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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.

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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

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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 mesonephros (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

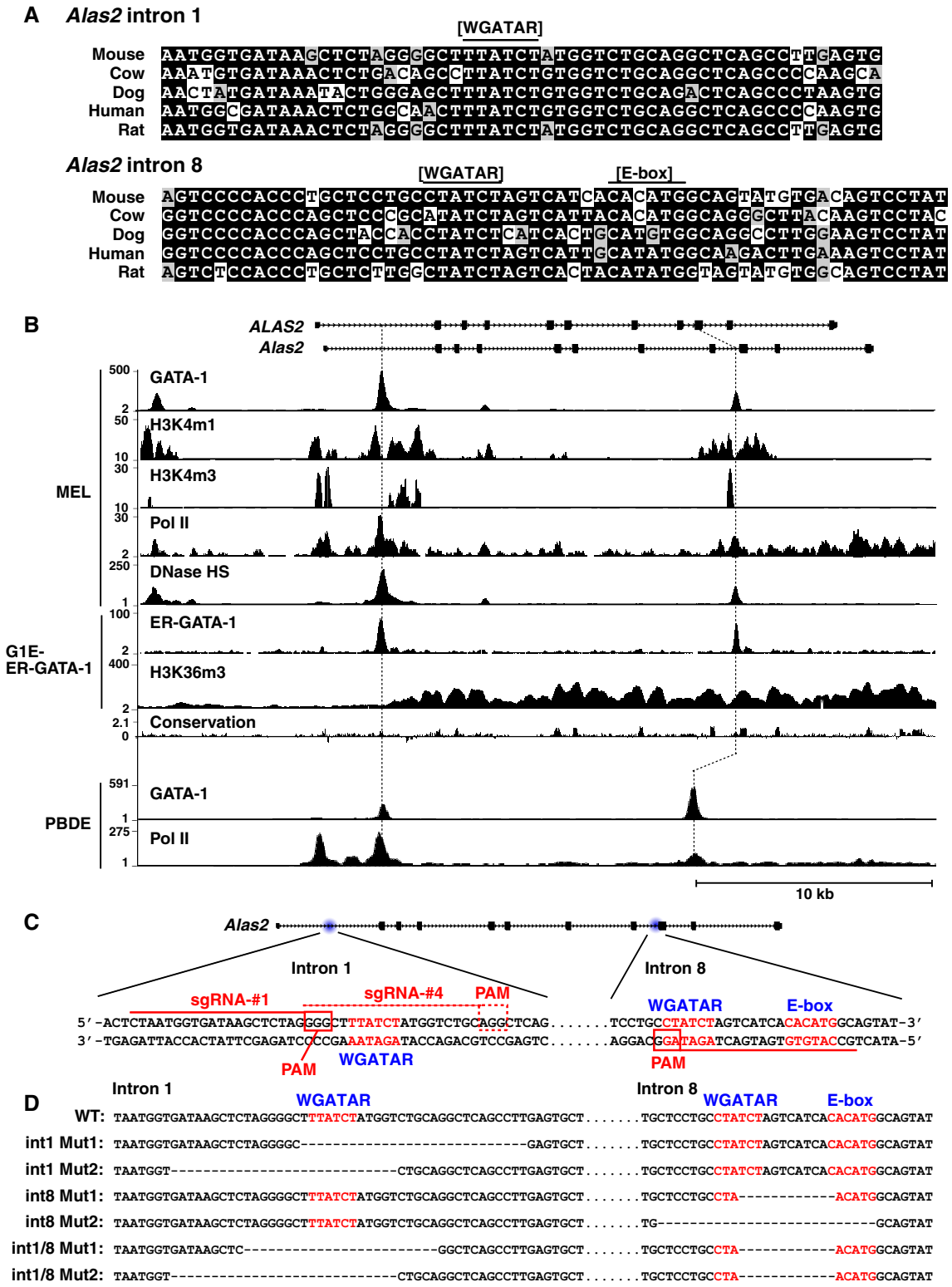


Figure 1. CRISPR/Cas9-mediated deletion of two GATA motif-containing intronic sites in *Alas2*.

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, GSM1003744, GSM1003753, GSM1014191, 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 elicit 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 *Slc4a1*, 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 *Ahsp* 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.

ALAS-2 catalyzes the production of the heme precursor 5-aminolevulinic acid (5-ALA) [40]. As 5-ALA is cell permeable [41], 5-ALA treatment of cells can replace the metabolite missing from double-mutant cells, thus bypassing the heme biosynthetic defect. 5-ALA induced double-mutant clone 1 to turn pink/red 48 h post-β-estradiol treatment (Fig 4A). To quantitate the impact of the double mutation and 5-ALA on heme biosynthesis, we analyzed the electronic absorption spectrum of the cytosolic fraction of β-estradiol-treated wild-type or double-mutant cells treated with or without 5-ALA. An intense oxyhemoglobin spectrum was observed in cytosolic lysate from β-estradiol-treated wild-type cells, with or without 5-ALA, and in β-estradiol-treated double-mutant cells with 5-ALA (Fig 4B and C). Very little oxyhemoglobin was detected in β-estradiol-treated double-mutant cells without 5-ALA. The oxyhemoglobin and heme concentrations per cell were calculated; oxyhemoglobin and heme levels were 30-fold lower in β-estradiol-treated double-mutant cells without 5-ALA than in β-estradiol-treated wild-type cells. 5-ALA rescued heme biosynthesis in double-mutant cells upon induction of erythroid maturation; oxyhemoglobin and heme levels were 20-fold higher in ALA- and β-estradiol-treated double-mutant cells than in β-estradiol-treated double-mutant cells. The total heme concentration measured by pyridine hemochromogen assay was comparable to the oxyhemoglobin concentration in all cells, consistent with hemoglobin constituting the majority of erythroid cell heme (Fig 4C, lower graph).

