Mechanism governing heme synthesis reveals a GATA factor/heme circuit that controls differentiation

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Abstract

Metal ion-containing macromolecules have fundamental roles in essentially all biological processes throughout the evolutionary tree. For example, iron-containing heme is a cofactor in enzyme catalysis and electron transfer and an essential hemoglobin constituent. To meet the intense demand for hemoglobin assembly in red blood cells, the cell type-specific factor GATA-1 activates transcription of Alas2, encoding the rate-limiting enzyme in heme biosynthesis, 5-aminolevulinic acid synthase-2 (ALAS-2). Using genetic editing to unravel mechanisms governing heme biosynthesis, we discovered a GATA factor- and heme-dependent circuit that establishes the erythroid cell transcriptome. CRISPR/Cas9-mediated ablation of two Alas2 intronic cis elements strongly reduces GATA-1-induced Alas2 transcription, heme biosynthesis, and surprisingly, GATA-1 regulation of other vital constituents of the erythroid cell transcriptome. Bypassing ALAS-2 function in Alas2 cis element-mutant cells by providing its catalytic product 5-aminolevulinic acid rescues heme biosynthesis and the GATA-1-dependent genetic network. Heme amplifies GATA-1 function by downregulating the heme-sensing transcriptional repressor Bach1 and via a Bach1-insensitive mechanism. Through this dual mechanism, heme and a master regulator collaborate to orchestrate a cell type-specific transcriptional program that promotes cellular differentiation.

Keywords Bach1; GATA factor; heme; network; transcriptome

Subject Categories Development & Differentiation; Transcription

Introduction

Metal ions regulate organismal development and homeostasis by controlling enzymes, sequence-specific DNA-binding proteins, and a plethora of cellular processes. While the role of metal ions in biochemical, physiological, and pathological regulation has received considerable attention, fundamental mechanistic and systems-level questions remain unanswered. For example, how do changes in metal ion availability impact transcriptomes and epigenomes that govern the establishment/maintenance of cell type-specific phenotypes and phenotypic plasticity?

Multiple transcriptional and posttranscriptional gene expression mechanisms are metal ion dependent. Through conformational transitions in the zinc- and heavy metal-binding metal-regulatory transcription factor 1 (MTF1), metal ions activate DNA binding [1]. Iron-sulfur clusters within certain transcription factors confer regulation by sensing oxidative stress and gases including nitric oxide [2]. Iron is a requisite cofactor for dioxygenases/histone demethylases that mediate gene regulation through chromatin modification [3,4]. Though heme iron has been studied extensively, based on its functions as an enzyme cofactor and a hemoglobin component in erythroid cells, heme is an important transcriptional regulator. Heme binding to the transcriptional repressor Bach1 stimulates its degradation via the proteasome [5]. In heme-deficient erythroid cells, Bach1 accumulates, occupies chromatin, and represses target gene transcription. In erythroid cells synthesizing vast amounts of hemoglobin, Bach1 is limiting, which favors globin gene expression and contributes to a balance between globin chain and heme biosynthesis [6].

Posttranscriptionally, EIF2α kinase (HRI) inhibits globin and heme biosynthetic enzyme translation upon heme deficiency. Heme binding to HRI opposes this mechanism [7]. In heme deficiency lacking HRI, globin chains accumulate, which elicits cytotoxicity. Iron-dependent translational control is also achieved through the
iron response element (IRE)/iron regulatory protein (IRP) system. IRP binding to IREs on mRNAs encoding ferritin and ALAS-2 represses translation in low iron [8], thus contributing to a balance between iron levels and heme biosynthesis.

Heme biosynthesis in erythroid cells requires GATA-1 [9,10], a master regulator of erythropoiesis [11,12]. GATA-1 activates transcription of genes encoding hemoglobin subunits, heme biosynthetic enzymes, including ALAS-2, and diverse red cell constituents [13–16]. Although heme downregulates the globin gene repressor Bach1 [5,17,18], whether heme has a vital role in establishing/maintaining the erythroid transcriptome is unknown.

It is instructive to consider how GATA-1- and heme-dependent mechanisms might converge to establish the erythroid cell transcriptome and constellation of erythroid cell phenotypes. We hypothesized that Alas2 transcription is regulated by two GATA-1-occupied cis elements that we identified based on sequence and chromatin attributes. CRISPR/Cas9-mediated excision of both Alas2 cis elements severely reduced Alas2 transcription, impairing heme biosynthesis. This system revealed GATA-1/heme-regulated genes that constitute an important sector of the erythroid cell transcriptome. While a subset of the GATA-1/heme-activated genes were Bach1 sensitive, a distinct cohort was Bach1 insensitive. GATA-1 strongly upregulated Bach1 transcription, and Bach1 accumulated only in heme-deficient cells. GATA-1 induction of globin chains, ALAS-2/heme biosynthesis, and Bach1, with heme repressing Bach1, constitutes a type I incoherent feed-forward loop, an essential component of a complex network that establishes/maintains the erythroid cell transcriptome. Our results establish the cis regulatory mechanism governing heme biosynthesis, a complex network in which heme interfaces with a GATA factor to establish/maintain a cell type-specific transcriptome, and a new molecular mechanism by which heme sculpts a transcriptome.

Results
Exploiting cis regulatory mechanisms to reengineer heme biosynthesis

A GATA-2-activated cis element (+9.5) within a Gata2 intron consists of an E-box-8-bp spacer-GATA motif [19–21]. Targeted disruption of the +9.5 in the mouse revealed its importance for activating Gata2 transcription in hemogenic endothelium and hematopoietic stem/progenitor cells (HSPCs), promoting hematopoietic stem cell (HSC) emergence in the aorta gonad mesenchros (AGM) region of the embryo, establishing the fetal liver HSPC compartment, and conferring vascular integrity [22,23]. A conditional Gata2 knockout using a +9.5 site-containing DNA segment driving Cre recombinase yielded similar fetal liver HSPC and vascular phenotypes [24]. “+9.5-like” cis elements share +9.5 sequence/chromatin attributes and mediate GATA-2-dependent activation of the associated gene [25].

