Introduction

GWAS have identified hundreds of loci associated with common human diseases and other clinically relevant traits. Most of these DNA-sequence variants map to noncoding regions of the human genome. The functional characterization of genotype-phenotype associations implicating noncoding variants remains a major bottleneck. Some noncoding variants influence phenotypic variation by modulating the activity of cell- or tissue-specific gene regulatory elements (1–3). The statistical enrichment of GWAS-implicated SNPs in regulatory sequences predicted from epigenomic profiling suggests a promising strategy for fine mapping (4, 5). However, relatively few examples of regulatory mechanisms at individual loci have been described in detail, limiting the ability to design informative high-throughput experiments to characterize causal variants and genes.

Erythropoiesis — the differentiation of hematopoietic stem cells into mature enucleated red blood cells (rbc) — is an auspicious system for dissecting how noncoding genetic variants influence phenotypes. The process is largely cell autonomous and driven by a small set of master transcription factors. Well-established cell-culture protocols exist to monitor proliferation and differentiation. Furthermore, GWAS have already revealed more than 100 loci associated with the number, size, or hemoglobin content of rbc (6, 7). Fine mapping these genetic associations with rbc traits promises not only to provide new illustrations of how noncoding variants influence complex phenotypes through effects on gene expression, but to also reveal genes that control rbc biology in health and disease.

Results

eQTL mapping in erythroblasts identifies cell-specific associations with gene expression. We mapped expression quantitative trait loci (eQTLs) in ex vivo–differentiated human erythroblasts, the nucleated precursors of mature rbc (8). To increase statistical power, we focused the eQTL search on 479 genes that display allelic imbalance (AI) (P < 2 × 10^{-9}) (Methods and Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI94378DS1). For each of these 479 genes, we tested to determine whether nearby SNPs (within 100 kb) were associated with their expression levels and had genotypes consistent with the observed AI effect (Figure 1A and Methods). We observed a strong enrichment of eQTLs among variants located near AI genes (Figure 1B). In total, we identified 6,325 significant eQTLs associated with the expression of 174 different genes at a false discovery rate (FDR) of less than 0.05 (Figure 1C and Supplemental Table 2). We observed further enrichment of erythroblast eQTLs within erythroid enhancers identified by DNase I hypersensitive site (DHS) and histone tail modification analyses and ChIP-sequencing (ChIP-seq) binding sites for the erythroid master transcriptional regulators GATA1 and TAL1 as well as the short binding motifs (12–18 bp) for GATA1 and GATA1::TAL1 (Figure 1C). We noted that the cooccurring GATA1::TAL1 motifs showed the greatest inflation among these annotations. Thus, epigenome features prioritize variants that control gene expression in human erythroblasts. Variants associated with rbc traits by GWAS were...
**Figure 1. eQTL mapping in erythroblasts.** (A) To identify eQTLs in erythroblasts (n = 24), we first focused on genes that show AI in at least 1 sample (n = 479 AI genes). Then we tested to determine whether SNPs located within 100 kb of these AI genes were associated with their expression level (left panel) and whether their genotypes were consistent with the expected AI ratio of reference allele/alternate allele (right panel). In this example, we highlight the candidate eQTL variant rs7287869 that is associated with the expression of the AI gene FAM118A. (B) Quantile-quantile plot of eQTL P values for variants located within 100 kb of 479 AI genes in human erythroblasts (black). Given that this analysis is limited to AI genes, we expected to observe a strong inflation of the eQTL test statistics (λ = 1.25). In comparison, the inflation is reduced (λ = 1.14) when analyzing variants located near 479 randomly selected non-AI genes (gray). This residual inflation could be explained if some of these genes have real eQTLs in the absence of AI or if they have AI effects that merely miss statistical significance. We generated subsets of SNPs overlapping erythroid enhancers (blue), GATA1 and TAL1 ChIP-seq peaks inside erythroid enhancers (purple), GATA1- or GATA1- TAL1–binding motifs inside erythroid enhancers (red and yellow, respectively), or all GATA1- or GATA1- TAL1–binding motifs (light and dark green, respectively). These subsets of variants show substantial enrichment (as summarized by the λ statistic) when compared with all SNPs (black). (C) Manhattan plot of eQTL P values. The dashed line corresponds to FDR q value = 0.05. (D) Number of genes that share at least 1 eQTL between erythroblasts and the GTeX tissues (at P < 0.001). The dashed line corresponds to the mean percentage of shared eGenes (mean = 20.8%).

