 3. Context-dependent heme antagonism of GATA1.

A. Heme antagonism of GATA1 via HERM. Hypothetical mechanism of HERM-binding transcriptional activator that elevates GATA1 target gene transcription in heme-deficient cells, with heme opposing this mechanism.

B. Heme accumulation inhibits GATA1 expression. Depletion of Flvcr1, the plasma membrane heme exporter, increases intracellular heme, which inhibits *Gata1* RNA and GATA1 protein expression.

C. HRI-dependent induction of ATF4 activates transcription in heme-deficient cells. In a low-heme environment, HRI is activated and phosphorylates the translational elongation factor, eIF2α, which increases ATF4 translation, enabling ATF4 to activate target genes transcription.

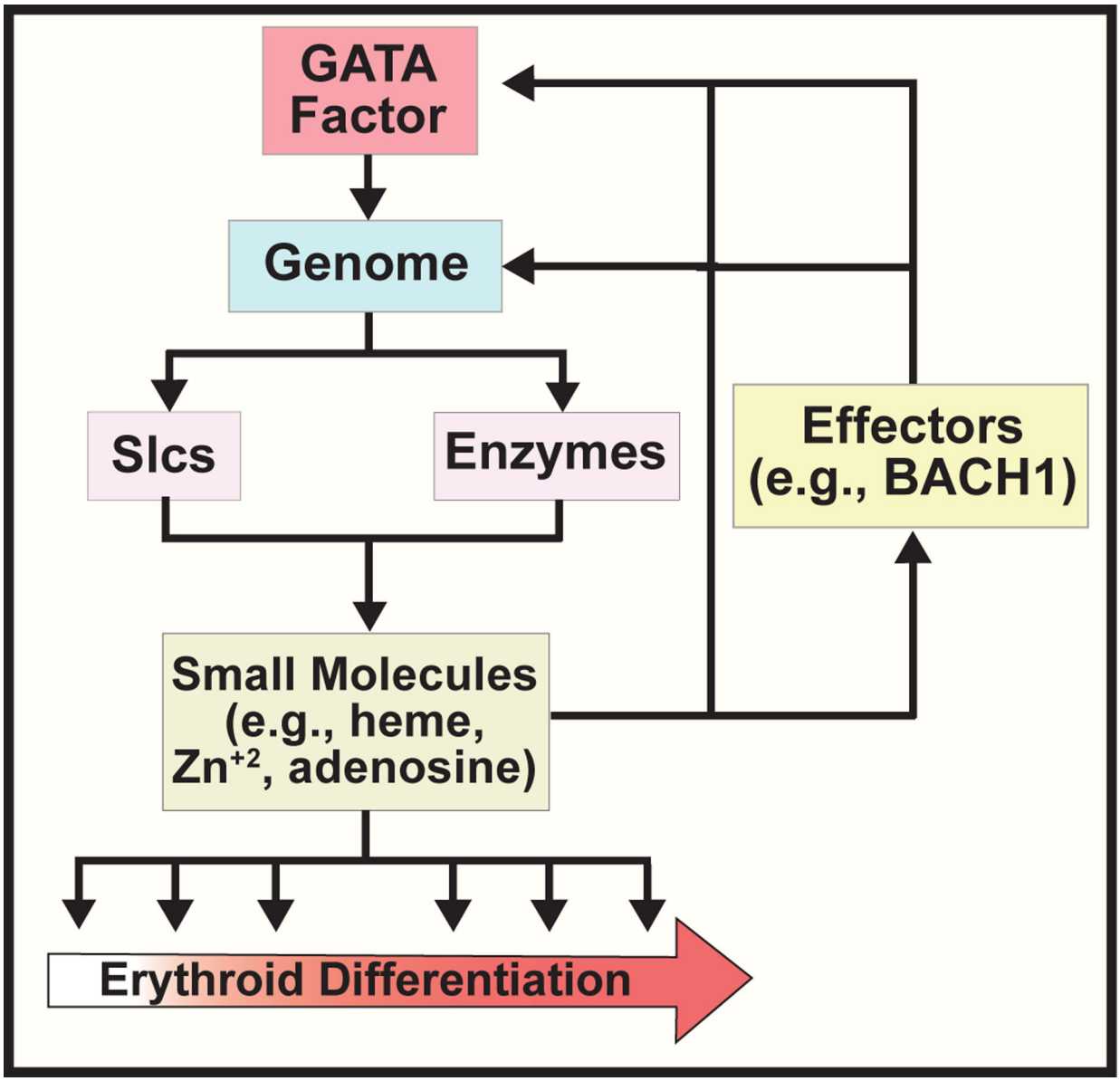

Figure 4. Feedback loops involving GATA factor, genome, and small molecules control cellular differentiation.

GATA factor dependent genome regulation controls the expression of transporters (SLCs) and biosynthetic enzymes for small molecules. The small molecules then function in a feedback loop to control GATA factor and genome function via protein effectors, e.g. BACH1.