Alas2 intron 8 contains a +9.5-like cis element (Fig 1A), and Alas2 is expressed in erythroid cells containing GATA-1, but not GATA-2. Although GATA-1 occupies +9.5-like elements [13,25], we are unaware of nonredundant GATA-1 function through such endogenous sites. As millions of GATA motifs reside in genomes [26–28], GATA motif function is not predictable based on established parameters, including chromatin occupancy. Since GATA-1 directly activates Alas2 transcription [29,30], and cis elements mediating GATA-1-dependent Alas2 activation were unknown, we tested whether GATA-1 functions through the Alas2 +9.5-like element in erythroid cells, analogous to GATA-2 function through the +9.5 in hematopoietic precursor cells. Another GATA binding cis element in Alas2 intron 1 contains a GATA motif, but lacks a +9.5-like composite element, and is associated with sideroblastic anemia [31,32]. ChIP-seq data revealed GATA-1 occupancy of intron 1 and 8 cis elements in erythroid cells, which harbor enhancer attributes (DNase hypersensitivity, histone H3 monomethylation at lysine 4, and Pol II occupancy) (Fig 1B).

To rigorously test whether the Alas2 cis elements are functionally important, we used CRISPR/Cas9 to generate erythroid cells lacking one or both cis elements. This analysis was conducted in G1E-ER-GATA-1 cells, normal proerythroblast-like cells derived from murine GATA-1-null embryonic stem cells [33]. G1E-ER-GATA-1 cells stably express physiological levels of a conditional GATA-1 allele (ER-GATA-1) encoding an estrogen receptor hormone binding domain fused to GATA-1 [34,35]. Estradiol activation of ER-GATA-1 induces rapid and synchronous erythroid maturation and recapitulates a physiological program in which proerythroblasts mature to orthochromatic erythroblasts prior to enucleation [16,33,35]. Alas2 resides on chromosome X, and G1E-ER-GATA-1 cells harbor one Alas2 allele. One sgRNA targeting intron 8 and two sgRNAs targeting intron 1 were designed and co-expressed in G1E-ER-GATA-1 cells with a Cas9-expression vector (Fig 1C). Clonal lines harboring intron 1 (int1 Mut1 and 2 obtained with sgRNA vector #4 and #1, respectively) (Fig 1D) or intron 8 mutations were generated (int8 Mut1 and 2). Double-mutant lines harboring deletions of intron 1 and intron 8 GATA motifs (int1/8 Mut1 and 2) were derived from int8 Mut1 cells transfected with intron 1-targeting sgRNA vector #4 and #1, respectively. Mutations were identified by sequencing genomic DNA amplicons (Fig EV1).

ER-GATA-1 activation in wild-type and intron 8 mutant cells induced erythroid maturation within 48 h, which involved a considerable reduction in cell size, concomitant with development of pink/red color, reflecting hemoglobinization (Fig 2A). β-estradiol treatment of double-mutant cells decreased cell size similar to wild-type cells, suggesting that the double-mutant cells were competent to undergo at least certain steps of erythroid maturation (Fig 2B). However, the intron 1 and double-mutant cells remained pale (Fig 2A). Since β-estradiol-treated double-mutant cells had a slightly different cytoplasmic morphology (Fig 2B), we quantitated cell proliferation in WT and double-mutant cells. The proliferative rate of untreated and β-estradiol-treated double-mutant and WT cells was indistinguishable (Fig 2C). Despite little or no difference in ER-GATA-1 protein levels (Fig 2D), induction of erythroid maturation with intron 1 and intron 1/8 double mutants was not associated with the expected large increase in Alas2 expression (Fig 2E, left). By contrast, the intron 8 mutation alone did not significantly alter ER-GATA-1-mediated induction of Alas2 expression (Fig 2E, left). However, in the context of the double cis element mutant, the intron 8 GATA motif contributed to the very low level Alas2 expression (Fig 2E, right), suggesting a cooperative function of the enhancers.

Although ChIP analyses indicated that GATA-1 occupied intron 1 and 8 sites, the intronic cis elements mediating GATA-1 occupancy of the Alas2 locus and induction of Alas2 transcription were
Figure 1. CRISPR/Cas9-mediated deletion of two GATA motif-containing intronic sites in Alas2.

A  Sequence alignment of GATA and E-box motifs in Alas2 intron 1 or 8 demonstrating conservation among mammals.

B  DNase hypersensitivity and ChiP-seq profiles for GATA-1 or Pol II occupancy and histone modifications at Alas2 and ALAS2 (accession numbers: GSM912907, GSM912895, GSM100374, GSM100373, GSM101491, GSM923572, GSM946526, GSM935465, and GSM935462).

C  CRISPR/Cas9 strategy to delete GATA motifs in intron 1 or 8 of Alas2 gene in G1E-ER-GATA-1 cells. PAM: Protospacer adjacent motif.

D  DNA sequences at Alas2 introns of wild-type (WT) and mutant clones.
unknown. We tested whether the cis element mutations abrogated ER-GATA-1 occupancy (Fig 2F). ER-GATA-1 occupied both sites in wild-type cells. The intron 1 GATA motif deletion abrogated ER-GATA-1 occupancy at intron 1, but not intron 8. The intron 8 GATA motif deletion abrogated intron 8, but not intron 1, occupancy. The double mutation abrogated ER-GATA-1 occupancy at both sites. As both cis elements mediated ER-GATA-1 occupancy, but only the intron 1 cis element contributed greatly to ER-GATA-1-dependent transcriptional activation, this analysis decoupled ER-GATA-1 occupancy from function.