Given that 5-ALA rescued heme biosynthesis in double-mutant cells, we asked whether 5-ALA rescued defective gene expression. 5-ALA treatment of double-mutant cells restored *Hbb-b1* and *Hba-a1* mRNA levels (Fig 5A). The mRNA levels of other ER-GATA-1 target genes and control genes were quantitated, and the fold changes upon 5-ALA/β-estradiol-treated cells relative to β-estradiol-treated cells were calculated (Fig 5A and B). Whereas the fold changes of *Alas2*, *Hbb-b1*, and *Hba-a1* mRNAs in response to 5-ALA treatment of double-mutant cells were significantly higher than wild-type cells, the fold changes of *Slc4a1*, *Epb4.9*, *Ahsp*, *Gata2*, *Eif2s1*, and *Eif3k* mRNAs in double-mutant cells were unaffected. We also quantitated *Alas2*, *Hbb-b1*, and *Hba-a1* primary transcripts, using constitutively expressed *Eif2s1* and *Eif3k* as controls. In double-mutant cells, β-estradiol and 5-ALA did not increase *Alas2* primary transcripts as expected (Fig 5C). 5-ALA significantly increased *Hbb-b1* and *Hba-a1* primary transcripts in double-mutant vs. wild-type cells, whereas *Eif2s1* and *Eif3k* primary transcripts were unaffected (Fig 5C and D). These results demonstrate that 5-ALA and heme confer expression of key constituents of the erythroid cell transcriptome.

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

A Representative pellets of WT and mutant G1E-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 (int1/8 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 unpaired Student's *t*-test using Microsoft Excel.