Also overrepresented among significant erythroblast eQTLs (Supplemental Figure 1 and Supplemental Table 2) (7).

We compared our eQTL results with the GTeX data set (9). Although GTeX does not include erythroblasts, it is a powerful resource for confirming eQTL effects that are shared across cell types. Of the 5,924 erythroid eQTLs for which results were available in GTeX, 4,502 (76%) were replicated at P < 0.001 in at least 1 tissue. On average, human erythroblasts and individual GTeX tissue share 1,755 eQTLs that control the expression of 32 genes (Figure 1D and Supplemental Figures 2–4). We found 63 genes with candidate erythroblast-specific eQTLs (Supplemental Table 3). Overall, genes with eQTLs in erythroblasts were enriched for genes implicated in heme biosynthesis (P < 6.6 × 10⁻⁷) and mouse rbc phenotypes (P < 8.9 × 10⁻⁴) (Supplemental Table 4).

**ATP2B4** eQTLs and rbc traits. Our eQTL search highlighted many genes without known functions in erythropoiesis (e.g., MTHFR, GSTM3, RCBTB1) (Figure 1B and Supplemental Tables 1 and 2). Because many erythroid eQTLs are also associated with rbc traits, our results are useful for prioritizing candidate causal genes at these GWAS loci even if these genes have no known roles in rbc biology (e.g., SUCO, PTGHH, CDHI) (Supplemental Table 2). Among the top genes with erythroblast-specific eQTLs, we were particularly interested in ATP2B4 (also known as PMCA4) because it encodes the main calcium ATPase of rbc (Figure 2A and Supplemental Figure 5) and because the ATP2B4 locus is characterized by an interesting erythroid-specific chromatin landscape (see below). GTeX has identified eQTLs for ATP2B4, but these variants are in weak linkage disequilibrium (LD) with the erythroblast-specific eQTLs (r² < 0.09 in the 1000 Genomes Project, http://www.internationalgenome.org/) and are not associated with ATP2B4 expression levels in erythroblasts (P > 0.05 after correction for multiple testing) (Supplemental Figure 6). We noted that the same SNPs associated with the expression of ATP2B4 in human erythroblasts had previously been associated with mean corpuscular hemoglobin concentration (MCHC, a measure of rbc hydration) and susceptibility to severe malaria infection by GWAS (10–13), implicating ATP2B4 as the likely causal gene for these rbc-related phenotypes. Additional support for our results comes from a recent report that showed that the same SNPs are associated with ATP2B4 protein levels in human rbc (14).

To test the role of Atp2b4 in rbc phenotypes, we analyzed blood from mice with a targeted deletion of this gene. Atp2b4-knockout mice are viable, but characterized by male infertility and protection against pathological cardiac hypertrophy (15, 16).

We found that MCHC was elevated in these Atp2b4⁻/⁻ mice (Figure 2B), consistent with the observation that the allele associated with low ATP2B4 expression in erythroblasts is associated with higher MCHC in humans (12). These results corroborate that Atp2b4 plays a causal role in maintaining MCHC in vivo.

To extend the characterization of variants at the ATP2B4 locus and their effects on rbc phenotypes, we used the first release of the UK Biobank (UKBB) (http://www.ukbiobank.ac.uk/) to test the association between ATP2B4 erythroblast-specific eQTLs and 8 rbc traits (Supplemental Table 5). We observed a strong association between the A allele of rs7551442 and increased MCHC, replicating the signal from previous GWAS (P = 2.6 × 10⁻⁹, Figure 2, C and D, and Supplemental Figure 7) and consistent with a recent report (7). We also detected an association of this allele with decreased rbc distribution width (RDW) (P = 1.2 × 10⁻²²) and increased hemoglobin levels (P = 2.1 × 10⁻⁷) (Figure 2, C and D). The ATP2B4 genetic association signals with rbc traits in the UKBB were essentially identical to the ATP2B4 erythrocyte eQTL association signals in human erythroblasts (Figure 2, C and D). This concordance supports the hypothesis that the variants act on rbc traits and malaria susceptibility through an effect on the expression of ATP2B4 in erythrocyte cells.