Heme amplifies GATA-1-mediated transcriptional activation

GATA-1 activation of genes encoding heme biosynthetic enzymes and globin chains [36] illustrates the interconnectedness of mechanisms governing heme biosynthesis and globin expression. Sustaining globin production in a heme-deficient state and presumably sustaining heme biosynthesis with a dearth of globin chains elicits cytotoxicity [37]. Given the vital need to coordinate heme and globin production, the GATA-1 requirement for hemoglobin biosynthesis, and GATA-1 regulation of other erythroid cell constituents, we reasoned that heme biosynthesis may be more broadly linked to establishment/maintenance of the erythroid cell transcriptome. Defective heme biosynthesis would therefore alter diverse erythroid phenotypes. One facet of this link involves Bach1 upregulation upon heme deficiency and Bach1-mediated repression of β-like globin transcription [17]. Whether Bach1 counteracts GATA-1-mediated activation and whether this mechanism is restricted to β-like globin gene regulation or broadly impacts the erythroid cell transcriptome are unknown.

*Alas2* intron 1 and intron 1/intron 8 cis element mutations, which severely reduced ER-GATA-1-mediated activation of *Alas2* expression (Fig 2E), decreased expression of *Hbb-b1* and *Hba-a1* (Fig 3), encoding major forms of β- and α-globin, respectively. We assessed whether these mutations influenced expression of GATA-1 target genes encoding other erythroid cell constituents. ER-GATA-1-mediated activation of *Sle4a1*, encoding an erythroid cell anion transporter, was slightly less in single-mutant vs. control cells and greatly reduced (9-fold) in double-mutant cells (Fig 3). By contrast, intron 1 and the double mutations did not alter ER-GATA-1-mediated repression of *Gata2* nor ER-GATA-1-mediated activation of *Epb4.9* and *Ahspl* expression, encoding the erythroid cytoskeletal protein dematin [38] and α-globin chaperone [39], respectively. As *Alas2* cis element mutations influenced a subset of GATA-1 target genes, heme deficiency compromised only select GATA-1 functions.

**Figure 2.** GATA motif deletions abrogate *Alas2* expression during erythroid maturation.

- **A** Representative pellets of WT and mutant GLE-ER-GATA-1 cell clones treated with or without β-estradiol for 48 h.
- **B** Wright–Giemsa staining of untreated and β-estradiol-treated (48 h) WT clone 1 (WT1) as well as double-mutant clone 1 (mt1/8 Mut1).
- **C** Growth curves in WT1 and double-mutant clone 1 cells treated with or without β-estradiol (n = 3, mean ± SE).
- **D** Western blotting of ER-GATA-1 and α-tubulin in WT and mutant clones.
- **E** Real-time RT–PCR of *Alas2* mRNA in untreated or β-estradiol-treated WT and mutant clones. *****P < 0.001 with respect to all values of WT1-3 (n = 4, mean ± SE)** (left panel). *Alas2* mRNA in intron 1 mutant clones was compared to that in intron 1/8 double-mutant clones. *****P < 0.001 with respect to all values of intron 1 mutants 1 and 2 (right panel).**
- **F** Quantitative ChIP analysis of ER-GATA-1 occupancy at the GATA sites of *Alas2* introns, HS2 of the β-globin locus control region, and the Hbb-b1 promoter in WT clone 1 and mutant clones. *Alas2* exon 2/intron 2 junction site was used as a negative control. *****P < 0.001 with respect to WT1 (n = 4, mean ± SE).**

Data information. **P-values** were calculated by a two-tailed paired Student’s t-test using Microsoft Excel.
Figure 2.

A

B

C

D

E

F

Figure 2.

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genes in the Alas2 double-mutant cells, and rescue by 5-ALA, provides strong evidence that these genes are heme-regulated. Thus, Alas2-enhancer/5-ALA-activated or -repressed genes were deemed heme-activated and heme-repressed, respectively, and heme amplifies GATA-1 activity to regulate these genes.

A large cohort of Alas2-enhancer-regulated genes were not rescued by 5-ALA (Fig 6C). It is instructive to consider the following mechanisms to explain the lack of rescue of these genes. First, 5-ALA only partially rescues heme levels in double-mutant cells (Fig 4B), and full restoration of heme might be required to restore the normal gene expression pattern. Second, considering that the Alas2 enhancers function to establish and/or maintain transcription of these genes, loss of the enhancers would abrogate one or both of these mechanisms. It is reasonable to postulate that this would lead to irreversible epigenetic changes that hinder heme-mediated reactivation. Third, the enhancer deletions might perturb higher-order locus positioning in nuclear subdomains, thus indirectly impacting upon transcriptional activity. Regardless of the specific mechanism responsible for the lack of rescue of a cohort of the Alas2 enhancer-regulated genes, the genomic analysis provided strong evidence that heme amplifies GATA-1 function at a substantial gene ensemble (94) in erythroid cells.

In the 94 GATA-1/Alas2-enhancer/5-ALA co-regulated genes, only two genes were Alas2-enhancer-repressed but 5-ALA-activated, or Alas2-enhancer-activated but 5-ALA-repressed. The remaining 92 GATA-1/Alas2-enhancer/5-ALA co-regulated genes were parsed into 4 groups: (i) GATA-1 activated/heme activated; (ii) GATA-1 activated/heme repressed; (iii) GATA-1 repressed/heme activated; (iv) GATA-1 repressed/heme repressed (Fig 6D). GATA-1/heme activated Hmox1, Hbb-b1, and Hba-a1, consistent with the real-time RT–PCR data (Figs 5A and 7C). The GATA-1/heme-activated genes were subjected to Gene Ontology (GO) analysis. This revealed functional classifications of “hemoglobin complex” ($P = 5.0 \times 10^{-9}$), “hematopoiesis” ($P = 0.0073$), “purine nucleotide binding” ($P = 0.0024$), and “microtubule-based movement” ($P = 0.0037$) in the GATA-1/heme-activated cohort (Table EV1) suggesting a propensity for these genes to be involved in erythroid cell function and differentiation. Whereas Slc48a1 (HRC-1) and Mfsd7B (FLVCR1) encoding heme transporters were heme-activated, most heme and iron transporter genes and genes mediating heme biosynthesis were not heme-regulated (Table EV2). Thus, heme deficiency influenced a subset of genes dictating heme or iron homeostasis.