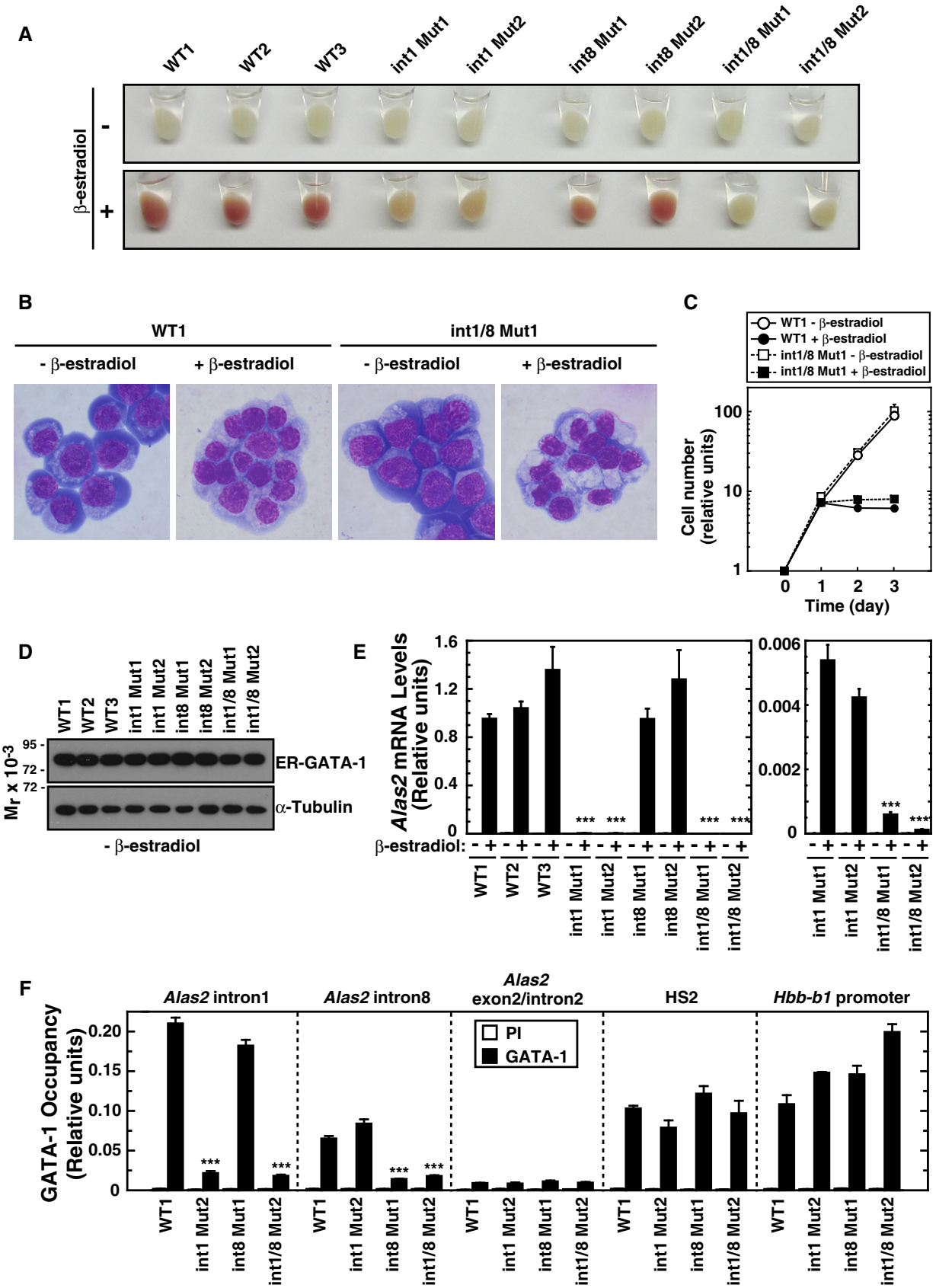


Figure 2.

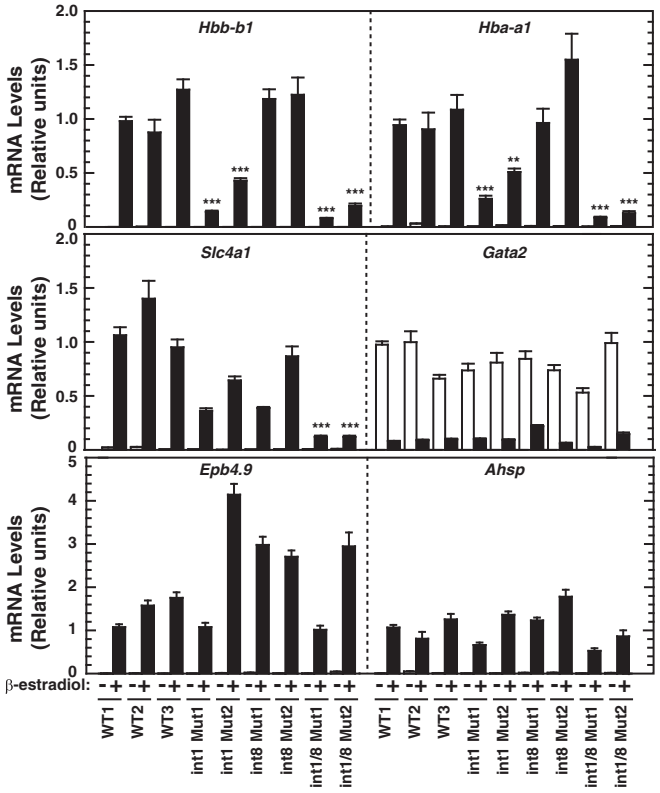


Figure 3. Deregulation of select GATA-1 target genes in *Alas2 cis* element double-mutant cells.
Real-time RT–PCR analysis of mRNA levels of ER-GATA-1 target genes in untreated or β-estradiol-treated (48 h) WT and mutant clones. $^{**}P < 0.01$; $^{***}P < 0.001$ with respect to all values of WT1-3 ($n = 4$, mean ± SE). *P*-values were calculated by a two-tailed unpaired Student's *t*-test using Microsoft Excel.

Dual mechanism of integrating heme and GATA factor functions to establish a cell type-specific transcriptome

Although heme deficiency deregulated select GATA-1-regulated genes, the extent to which heme influences GATA-1 function was unclear. We used RNA-seq to compare transcriptomes of untreated WT1, β-estradiol-treated WT1, β-estradiol-treated double-mutant, and β-estradiol/5-ALA-treated double-mutant cells. GATA-1-regulated genes were identified by comparing untreated and β-estradiol-treated WT1 transcriptomes. Two strategies delineated heme-regulated genes: (i) Comparing gene expression in β-estradiol-treated WT1 cells with β-estradiol-treated double-mutant cells identified *Alas2*-enhancer-regulated genes and (ii) comparing gene expression in β-estradiol-treated double-mutant cells with β-estradiol/5-ALA-treated double-mutant cells identified 5-ALA-regulated genes. Genes with FDR < 0.05 were deemed significant and subjected to subsequent analyses. The GATA-1 regulatory effect (number of GATA-1-regulated genes) was considerably greater than *Alas2*-enhancer and 5-ALA regulatory effects, and the *Alas2*-enhancer regulatory effect correlated well with the 5-ALA regulatory effect (Coefficient of determination (r^2) = 0.57; Fig 6A). Using genes differentially expressed >2-fold, each regulatory mode was subdivided into activated and repressed genes (Fig 6B). GATA-1/*Alas2*-enhancer/5-ALA co-regulated 94 genes (Fig 6C). The dysregulation of these 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 re-activation. 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* (*HRG-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