An erythroid-specific regulatory element is required for ATP2B4 expression. Characterization of DHSs at the ATP2B4 locus revealed an intronic DHS peak that was present in both adult and fetal erythroblasts (Figure 2, C and D) (17). This DHS peak was also present in the K562 erythroleukemic cell line, but absent from 229 other cell types and tissues, including CD34+ hematopoietic stem/progenitor cells (HSPCs) (Supplemental Figure 8) (18, 19). Further annotation of this DHS revealed that it overlapped with an erythroblast enhancer chromatin signature as defined in primary human erythroid precursors (17) and with several GATA1-biding motifs, and harbored multiple erythroblast-specific eQTLs in strong LD that were associated with ATP2B4 expression and rbc phenotypes (Figure 2D and Figure 3A). Supporting the regulatory potential of this DHS in erythroid cells, a recent analysis of ENCODE data found that it showed allele-specific transcription factor binding only in K562 cells (20).

We posited that this region may harbor the causal variant or variants influencing ATP2B4 expression, rbc traits, and malaria susceptibility. To test this hypothesis, we deleted this region in a human erythroid precursor cell line (HUDEP-2) using the CRISPR-Cas9 system (21). We introduced 2 independent pairs of guide RNAs, one of which results in a 927-bp deletion and the other in a 889-bp deletion (Supplemental Table 6). We observed a dose-dependent reduction in ATP2B4 expression upon enhancer deletion, with biallelic deleted clones displaying only 3% residual ATP2B4 expression.

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**RESEARCH ARTICLE**

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To test the requirement of the enhancer element in nonerythroid cells, we generated 293T cells (human embryonic kidney cell line) expressing the ATP2B4 protein. There was an intermediate phenotype in monoallelic enhancer deleted cells (Figure 3B). A clone in which one copy of the enhancer was deleted and the other copy was inverted showed an expression pattern similar to that of the monoallelic enhancer deleted clones, suggesting that the enhancer can function independently of orientation in situ (Figure 3B).

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5 GATA1 or composite half-E-box/GATA1–binding motifs, binding sites for the transcription factors GATA1 and TAL1 (Figure 3A). We introduced into HUDEP-2 cells a pair of guide RNAs to generate a 98-bp deletion that removed the 3 SNP positions and these GATA1 motifs (Figure 3A and Supplemental Table 6). We observed that clones with biallelic deletion of this 98-bp segment had 83% reduction in expression of ATP2B4 when compared with ATP2B4 expression in WT cells (Figure 3D). To fine map the gene-expression regulatory element, we introduced 9 individual guide

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**Figure 3. Genome editing at the ATP2B4 erythroid enhancer.**  
(A) Upper panel shows deletions engineered around the ATP2B4 regulatory region. Lower panel shows sgRNA cleavage sites inside the 98-bp core enhancer. The alternative allele of rs10751451 creates a GATA1 motif (purple box). Boxes separated by dashed lines correspond to GATA1-TAL1 motifs.  
(B) ATP2B4 expression measured in HUDEP-2 cells. Combined analysis of 2 sgRNA pairs that result in overlapping 927-bp and 889-bp deletions. Nondeletion (n = 13), monoallelic deletion (n = 13), and biallelic deletion (n = 14) are clones with, respectively, 0, 1, or 2 ATP2B4 enhancer alleles deleted. None of these clones has an inversion allele. One clone was identified with 1 deletion allele and 1 inversion allele. Four clones exposed to nontargeting sgRNAs are shown for comparison. Gene expression is normalized to mean of nondeletion clones for the same sgRNA pair. Bars and whiskers show mean and SD. The difference in ATP2B4 expression levels between nondeletion and nontargeting clones is not significant.  
(C) ATP2B4 expression in 293T cell clones exposed to enhancer targeting sgRNA pairs, but without deletion (n = 4) or with biallelic deletion (n = 3). (D) ATP2B4 expression in HUDEP-2 cells with 98-bp core enhancer deletion. Control (n = 2) refers to 1 nontargeting control clone and unedited parental cells as compared with biallelic deletion clones (n = 11). (E) ATP2B4 expression in HUDEP-2 cells with individual sgRNAs specifying cleavages within the 98-bp core enhancer. Each dot indicates an edited bulk population that is an independent transduction of cells. Mean and SD are plotted for each of 4 biological replicates. Gene expression is normalized to unedited cells. In B and E, we used 1-way ANOVA with Bonferroni’s correction for the number of comparisons tested. In C and D, we used Welch’s t test. All P values are 2 tailed. **P < 0.01; ****P < 0.0001.
RNAs to produce small insertions-deletions (indels) at their cleavage sites within the 98-bp enhancer core (Figure 3A and Supplemental Table 6). We observed a significant reduction of ATP2B4 expression with one of the 9 guides (sg8 in Figure 3E). This guide RNA cleaves the enhancer directly over a GATA1-binding motif. Overall, these results demonstrate a hierarchical requirement for trait-associated sequences at the erythroid-specific enhancer of ATP2B4.