Although Hbb-b1 expression decreased in double-mutant cells, ER-GATA-1 occupancy at β-globin locus control region HS2 and the Hbb-b1 promoter was unaffected (Fig 2F). Thus, repression did not involve reduced ER-GATA-1 occupancy. Prior reports indicated that Bach1 represses Hbb-b1 and Hba-a1 expression in a heme-dependent manner [17,42]. Whereas little to no Bach1 was detected in wild-type cells, Bach1 was detected in β-estradiol-treated double-mutant cells (Fig 7A). In β-estradiol/5-ALA-treated double-mutant cells, Bach1 decreased greatly. Bach1 expression was upregulated in β-estradiol-treated wild-type and double-mutant cells, with or without 5-ALA (Fig 7B). Thus, GATA-1 activates Bach1 expression upon erythroid differentiation, and heme controls Bach1 protein levels.

To assess the influence of GATA-1 and heme on Bach1 function, we quantitated expression of the Bach1-repressed gene Hmox1. GATA-1 induced Hmox1 expression in WT1 cells, but not...
double-mutant cells (Fig 7C). Heme deficiency, which upregulated Bach1, repressed Hmox1 expression (Fig 7C). 5-ALA-mediated rescue of heme biosynthesis, which downregulated Bach1 (Fig 7A), induced Hmox1 expression (Fig 7C). To test whether elevated Bach1 in β-estradiol-treated double-mutant cells suppresses expression of heme-regulated GATA-1 target genes identified by the RNA-seq, cells were infected with retrovirus expressing shRNA targeting luciferase or Bach1 (Fig 7D). In Bach1-knockdown double-mutant cells, Fbxo30, Tbcel, and Slc7a11 mRNA (Fig 7E) and primary transcript (Fig 7F) levels increased. Despite being GATA-1/heme-activated, surprisingly, Sgstm1, Gm2016, and Slc30a1 mRNA levels were unaffected (Fig 7E). Hbb-b1 and Hba-a1 mRNA levels were upregulated 4.4- and 2.8-fold, respectively, by Bach1 knockdown, implicating Bach1 in globin gene repression upon heme deficiency (Fig 7E). Bach1 suppression of GATA-1-mediated activation of these genes illustrates a new mode of GATA factor function. Hbb-b1, Hba-a1, Fbxo30, Tbcel, and Slc7a11 were deemed Bach1 sensitive, and Sgstm1, Gm2016, and Slc30a1 Bach1 insensitive. As the Bach1 knockdown did not alter Sgstm1, Gm2016, and Slc30a1 primary transcripts, these genes were transcriptionally repressed in a Bach1-independent manner (Fig 7F). To determine whether Bach1 sensitivity related to the magnitude of the GATA-1-dependent transcriptional response, we quantitated expression of Bach1-sensitive and Bach1-insensitive GATA-1 target genes. The magnitude of GATA-1-mediated activation of the genes in β-estradiol-treated WT1 and double-mutant cells did not predict Bach1 sensitivity (Fig 7G).

To further evaluate the mechanism of Bach1 sensitivity, we analyzed GATA-1 and Bach1 occupancy at five GATA-1/heme-activated and Bach1-sensitive genes. ChIP-seq data from human peripheral blood-derived erythroblasts (PBDE) [43] and K562 cells [13] revealed that GATA-1 and Bach1 occupied similar sites at three genes (HBB, HBA1, and SLC7A11). Only Bach1 occupied FBXO30, and only GATA-1 occupied TBCEL (Fig EV2A). These results indicated that GATA-1 and Bach1 directly regulate four out of five GATA-1/heme-activated and Bach1-sensitive genes. A genomewide comparison between GATA-1 and Bach1 occupancy in K562 cells was also conducted (Fig EV2B and Table EV3). GATA-1 and Bach1 occupied 3,132 and 2,976 genes, respectively, among a total of 57,820 GENCODE-annotated genes. GATA-1 and Bach1 co-occupied 778 genes, which was highly significant ($P < 2.2 \times 10^{-16}$; naive Fisher’s exact test). GATA-1 and Bach1 co-occupancy of numerous chromatin sites suggests that Bach1 function at GATA-1 target genes represents a common mechanism.

In principle, the distinct regulatory modes may reflect different requirements for chromatin modification at different target genes.
Whereas ER-GATA-1 induced trimethylated histone H3 at lysine 4 (H3K4me3), a mark associated with active promoters, at the Hbb-b1 promoter, H3K4me3 was not reduced in β-estradiol-treated double-mutant as compared with WT1 cells (Fig EV3). Although H3K4me3 levels were slightly increased in β-estradiol-treated WT1 cells, the levels were quite high at promoters of Bach1-sensitive (Fbxo30) and Bach1-insensitive genes (Sqstm1 and Slc30a1) in WT1 and double-mutant cells. ER-GATA-1 increased trimethylated histone H3 at lysine 36 (H3K36me3), a mark associated with active transcription, at open reading frames of Bach1-sensitive or Bach1-insensitive genes in WT1 cells; this mark decreased at Bach1-sensitive and Bach1-insensitive loci in double-mutant cells, with the exception of Sqstm1. These results indicate that GATA-1 target genes differing in Bach1 sensitivity are not discriminated by mechanisms establishing/maintaining H3K4me3 and H3K36me3. Furthermore, GATA-1 target gene sensitivities to FOG-1, TAL1, Lmo2, and Mi2β knockdowns do not predict the unique heme or Bach1 regulation described herein (Fig 8A), highlighting this new mode of GATA factor regulation.