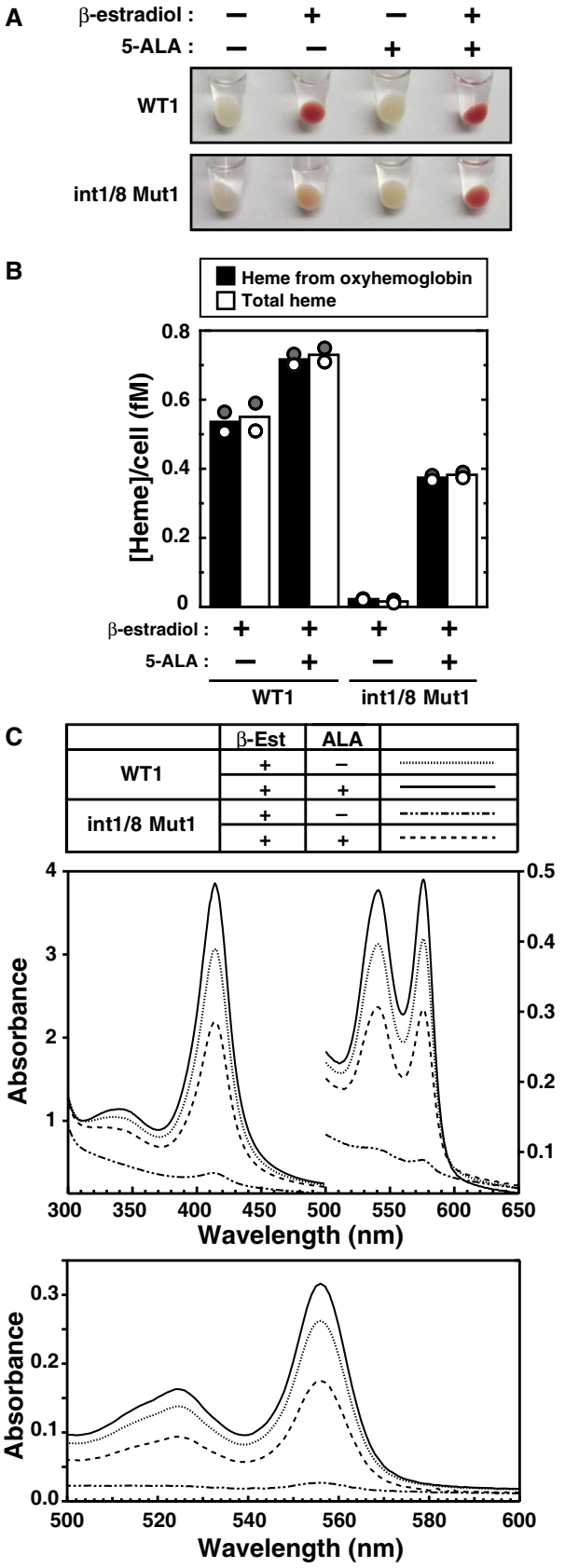


Figure 4. 5-ALA rescues heme biosynthesis in *Alas2* double-mutant cells.

A Representative cell pellets of untreated or β-estradiol-treated (48 h) WT clone 1 and double-mutant clone 1, incubated with or without 5-ALA.

B Concentration of heme per cell expressed in femtomolar (fM) quantities. The concentration of heme from oxyhemoglobin was quantitated using known extinction coefficients for human hemoglobin monomers. The total heme concentration was quantified using the pyridine hemochrome assay (n = 2; bars show the mean; individual data points are shown; each data point was based on two measurements).

C Visible absorption spectra of cleared cytosolic fraction (upper panel) and the pyridine hemochromogen (lower panel) of β-estradiol-treated WT clone 1 or double-mutant clone 1, incubated with or without 5-ALA.

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, *Sqstm1, 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 *Sqstm1, Gm2016*, and *Slc30a1* Bach1 insensitive. As the Bach1 knockdown did not alter *Sqstm1, 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 ($P < 2.2 \times 10^{-16}$; naïve 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.