Finally, given ATP2B4’s role in rbc calcium homeostasis, we measured intracellular calcium concentration by ratiometric imaging in unedited HUDEP-2 cells as well as cells with a deletion of the ATP2B4 enhancer element. At baseline or upon stimulation (see Methods), we found higher intracellular calcium levels in ATP2B4-edited HUDEP-2 cells, indicating that cells that do not express ATP2B4 cannot efficiently pump calcium outside of the cytoplasm (Figure 4, A and B). In response to endoplasmic reticulum calcium release by caffeine stimulation, ATP2B4-deficient cells demonstrate exaggerated cytoplasmic calcium accumulation and persistence (Figure 4C). These results provide a physiological link between common regulatory SNPs at ATP2B4, a gene that encodes a major calcium pump, an ion homeostatic defect in erythroid cells, and human complex phenotypes, such as rbc hydration and susceptibility to severe malaria infection.

Discussion

Few GWAS discoveries have been investigated at the molecular and cellular levels. To explore the genetic architecture of regulatory variants that control rbc traits in humans, we undertook an eQTL search in human in vitro–differentiated erythroblasts. Although we identified more than 4,500 eQTLs that replicated...
in the GTEx data set, we acknowledge that some of our findings might be false-positive associations; independent replication studies in the same cell type are needed. Furthermore, because we limited our eQTL analyses to genes with AI and variants located within 100 kb of these genes in order to increase statistical power, we would have missed genes without exonic variants (necessary to monitor AI) or that are controlled by long-range regulatory variants. Despite these limitations, we found strong eQTLs for ATP2B4, validating our experimental design. Our own functional results and the recent report that the same SNPs are associated with ATP2B4 protein levels in human rbc (14) strongly argue that this is a true association signal.

Using human erythroblasts, knockout mice, and erythroid cells amenable to genome editing, we undertook the detailed characterization of the ATP2B4 locus and its roles in rbc biology. This comprehensive approach allowed us to identify the causal regulatory variants within an erythroid-specific enhancer, to confirm ATP2B4 as the causal gene, and to highlight the calcium homeostasis defect as one possible effector pathway responsible for the association with MCHC and malaria susceptibility (Figure 4D). Excess intracellular calcium activates a calcium-activated potassium channel (the Gardos channel), resulting in potassium efflux, rbc volume loss, and elevated MCHC. Hydration of rbc has been linked with clinical severity in the hemoglobin disorder sickle cell disease (22) and with infectivity by the malaria agent Plasmodium falciparum (23). Supported by our genetic and mechanistic results, the development of therapies that specifically modulate ATP2B4 activity could have a broad impact on rbc diseases that affect millions of individuals worldwide.

Methods

Cell culture, RNA sequencing, and DNA genotyping. The cell culture protocol to proliferate and differentiate human CD34+ HSPCs into erythroblasts has been described before (8, 24). We purchased human fetal (fetal liver, n = 12) and adult (bone marrow, n = 12) CD34+ HSCs from DV Biologics and Lonza, respectively. This sample size was limited our eQTL analyses to genes with AI and variants located within 100 kb of the AI genes. Second, we hypothesized that samples that are homozygous for the tested SNP (either reference/reference or alternate/alternate) should not show AI, whereas heterozygote samples should have AI. In other words, the reference allele:alternate allele ratio in heterozygote samples should be further from the expected 50:50 ratio than the ratio observed in homozygote samples. We tested this hypothesis using a 1-sided t test. Because the linear regression and t test P values were not correlated, we metaanalyzed these statistics using Fisher’s method to obtain a final P value. In situations of perfect LD between the potential regulatory and exonic variants, that is, when there are no homozygous samples at the exonic variants that are heterozygous at the regulatory variants, we cannot perform the concordance t test and simply report the linear regression results. We used a FDR methodology to correct for multiple testing, considering SNPs with a q value less than 0.05 as significant eQTLs.