**Discussion**

Elucidating cis regulatory mechanisms governing heme biosynthesis yielded the serendipitous discovery that heme is required for the master regulator GATA-1 to establish/maintain the transcriptome of the erythroblast—the precursor to the red blood cell (Fig 8).
Figure 6. GATA-1/heme regulation of the erythroid cell transcriptome.

A GATA-1-regulated genes

β-estradiol: — + — +
5-ALA: — + — +
WT1
int1/8
Mut1

β-estradiol: — + — +
5-ALA: — + — +
WT1
int1/8
Mut1

Number of genes

150 100 50

β-estradiol: — + — +
5-ALA: — + — +
WT1
int1/8
Mut1

β-estradiol: — + — +
5-ALA: — + — +
WT1
int1/8
Mut1

B Pie charts display the percentage of activated and repressed genes (FDR < 0.05 and expressed differentially > 2-fold) in GATA-1-, Alas2-enhancer-, or 5-ALA-regulated genes determined by RNA-seq analysis.

C Venn diagram depicting relationships between GATA-1-, Alas2-enhancer-, and 5-ALA-regulated genes.

D Pie chart depicting the gene numbers in 4 categories for genes regulated by GATA-1, Alas2-enhancer, and 5-ALA.
heme biosynthesis is normal, GATA-1 establishes/maintains a rich target gene ensemble that promotes erythropoietic survival and maturation [11–16], and, in other contexts, promotes megakaryopoiesis or the generation and/or function of mast cells and eosinophils [44–48]. We generated heme-deficient erythroid cells by deleting two GATA-1-occupied cis elements that exhibited considerable differences in their activities. The quantitatively greater enhancer activity of the intron 1 element was not predictable from sequence features and chromatin attributes, thus highlighting the importance of analyzing cis element function at endogenous loci.

Using our heme-deficient cell system with conditional heme rescue, we demonstrate that heme deficiency renders an important sector of GATA-1 target genes GATA-1 insensitive. The genes comprising this sector encode erythroid cell constituents vital for homeostasis and red blood cell genesis from progenitor cells. Heme export is required for erythropoiesis and protects erythroblasts from heme-mediated toxicity [49,50]. As heme permits GATA-1 to establish the erythroblast transcriptome, heme levels must suffice for GATA-1 function, as well as its canonical role in hemoglobin assembly, while not being excessive, which is cytotoxic.

GATA factors utilize coregulators to confer activation or repression [27] and are controlled by signaling mechanisms that induce phosphorylation [51–53], acetylation [54], methylation [55], and sumoylation [56]. How biochemical constituents, such as metal ions and metabolic intermediates, impact specific steps in GATA factor function is largely unexplored. In principle, such constituents might influence signaling mechanisms targeting GATA factors or protein interactors that control GATA factor activity. Analogous to the context-dependent influence of heme on GATA-1 function, GATA-1 sumoylation [56] and GATA-2 phosphorylation [53] influence only a subset of their respective target genes. Heme deficiency did not alter GATA-1 sumoylation (Fig 7A, top) indicating that the heme activity cannot be explained by enhanced sumoylation. As heme only influenced a cohort of GATA-1 target genes, it did not regulate GATA-1 functions essential at all target loci. An intriguing mechanistic insight emerged from the finding that 5-ALA did not rescue expression of all genes downregulated by heme deficiency (Fig 6). As the establishment and maintenance of GATA-1 target gene transcription can be differentially regulated [57], a subset of the genes repressed upon heme deficiency might not be reactivated due to differential mechanisms mediating maintenance vs. establishment of transcription. While GATA-1/heme suffices to maintain expression of certain genes repressed upon heme deficiency, these repressed genes might adopt chromatin attributes that are refractory to GATA-1/heme-mediated establishment of an active transcriptional state.

GATA-1 induction of globin chains, ALAS-2/heme biosynthesis, and Bach1, with heme repressing Bach1, constitutes a type I incoherent feed-forward loop [58]. This loop is a core of a complex network that establishes/maintains the erythroid cell transcriptome. One component of the mechanism by which heme amplifies GATA-1 activity involves downregulating the heme-sensing repressor Bach1. In heme deficiency, Bach1 accumulated and repressed a subset of GATA-1/heme target genes. Disruption of heme biosynthesis and 5-ALA rescue in cells with or without GATA-1 and Bach1 indicates a balance between GATA-1 Bach1 and dictates transcription at select GATA-1 target genes.

This relationship between GATA-1 and Bach1 could not have been predicted from existing knowledge of Bach1 function. Whereas Bach1 occupancy at the HS2 DNase I hypersensitive site of the β-globin locus control region decreased significantly during the initial 8 h of hemo treatment of MEL cells, Hbb-b1 expression was not activated [17]. Sun et al [17] suggested that this reflects lack of recruitment of the activator p45/NF-E2 to HS2. p45/NF-E2 regulates β-like globin expression in MEL cell systems [59–61], but p45/NF-E2 knockout mice do not exhibit defective β-like globin gene expression [62,63]. In our study, Hbb-b1 expression was considerably higher in WT cells in comparison with heme-deficient double-mutant cells (Figs 3 and 5A and C). Although it is formally possible that NF-E2/p45 levels/activity are altered in the double-mutant cells, thus contributing to decreased Hbb-b1 expression, we demonstrated that Bach1 accumulated in heme-deficient, double-mutant G1E-ER-GATA-1 cells. Importantly, Bach1 knockdown in this context almost completely restored Hbb-b1 expression (Fig 7E). Our results indicate that Bach1 represses Hbb-b1 expression in a heme-dependent manner.