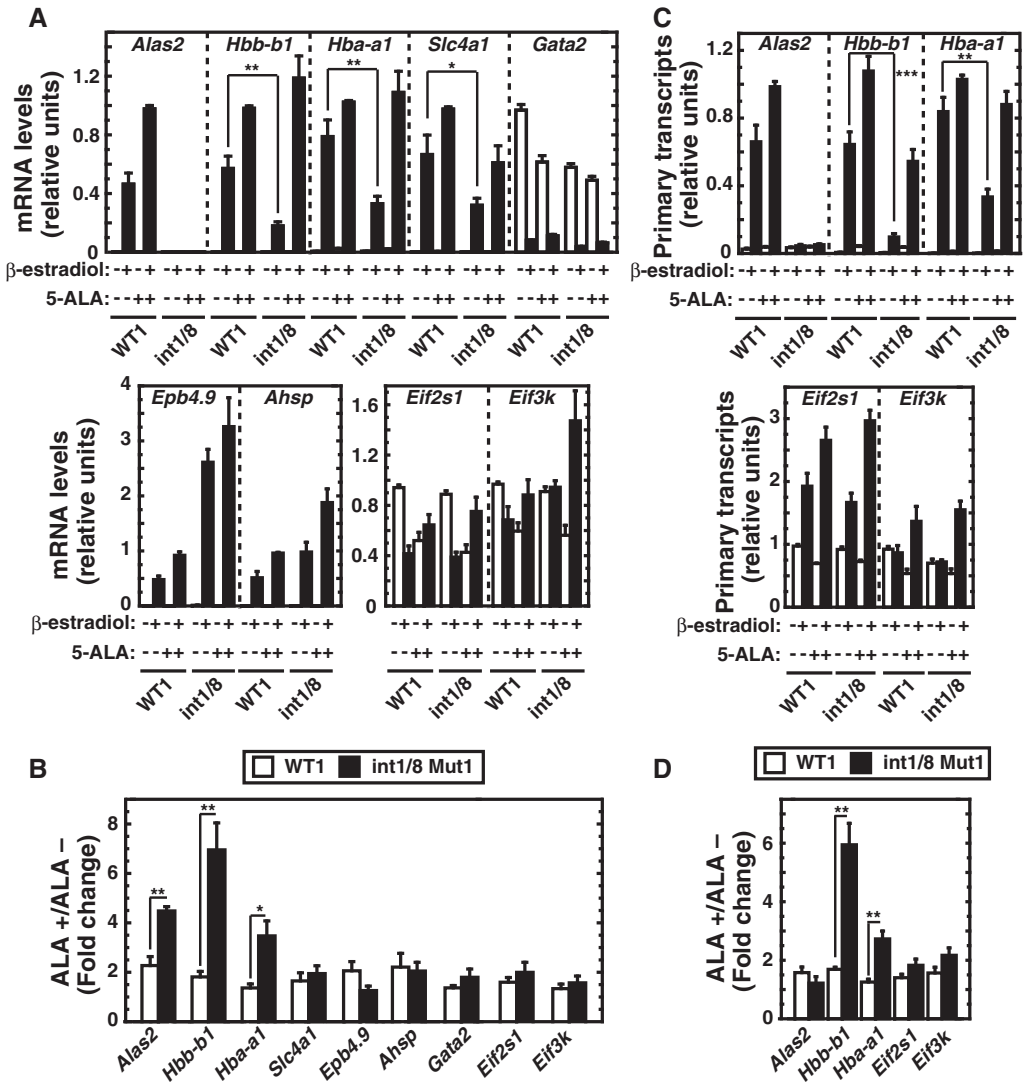


Figure 5. 5-ALA rescues GATA-1 activity in *Alas2* double-mutant cells.

A Real-time RT–PCR analysis of mRNA levels of ER-GATA-1 target genes and two housekeeping genes, *Eif2s1* and *Eif3k* (n = 4, mean ± SE).

B Fold changes of mRNA levels after 5-ALA treatment relative to no treatment in β-estradiol-treated WT clone 1 or double-mutant clone 1 (n = 4, mean ± SE).

C Real-time RT–PCR analysis of primary transcripts of ER-GATA-1 target genes (n = 4, mean ± SE).

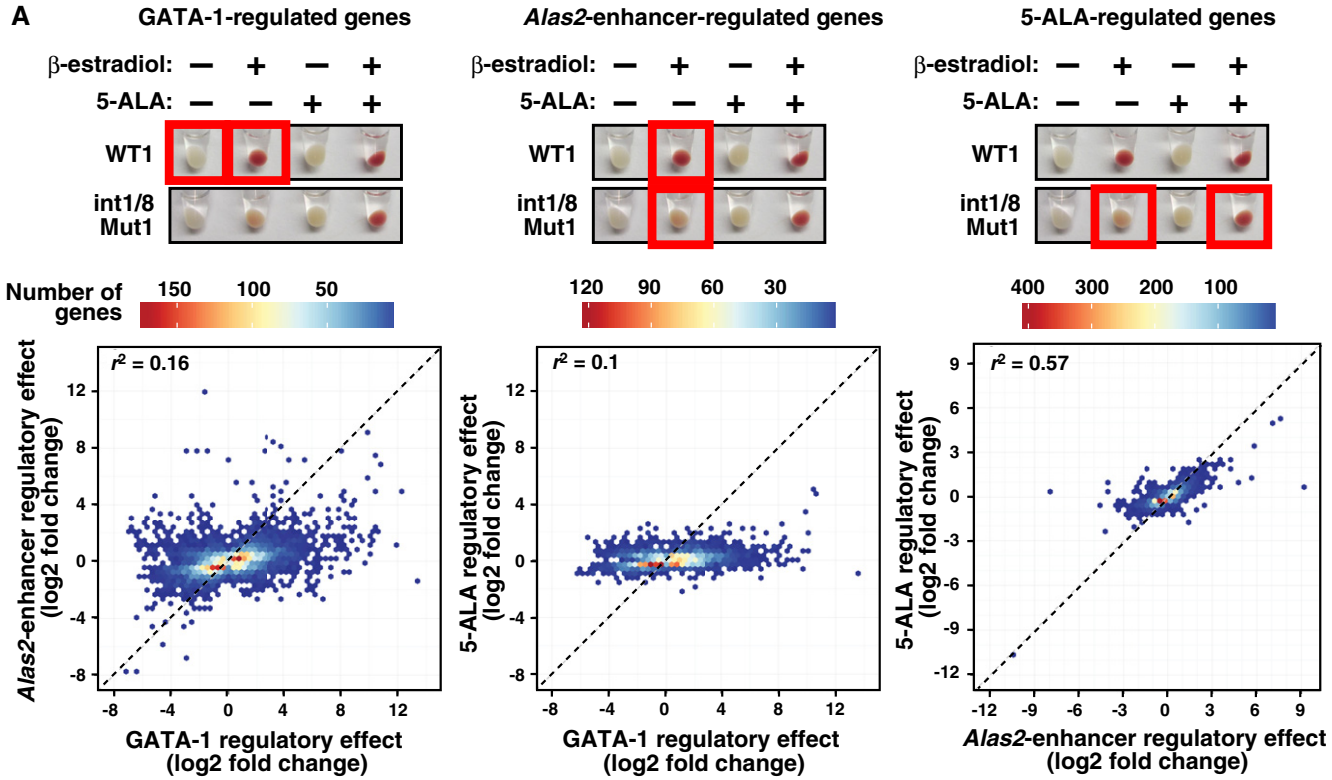
D Fold changes of primary transcripts with 5-ALA treatment in comparison with no treatment in β-estradiol-treated cells (n = 4, mean ± SE).