Replication of eQTLs in GTEx. We used the GTEx database to replicate the eQTLs that we identified in human erythroblasts (9). GTEx does not include erythroblasts, but we reasoned that it would still replicate the eQTLs that we identified in human erythroblasts (9). GTEx does not include erythroblasts, but we reasoned that it would still replicate the eQTLs that we identified in human erythroblasts (9). GTEx does not include erythroblasts, but we reasoned that it would still replicate the eQTLs that we identified in human erythroblasts (9). GTEx does not include erythroblasts, but we reasoned that it would still replicate the eQTLs that we identified in human erythroblasts (9). GTEx does not include erythroblasts, but we reasoned that it would still replicate the eQTLs that we identified in human erythroblasts (9). GTEx does not include erythroblasts, but we reasoned that it would still replicate the eQTLs that we identified in human erythroblasts (9). GTEx does not include erythroblasts, but we reasoned that it would still replicate the eQTLs that we identified in human erythroblasts (9).

AI and eQTL mapping. We measured AI at each heterozygous geno-type covered by RNA-seq in the 24 human erythroblast samples. We only considered SNPs directly genotyped or with high imputation quality (R² > 0.6). We removed duplicated reads using the Picard MarkDuplicates tool (v. 1.96). We counted each read using the samtools (v. 1.1) mpileup software and genome build hg19 and kept uniquely mapping reads using the -q 50 argument (mapping quality > 50) and sites with base quality greater than 10. We further restricted the analysis to uniquely mapping sites as per the ENCODE 50-mer mappability track (score = 1) and removed sites showing mapping bias in simulations (28). We excluded sites with less than 30 overlapping reads. For a given heterozygous SNP, we determined the statistical significance of AI, that is, the difference between the observed and expected ratio of reference allele/alternate allele, with a binomial test. To account for read-mapping bias, we summed all reads overlapping all heterozygous SNPs in the RNA-seq data set and calculated the expected ratio for each combination of alleles in each sample independently. For SNPs with high sequencing coverage, we downsampled the number of reads that fell in the top 25th coverage percentile so that the most covered sites did not bias the expected ratio (29). We used Bonferroni’s correction to account for the number of tests performed: the significance threshold for this AI experiment was α = 2 × 10⁻⁵.

Next, we mapped the regulatory variants responsible for differential gene-expression phenotypes. Given our limited sample size, we focused on genes that showed AI, reasoning that the likelihood of finding significant eQTL was higher in this subset of genes. We developed a method that combines statistical evidence of AI and eQTL effects. First, we tested by linear regression the association between SNP genotypes (additive model) and gene-expression levels (expressed as log₁₀[FPKM + 1], where FPKM indicates fragments per kilobase of transcript per million reads), adjusting for cell type (fetal or adult). For these analyses, we only considered SNPs located within 100 kb of the AI genes. Second, we hypothesized that samples that are homozygous for the tested SNP (either reference/reference or alternate/alternate) should not show AI, whereas heterozygote samples should have AI. In other words, the reference allele:alternate allele ratio in heterozygote samples should be further from the expected 50:50 ratio than the ratio observed in homozygote samples. We tested this hypothesis using a 1-sided t test. Because the linear regression and t test P values were not correlated, we metaanalyzed these statistics using Fisher’s method to obtain a final P value. In situations of perfect LD between the potential regulatory and exonic variants, that is, when there are no homozygous samples at the exonic variants that are heterozygous at the regulatory variants, we cannot perform the concordance t test and simply report the linear regression results. We used a FDR methodology to correct for multiple testing, considering SNPs with a q value less than 0.05 as significant eQTLs.