MaFK is a heterodimeric partner of Bach1 and p45/NF-E2, which occupies HS2 when Hbb-b1 expression is both repressed and activated [64]. Upon hemin-induced MEL cell maturation, p45/NF-E2 replaces Bach1 at HS2, which correlates with Hbb-b1 activation. After maturation, MaFK occupancy was unaffected. As expected, heme downregulated Bach1 chromatin occupancy [17], since heme was known to stimulate Bach1 proteolysis via the ubiquitin–proteasome system [18]. While these mechanistic studies implicate Bach1 in repressing β-like globin gene expression in MEL cells, how GATA-1 might interface with the respective mechanisms was unclear.

Figure 7. Bach1-dependent and Bach1-independent modes of heme-dependent GATA-1 function.

A Western blot of ER-GATA-1, Bach1, and β-actin in untreated or β-estradiol-treated (48 h) WT clone 1 and double-mutant clone 1 incubated with or without 5-ALA. The upper band of the ER-GATA-1 blot represents sumoylated ER-GATA-1, which increases as active ER-GATA-1 becomes nuclear-localized. A darker exposure of the Bach1 blot is also shown.

B Real-time RT–PCR analysis of Bach1 mRNA levels (left panel) and primary transcripts (right panel) (n = 4, mean ± SE).

C Real-time RT–PCR quantitation of Hmox1 mRNA levels (left panel) and primary transcripts (right panel) (n = 4, mean ± SE).

D Bach1 knockdown in β-estradiol-treated double-mutant clone 1 (n = 4, mean ± SE) Western blot analysis of ER-GATA-1, Bach1, and β-actin. A darker exposure of the Bach1 blot is also shown.

E Real-time RT–PCR analysis of mRNA levels of GATA-1/heme-activated genes in β-estradiol-treated WT1 or double-mutant clone 1 expressing shRNA targeting Bach1. P-values were calculated with respect to β-estradiol-treated control double-mutant clone 1 (n = 4, mean ± SE).

F Real-time RT–PCR analysis of primary transcripts of GATA-1/heme-activated genes in β-estradiol-treated WT1 or Bach1 knockdown double-mutant clone 1. P-values were calculated with respect to β-estradiol-treated control double-mutant clone 1 (n = 4, mean ± SE).

G Real-time RT–PCR analysis of mRNA levels of GATA-1/heme-activated genes in untreated or β-estradiol-treated WT1 and double-mutant clones. P-values were calculated with respect to β-estradiol-treated WT1 (n = 4, mean ± SE).

Data information: **P < 0.01, ***P < 0.001. P-values were calculated by a two-tailed unpaired Student’s t-test using Microsoft Excel.
Figure 7.
At a cohort of GATA-1/heme-regulated genes, heme amplification of GATA-1 function was Bach1 insensitive. Whereas heme deficiency reduced GATA-1-mediated activation of these genes, and Bach1 accumulated, a major reduction in Bach1 did not abrogate the inhibitory effect on GATA-1 function. These genes included Sqstm1, Gm2016, and Slc30a1. Sqstm1 encodes p62/sequestosome 1, a selective autophagy receptor that binds autophagic cargo, linking it to LC3/GABARAP autophagy machinery [65,66]. By binding ubiquitin, p62/sequestosome 1 promotes protein degradation via the proteasome [67,68]. While GATA-1 activates genes encoding components of the autophagy machinery, thus instigating autophagy upon erythroid maturation [43], Sqstm1 was not known to be GATA-1-regulated.

p62/sequestosome 1 is also a signaling adapter [69] and can activate the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor [70], a mediator of stress responses [71]. GATA-1/heme induction of p62/sequestosome 1 provides additional mechanistic insights into how GATA-1 instigates autophagy and links GATA-1

Figure 8. GATA factor/heme circuit as an essential determinant of a cell type-specific transcriptome that controls cellular differentiation.

A The relationships between heme, Bach1, FOG-1, TAL1, Lmo2, and Mi2β regulation of GATA-1 target genes in G1E-ER-GATA-1 cells. "Sensitive" implies significant deregulation upon heme deficiency, FOG-1-binding deficiency, or knockdown of the respective factor. TAL1, Lmo2, and Mi2β knockdowns were reported previously [76,77]. Expression of TAL1, Lmo2, and Mi2β decreased by 70%, 75%, and 50%, respectively, by the knockdown. FOG-1 sensitivity was determined using G1E-ER-GATA-1 (V205G) cells expressing mutated GATA-1 defective in FOG-1 binding [82].

B Cellular and molecular consequences of heme deficiency on GATA-1 function. Type I incoherent feed-forward loop [83] under normal heme conditions that controls heme biosynthesis and globin chain production. This loop also activates a p62-dependent pathway that can induce Nrf2 and confer cytoprotection [70], and promote autophagy, a process vital for erythroid cell maturation [84–87]. In heme deficiency, Bach1-sensitive and Bach1-insensitive (X?) mechanisms disrupt multiple mechanistic steps, thus impairing hemoglobin biosynthesis, cytoprotection, and autophagy. As impaired autophagy and associated molecular defects would compromise cell survival and/or maturation, this underscores the critical implications of the heme mechanism to amplify GATA-1 activity described herein. The red arrows indicate relationships derived from this study. The black arrows indicate relationships that were either known or predicted from existing knowledge.
and Nrf2 mechanisms. Nrf2 induces heme oxygenase-1 in response to cell stress, and heme oxygenase-1 catalyzes heme degradation [72]. Building upon the type I incoherent feed-forward loop (Fig 8B), (i) GATA-1 induces the heme biosynthetic enzyme Alas2; (ii) GATA-1/heme induces Bach1; (iii) Bach1 represses heme oxygenase-1; (iv) GATA-1/heme induces p62/sequestosome; (v) p62/sequestosome induces Nrf2; and (vi) Nrf2 induces heme oxygenase-1. This iterative cycle has important consequences for autophagy and cytoprotection in erythroid cells and likely for a broad cadre of systems. The other Bach-1-insensitive GATA-1/heme-activated genes encode a zinc transporter (Slc30a1 [73]) and a gene of unknown function (GM2016).