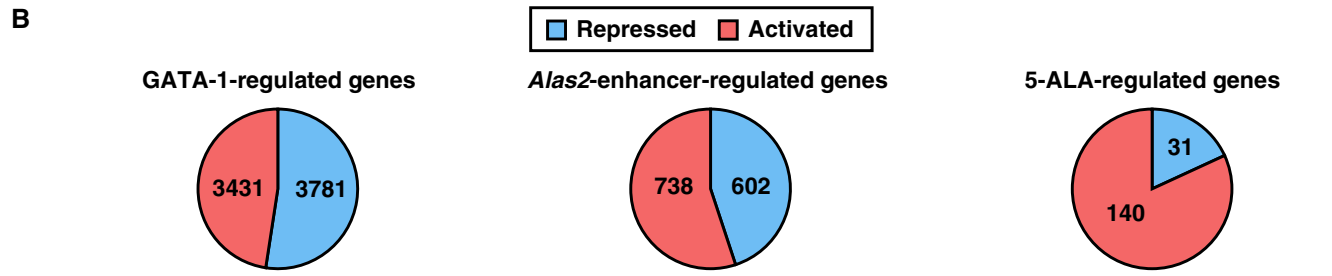
Data information: *P < 0.05; **P < 0.01; ***P < 0.001. P-values were calculated by a two-tailed unpaired Student's t-test using Microsoft Excel.

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). When





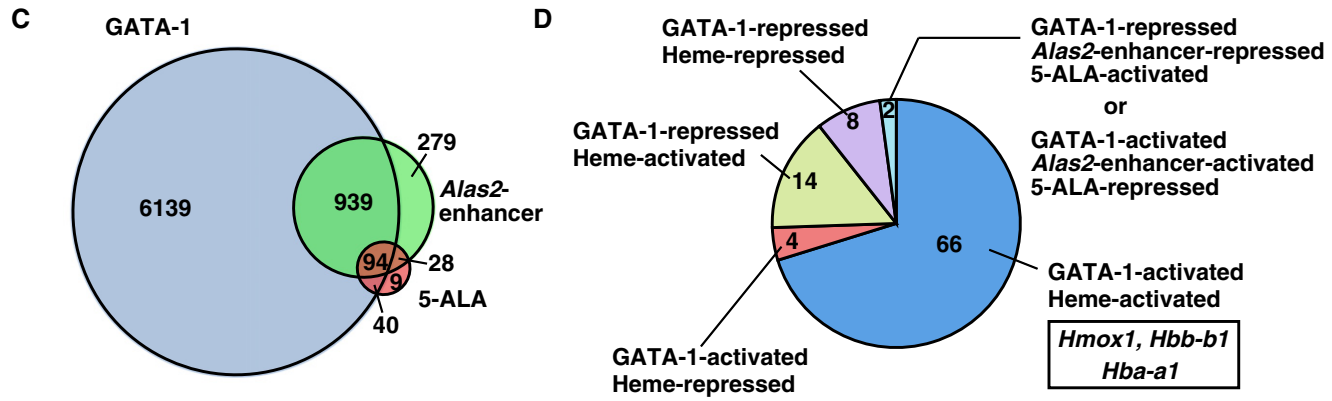


Figure 6. GATA-1/heme regulation of the erythroid cell transcriptome.