eQTL enrichment analyses. We used 3 sources of information to test the enrichment of erythroid eQTL within specific genomic annotations. First, we obtained the coordinates of erythroid-specific enhancers defined using DHSs and histone tail modifications (17). From the same study, we also obtained genomic coordinates of GATA1 and TAL1 peaks determined by ChIP-seq (17). Finally, we used Homer software to identify binding motifs for GATA1 (MA0035.2) and GATA1::TAL1 (cooccurring GATA1 and half-E box motifs, MA0140.2) across the
human genome, or specifically within erythroid enhancer regions (30, 31). We carried out gene ontology and pathway enrichment analyses using the TopGene suite (topgene.cchmc.org) (32).

**Analyses of rbc traits in Atp2b4−/− mice.** Mice (male only, n = 26) with complete inactivation of Atp2b4 have been generated and described elsewhere (15, 16). The mice are in mixed background of B6C3F1 x C57BL/6. All mice used were males between 9 and 13 weeks of age. Mice were anesthetized with isoflurane (2.5%), and blood was collected from the jugular vein by venipuncture. The samples were measured within 6 hours of collection at room temperature in the biological research unit at Cancer Research UK Manchester Institute. Evaluation of hematological parameters was carried out in 2 batches on a Sysmex XT-2000iv (Sysmex) automated hematology analyzer using a mouse profile. Quality control was carried out before running each batch of samples. No randomization was used, and experimenters who did the complete blood count analyses were blinded to the animals’ Atp2b4 genotypes.

**Replication of the association between ATP2B4 and rbc phenotypes in the UKBB.** We tested the association between genotypes at the ATP2B4 locus (2 Mb) and rbc traits in the July 2015 release of the UKBB data set. We excluded participants with blood cancer, leukemia, lymphoma, bone marrow transplant, congenital or hereditary anemia, HIV, end-stage kidney disease, dialysis, splenectomy, or cirrhosis and those with extreme rbc trait measurements (>8 SD from the mean). We limited our analysis to participants of British ancestry with imputed genotype data available. In total, we tested the association between 8 rbc traits (hemoglobin, hematocrit, rbc count, mean corpuscular volume, mean corpuscular hemoglobin, MCHC, RDW, and reticulocyte count) and genotypes (additive model) in 136,727 participants with PLINK1.9 (https://www.cog-genomics.org/plink2). This sample size provides more than 99% power to replicate the association between ATP2B4 SNPs and MCHC at α = 0.05. After applying exclusion criteria, we corrected the rbc traits for age, sex, recruitment center, and cell counter and then normalized the residuals using inverse normal transformation. As covariates, we included in the association tests the 10 first principal components calculated using FlashPCA (33).

**Generating ATP2B4 deletions in cell lines.** HUDEP-2 cells and 293T cells with stable expression of Cas9 were generated by lentiviral transduction (lentiCas9-Blast, Addgene plasmid ID 52962) and blasticidin and 1 μg ml−1 puromycin selection to a publicly available sequence deconvolution algorithm (37). Reverse transcription–quantitative PCR. RNA was extracted for each selected clone using a kit (QIAGEN). 1 μg of RNA per clone was converted to cDNA using the iScript cDNA kit. Real-time reverse-transcription–quantitative PCR (RT-qPCR) was subsequently performed using SYBR Select Master Mix (Thermo Fisher Scientific). Primers were designed to span exon 5 and exon 6 of the ATP2B4 gene and were empirically validated for efficiency by serial dilution analysis (Supplemental Table 7). Gene expression was normalized to that of GAPDH. All gene expression data reported represent the mean of at least 3 technical replicates.