Analogous to the heme-sensitive or heme-insensitive GATA-1 target genes and the Bach-sensitive or Bach-insensitive GATA-1/heme target genes, the coregulators FOG-1 [74,75], SetD8 [76], Mi2β [73], and LMO2 [77], and transcription factors FoxD3 [43] and TAL1 [77] mediate certain, but not all, GATA-1 actions. While the FOG-1-dependent genes Hbb-b1 and Hba-a1 were heme-activated, other FOG-1-dependent genes, Ahsp, Slc4a1, Hepb1, Pdss2, and Abcb10, as well as FOG-1-independent genes, Ebf4, Klf1, and Tac2, were not heme-regulated. With the exception of Hbb-b1 and Hba-a1, GATA-1-K137-sumoylation-dependent genes (Ahsp, Slc4a1, Abcb10, Hepb1, Pdss2, Tac2, Kit, and Lyl1) were not heme-regulated. The GATA-1/heme-activated gene Hbb-bh1 and GATA-1-repressed/heme-activated genes, Ephx2, Apoc1, P2rx1, and Rab44, were SetD8 sensitive. Mi2β-sensitive genes, Kit, Clec10a, Rgs19, and Clec4d, were not heme-regulated. Bach-sensitive (Fbxo30, Tbc1, and Slc7a11) and Bach-insensitive genes, Sgstm1, Gm2016, and Slc30a1, were SetD8 insensitive. Thus, heme and Bach1 regulation of GATA-1 function represents new modes of transcriptional control not predicted by established mechanisms (Fig 8A).

In summary, heme amplifies GATA-1 activity to establish a cell type-specific transcriptome. As a component of a complex network, a GATA-1/heme-dependent incoherent feed-forward loop ensures the identity and physiological function of the developing red blood cell and constitutes a new paradigm for GATA factor control of cellular differentiation and phenotypes. A dual mechanism involving Bach1-sensitive and Bach1-insensitive components underlies the heme activity to sculpt a genetic network that drives cellular differentiation. From systems biological/pathophysiological perspectives, it will be instructive to determine how parameters influence the heme/GATA factor circuit in homeostasis and disease, thereby dictating red blood cell development and function, and perhaps impacting a broader repertoire of GATA factor-expressing cell types.

Materials and Methods

Cell culture

G1E-ER-GATA-1 cells were cultured in Iscove’s modified Dulbecco’s medium ( Gibco) containing 15% FBS (Gemini), 1% penicillin/streptomycin (Gemini), 2 U/ml erythropoietin, 120 mM monothioglycerol (Sigma), and 0.6% conditioned medium from a Kit ligand-producing CHO cell line, and 1 µg/ml puromycin (Gemini). ER-GATA-1 activity was induced by addition of 1 µM β-estradiol (Steraloids) to the media. To rescue heme biosynthesis, 1 mM 5-aminolevulinic acid hydrochloride (5-ALA; Sigma) was added to the media.

Generation of genomic deletions in cells using CRISPR/Cas9 system

Guide sequences for gene targeting were designed with online tools (http://crispr.mit.edu/). A U6 promoter-driven sgRNA expression cassette was amplified by hemi-nested PCR and cloned into Small-cut pBluescript (Addgene). 10 µg sgRNA-expressing plasmids were co-nucleofected into 3 × 10^6 G1E-ER-GATA-1 cells with 10 µg Cas9-expressing plasmid [25] using Amaxa Nucleofector Kit R (Lonza), 72 h after transfection, genomic DNA of the cell population was extracted and examined to detect mutations using T7 endonuclease I that cleaves heteroduplexes consisting of wild-type and mutated DNA sequences. Genomic DNA flanking the target site was amplified by PCR and amplicons were denatured, reannealed, and incubated with T7 endonuclease I (New England Biolabs) for 10 min at 37°C. Clonal cell lines were isolated by dilution in a 48-well plate. Genomic DNA flanking the mutated site was amplified by PCR and mutations were detected by direct sequencing of the PCR products.

Western blotting

1 × 10^6 cells were boiled in SDS lysis buffer (50 mM Tris, pH 6.8, 2% β-mercaptoethanol, 2% SDS, 0.04% bromophenol blue, 10% glycerol) for 15 min. Samples were resolved by SDS–PAGE and detected with Pierce ECL 2 (Life Technologies). Following antibodies were used: rat anti-GATA-1 (Santa Cruz Biotechnology; sc-265), rabbit anti-Bach1, mouse anti-β-actin (Cell Signaling; 8H10D10), and mouse anti-α-tubulin (Calbiochem; CP06). Following secondary antibodies were used: goat anti-mouse-IgG-HRP, goat anti-rat-IgG-HRP (Santa Cruz Biotechnology; sc-2005, sc-2006), and protein A-HRP (Sigma; P8651).

RT-qPCR

Total RNA was purified from 2 × 10^6 cells with TRIzol (Life Technologies). 0.75 µg RNA was treated with DNase I (Life Technologies) for 15 min at room temperature. DNase I was inactivated by addition of EDTA and by heating at 65°C for 10 min. To synthesize cDNA, DNase I-treated RNA was incubated with 125 ng of a 5:1 mixture of oligo(dT) primers and random hexamer at 68°C for 10 min. RNA/primers were incubated with Moloney MLV reverse transcriptase (Life Technologies), 10 mM DTT, RNAsin (Promega), and 0.5 mM deoxynucleoside triphosphates at 42°C for 1 h, and then heat inactivated at 98°C for 5 min. Real-time PCRs were conducted with Power SYBR Green Master Mix (Applied Biosystems) using ViIA 7 Real-Time PCR system (Applied Biosystems).