A GATA-1-, Alas2-enhancer-, and 5-ALA-regulated genes were identified by comparing conditions depicted by red boxes. Hexbin plots comparing the regulatory effects (number of regulated genes) between GATA-1, *Alas2*-enhancer, and 5-ALA. All upregulated genes (FDR < 0.05 and log2 fold change > 0) and downregulated genes (FDR < 0.05 and log2 fold change < 0) were tested. Coefficient of determination (r^2) was shown in each plot.

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 erythroid precursor 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 hemin 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.

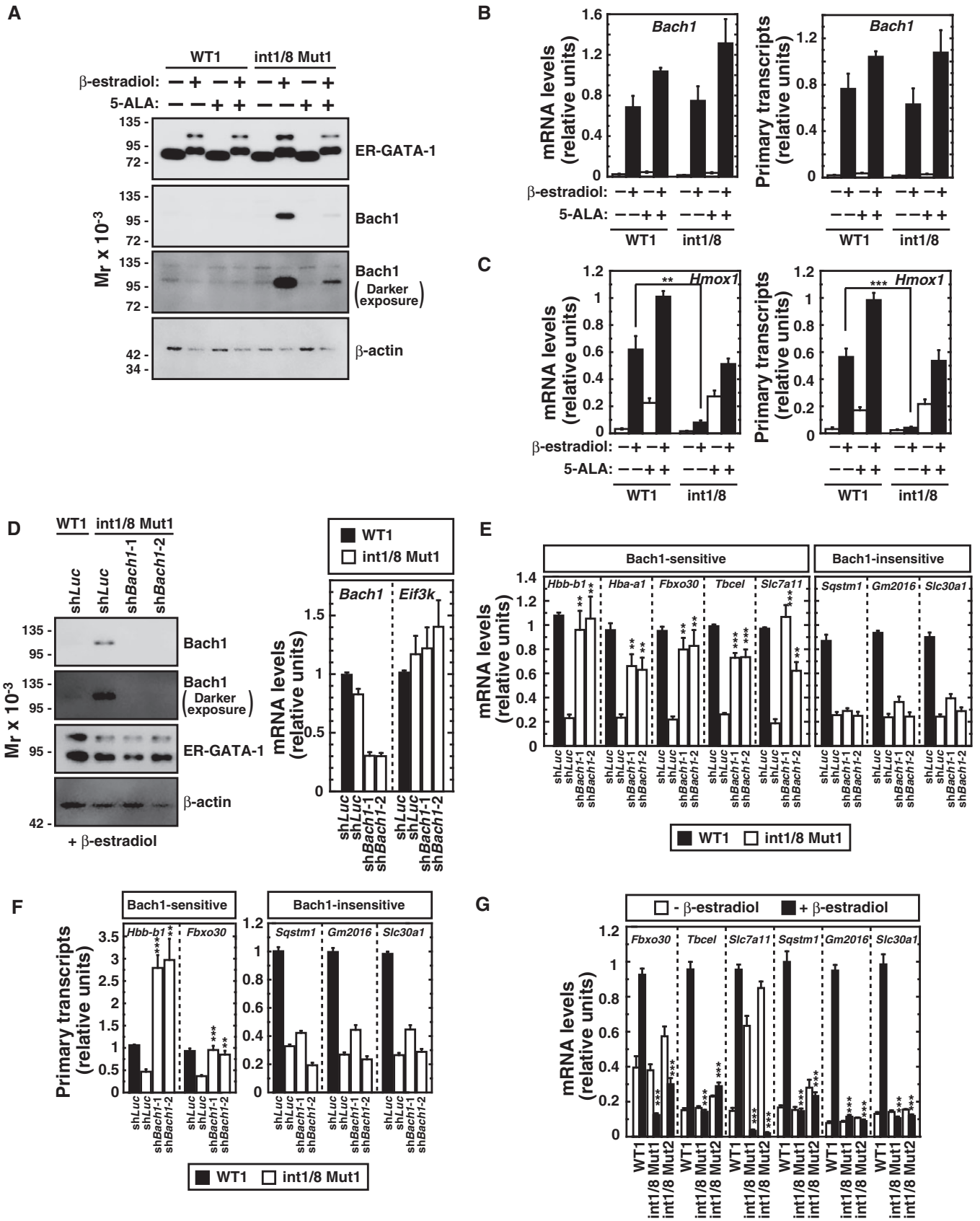

A
WT1
int1/8 Mut1
β-estradiol:
5-ALA:
Mr x 10-3
ER-GATA-1
Bach1
Bach1 (Darker exposure)
β-actin
B
Bach1
mRNA levels (relative units)
Primary transcripts (relative units)
β-estradiol:
5-ALA:
WT1
int1/8
C
Hmox1
D
shLuc
shBach1-1
shBach1-2
+ β-estradiol
Eif3k
E
Bach1-sensitive
Bach1-insensitive
Hbb-b1
Hba-a1
Fbxo30
Tbcel
Slc7a11
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Figure 7.