**Intracellular calcium monitoring.** Intracellular calcium levels in HUDEP-2 cell lines were monitored using Fura-Red, a ratiometric fluorescent calcium indicator, with a laser-scanning confocal microscope. Cells were first seeded (1 hour) in a coverslip-bottom chamber coated with Cell-Tak (Corning). Cells were then washed with HEPES-PSS and incubated with Fura-Red (10 μM) for 45 minutes at 37°C. Intracellular calcium levels were recorded on an LSM-Duo confocal microscope (Zeiss) with a 40× objective (Plan APO Oil DIC, 1.3 NA). Single emission fluorescence (LP575) was collected (10 FPS) upon alternate excitation (405 and 489 nm, solid state lasers) on a 512 × 256 field of view. Stacks of interleaved images (16 bits) were then analyzed using FIJI (ImageJ, NIH). Upon background subtraction, 20 × 20 pixel to produce interstitial deletions. Cells were also transduced with a pool of lentiviruses containing 10 unique nontargeting sequences (Supplemental Table 6). After transduction, bulk cultures were incubated for 7 to 10 days with 10 μg ml−1 blasticidin and 1 μg ml−1 puromycin selection to select for cells with edited alleles. Those bulk cultures transduced with tandem sgRNA lentiviruses were plated clonally at limiting dilution. 96-well plates with greater than 30 clones per plate were excluded to avoid mixed clones. After approximately 14 days of clonal expansion, genomic DNA was extracted using 50 μl QuickExtract DNA Extraction Solution per well (Epicentre). Clones were screened for deletion by conventional PCR, with 1 PCR reaction using primers internal to a segment to be deleted (nondeletion amplicon) and 1 gap-PCR reaction using primers across the deletion junction (deletion amplicon) that would produce a characteristic short amplicon in the presence of deletion (Supplemental Table 7). Clones bearing inversion alleles were also identified with one primer outside the segment to be deleted and the other primer inside the segment to be deleted, both in the same orientation with respect to the reference genome, as previously described. PCR was performed using the QIAGEN HotStarTaq 2× Master Mix and the following cycling conditions: 95°C for 15 minutes, 45 cycles of 95°C for 30 seconds, 60°C for 45 seconds, 72°C for 1 minute, 72°C for 10 minutes. Bipartite deletion clones were identified based on the presence of a deletion PCR band with absence of a nondeletion PCR band. Inversion clones were also identified as previously described. Compound deletion-inversion clones had 1 deleted allele and 1 inverted allele without the presence of nondeletion alleles. For disruption of individual GATA1 motifs, stable Cas9-expressing HUDEP-2 cells were transduced with lentiviruses carrying individual guide RNAs (lentiGuide-Puro) (Figure 3A and Supplemental Table 6).

Edited populations of cells were selected with puromycin and blasticidin and RNA was isolated 7 to 10 days following transduction. Genome editing with indel rates exceeding 75% was confirmed by isolating gDNA from each of these bulk populations of cells, performing a PCR reaction with primers flanking the edited region (Supplemental Table 6), and Sanger sequencing the amplicon with analysis according to a publicly available sequence deconvolution algorithm (37).

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circular regions of interest (ROIs) were manually positioned on cells and individual ROI mean fluorescence intensity was measured. Variations in intracellular calcium levels were expressed as the mean ratio of the fluorescence from calcium-bound (excitation wavelength 405 nm) and calcium-free (excitation wavelength 489 nm) Fura-Red of each ROI from 10 consecutive images.

Statistics. Statistical tests were performed using Welch’s t test, binomial test, linear regression, or 1-way ANOVA when appropriate (see specific Methods subsections and figure legends). Multiple testing correction was performed using Bonferroni’s method or an FDR procedure. Adjusted P values of less than 0.05 or q values of less than 0.05 were considered significant.

Study approval. Mouse experiments were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the University of Manchester Ethics Committee. Human genetic analyses were approved by the Montreal Heart Institute Ethics Committee, and informed consent was obtained from all participants.

Author contributions
SL, ESG, DEB, and GL conceived and designed the experiments. SL, ESG, MB, PGS, FS, AA, SP, and JL performed the experiments. SL, ESG, JL, DEB, and GL analyzed the data. RK, YN, EB, and DO contributed reagents and materials. SL, ESG, DEB, and GL wrote the manuscript with contributions from all authors.

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Address correspondence to: Daniel E. Bauer, Boston Children’s Hospital, Karp 8211, One Blackfan Circle, Boston, Massachusetts 02115, USA. Phone: 617.919.2508; Email: daniel.bauer@childrens.harvard.edu. Or to: Guillaume Lettre, Montreal Heart Institute, Research Centre (3rd floor), 5000 Belanger Street, Montreal, Quebec H1T 1C8, Canada. Phone: 514.376.3330; Email: guillaume.lettre@umontreal.ca.