Quantitative ChIP

1 × 10^7 cells were cross-linked in 1% (v/v) formaldehyde for 10 min at room temperature, sonicated, and immunoprecipitated with rabbit anti-GATA-1 antiserum, rabbit anti-H3K4me3 (Active motif; 39159), rabbit anti-H3K36me3 (Active motif; 61101), or preimmune serum. ChIP samples were quantitated using ViIA 7 Real-Time PCR system with Power SYBR Green Master Mix.
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Heme quantitation

Cells (7–8 \times 10^7 WT or double-mutant cells) were resuspended in cold hypotonic buffer (8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, 10 mM NaCl) and lysed using three successive rounds of freezing at –80°C, followed by thawing on ice. Cell lysates were centrifuged at 17,000 g for 30 min at 4°C. The supernatants were centrifuged at 100,000 g for 60 min at 4°C. Electronic absorption spectra of the cleared lysates were recorded on a double-beam Varian Cary 4 Bio spectrophotometer from 300 to 650 nm. The oxyhemoglobin concentration in the cleared lysates was calculated using extinction coefficients for human oxyhemoglobin monomers [78]. The total heme concentration in the cleared lysates was quantified using the pyridine hemochrome assay. Briefly, 200 µl of a stock solution comprised of 0.1 N NaOH and 33% pyridine (v/v) was combined with 100 µl of the cleared lysate to convert all heme into a complex with pyridine. After adding 2–5 mg of sodium dithionite, the absorption spectrum was recorded between 500 and 600 nm. Known extinction coefficients of the reduced pyridine hemochromogen of protoheme were used to calculate the heme concentrations [79].

Retroviral infection

Two distinct shRNAs targeting Bach1 transcripts were designed and cloned into MSCV-PIG (IRES-GFP) vector provided by Mitchell Weiss. Transfecting 293T cells with 15 µg of MSCV-PIG vector and pCL-Eco packaging vector produced retrovirus expressing shRNA targeting luciferase or Bach1. G1E-ER-GATA-1 cells (2 × 10^5) were added to 100 µl viral supernatant, polybrene (8 µg/ml), and HEPES buffer, and then spinoculated at 1,200 g for 90 min at 30°C. Three days post-infection, cells were treated with 4°C, followed by thawing on ice. Cell lysates were centrifuged at 17,000 g, C. The supernatants were centrifuged at 30 min at 4°C. Electronic absorption spectra of the cleared lysates were recorded on a double-beam Varian Cary 4 Bio spectrophotometer from 300 to 650 nm. The oxymyoglobin concentration in the cleared lysates was calculated using extinction coefficients for human oxymyoglobin monomers [78]. The total heme concentration in the cleared lysates was quantified using the pyridine hemochrome assay. Briefly, 200 µl of a stock solution comprised of 0.1 N NaOH and 33% pyridine (v/v) was combined with 100 µl of the cleared lysate to convert all heme into a complex with pyridine. After adding 2–5 mg of sodium dithionite, the absorption spectrum was recorded between 500 and 600 nm. Known extinction coefficients of the reduced pyridine hemochromogen of protoheme were used to calculate the heme concentrations [79].

Sequences of oligonucleotides for construction of shRNA plasmids

shBach1-1: 5’-TGCTTTGACAGTGAAGCAGCGGCTTCCAGTTTCTCAAGTTTATAGTGAAGCAAGATGTTAATACATGGAAGACTGACATGCATTCACTGCCTCGGA-3’

shBach1-2: 5’-TGCTTTGACAGTGAAGCAGCGGCTTCCAGTTTCTCAAGTTTATAGTGAAGCAAGATGTTAATACATGGAAGACTGACATGCATTCACTGCCTCGGA-3’

RNA sequencing

RNA samples from three biological replicates were used for the analysis. Total RNA was purified in TRIzol (Life Technologies). Samples were prepared using TruSeq RNA Sample Prep Kit (Illumina) and sequenced on an Illumina HiSeq 2000. Transcript quantification and differential expression were conducted using the software packages RSEM [80] and DESeq2 [81], respectively. RSEM v1.2.20 was provided with a reference transcript set consisting of all protein coding and long intergenic noncoding transcripts from the Ensembl release 79 annotation of the NCBI Build 38 mouse genome assembly. Default parameters and Bowtie v1.1.1 were used for transcript quantification with RSEM. Gene-level read counts were then given as input to DESeq2 v1.6.3 for differential expression analysis between pairs of conditions. DESeq2 was run with default parameters except for betaPrior = F, cooksCutoff = F, and alpha = 0.05. Genes with Benjamini–Hochberg FDR values < 0.05 were deemed to be differentially expressed between a given pair of conditions. The accession number for the RNA-seq data is GEO:GSE74371. Ensembl Gene IDs for various cohorts of differentially expressed genes were given as input to The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) for Gene Ontology (GO) analysis. Enriched GO terms were clustered and all significant GO terms (P < 0.05) were listed. The GO term categories, G0TERM_BP_FAT, G0TERM_MF_FAT, and G0TERM_CC_FAT, were used.

Expanded View for this article is available online.

Acknowledgements

This work was supported by NIH grants DK50107 (to E.H.B.) and HG007019 (to E.H.B. and C.N.D.) and Cancer Center Support Grant P30 CA014520. We thank Jin-soo Kim and Mitchell Weiss for Cas9 and MSCV vectors, respectively.

Author contributions

NT, EM, JNB, and EHB conceived and designed the experiments. NT and EM wrote the paper. NT, EM, JNB, and EHB performed the experiments. NT, EM, JNB, CND, and EHB analyzed the data. KI and DY contributed reagents/materials/analysis tools. NT, JNB, CND, and EHB wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References


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