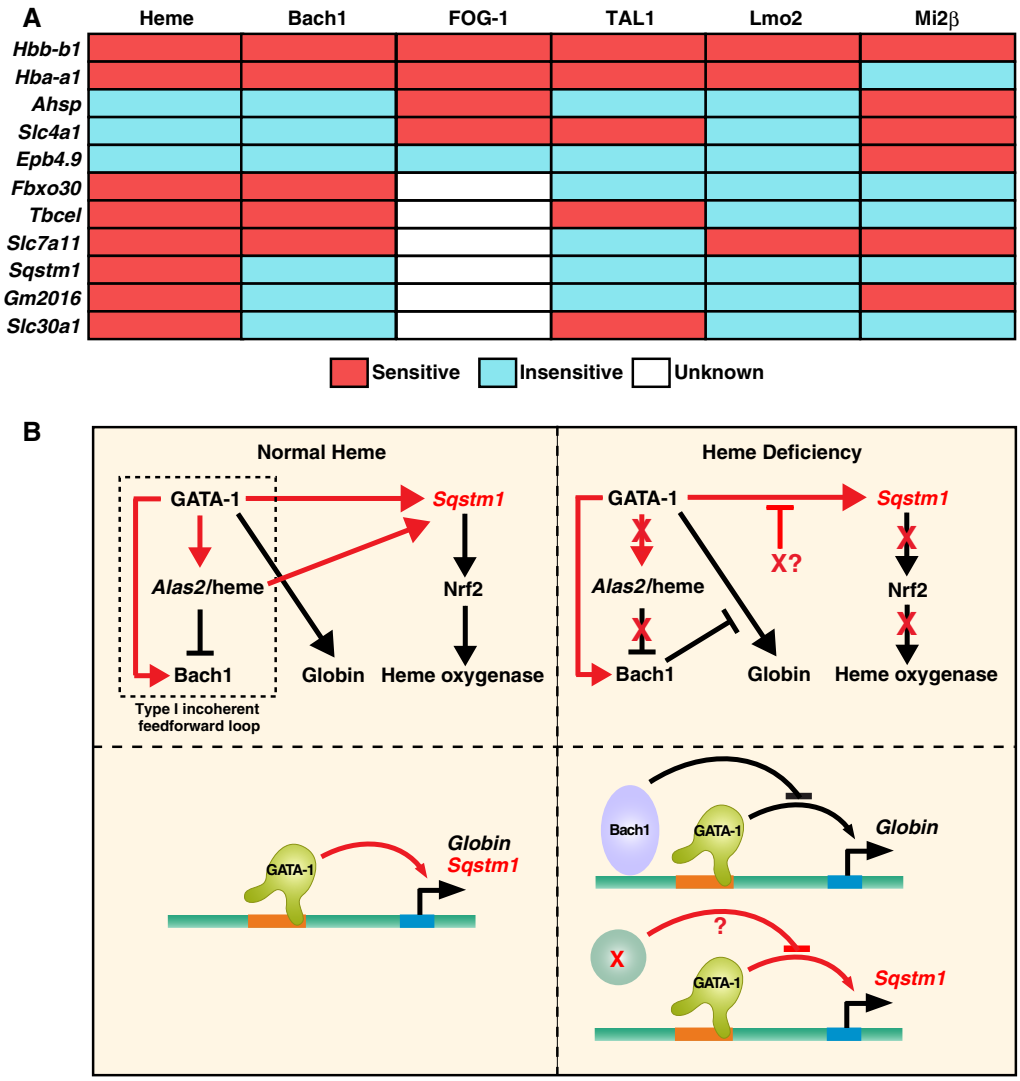

Figure 8. GATA factor/heme circuit as an essential determinant of a cell type-specific transcriptome that controls cellular differentiation.

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.

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

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 Bach1-sensitive or Bach1-insensitive GATA-1/heme target genes, the coregulators FOG-1 [74,75], SetD8 [76], Mi2β [75], and LMO2 [77], and transcription factors FoxO3 [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*, *Hebp1, Ptdss2,* and *Abcb10*, as well as FOG-1-independent genes, *Epb4.9, 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, Hebp1, Ptdss2, 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. Bach1-sensitive (*Fbxo30, Tbcel,* and *Slc7a11*) and Bach1-insensitive genes, *Sqstm1, 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 nM 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 SmaI-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.

Heme quantitation

Cells (7–8 × 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 β-estradiol, and after 48 h, cells were harvested for RT–qPCR and Western blotting analyses.

Sequences of oligonucleotides for construction of shRNA plasmids

sh*Bach1*-1: TGCTGTTGACAGTGAGCGCGCTTCCAGTTTCTCAAGTTTATAGTGAAGCCACAGATGTATAAACTTGAGAAACTGGAAGCATGCCTACTGCCTCGGA.

sh*Bach1*-2: TGCTGTTGACAGTGAGCGCGCCCGTATGCTTGTGTGATTATAGTGAAGCCACAGATGTATAATCACACAAGCATACGGGCATGCCTACTGCCTCGGA.

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, GOTERM_BP_FAT, GOTERM_MF_FAT, and GOTERM_CC_FAT, were used.

Expanded View for this article is available online.

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Author contributions

NT, EM, JNB, and EHB conceived and designed the experiments. NT and EM 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.

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