Altered translation of GATA1 in Diamond-Blackfan anemia

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Ribosomal protein haploinsufficiency occurs in diverse human diseases including Diamond-Blackfan anemia (DBA)1,2, congenital asplenia3 and T cell leukemia4. Yet, how mutations in genes encoding ubiquitously expressed proteins such as these result in cell-type– and tissue-specific defects remains unknown5. Here, we identify mutations in GATA1, encoding the critical hematopoietic transcription factor GATA-binding protein-1, that reduce levels of full-length GATA1 protein and cause DBA in rare instances. We show that ribosomal protein haploinsufficiency, the more common cause of DBA, can lead to decreased GATA1 mRNA translation, possibly resulting from a higher threshold for initiation of translation of this mRNA in comparison with other mRNAs. In primary hematopoietic cells from patients with mutations in RPS19, encoding ribosomal protein S19, the amplitude of a transcriptional signature of GATA1 target genes was globally and specifically reduced, indicating that the activity, but not the mRNA level, of GATA1 is decreased in patients with DBA associated with mutations affecting ribosomal proteins. Moreover, the defective hematopoiesis observed in patients with DBA associated with ribosomal protein haploinsufficiency could be partially overcome by increasing GATA1 protein levels. Our results provide a paradigm by which selective defects in translation due to mutations affecting ubiquitous ribosomal proteins can result in human disease.

Diamond-Blackfan anemia (DBA, OMIM 105650) is characterized by a specific reduction in the production of red blood (erythroid) cells and their precursors without defects in other hematopoietic lineages2–5. In more than 50% of cases, DBA is caused by heterozygous loss-of-function mutations (haploinsufficiency) in 1 of 11 genes encoding ribosomal proteins1. Moreover, recent studies have shown that haploinsufficiency of ribosomal proteins can contribute to other cell-type–specific diseases in humans, including congenital asplenia and T cell lymphocytic leukemia3,4. How mutations that halve the quantity of ubiquitously expressed ribosomal proteins result in such specific human disorders remains unknown. Numerous theories have been proposed for the pathogenesis of these diseases7. However, these models do not explain the cell-type specificity of DBA and other ribosomal disorders. The experimental evidence to support such pathogenic models for DBA is often contradictory8. Animal models of DBA do not faithfully mimic the disease, and the involvement of different molecular pathways in the hematopoietic defects observed is variable9,10.

We reasoned that identifying genetic causes for the remaining 50% of DBA cases might provide insight into the pathogenesis of this disorder. We recently identified mutations in the GATA1 gene using whole-exome sequencing11, the first nonribosomal gene to our knowledge to be identified in DBA. GATA1 encodes a key hematopoietic transcription factor essential for the specification of erythroid cells, as well as megakaryocytes and eosinophils, from early hematopoietic stem and progenitor cells11,12. In humans, GATA1 mRNA is alternatively spliced to produce two forms of the protein: a long (or full-length) form derived from inclusion of the second exon and a short form without this exon, which therefore lacks the N-terminal 83 amino acids11,13. The GATA1 mutations that we previously identified occur in the splice donor site of exon 2 and affect splicing by impairing the production of the mRNA encoding the full-length form.

It remained unclear whether the pathogenic mechanisms of these GATA1 mutations are similar to those of ribosomal protein haploinsufficiency or whether GATA1 mutations represent a distinct subset of DBA7. In an attempt to address this question, we undertook a systematic screening for new GATA1 mutations in over 200 additional patients with DBA. We identified a highly informative mutation in a male patient who had received a clinical diagnosis of DBA (Fig. 1, Supplementary Fig. 1 and Supplementary Table 1). This mutation changed the first translation initiation codon in GATA1, ATG, to an ACG codon (Fig. 1a). Consistent with X-linked inheritance, the patient’s asymptomatic mother was a carrier for this mutation (Fig. 1a). When expressed in human 293T cells, wild-type GATA1 cDNA predominantly produced the full-length form of the protein.

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observation that mammalian ribosomes are capable of initiating βGATA1s in extracts from primary differentiating erythroid cells in culture.

To gain further insight into the physiological relevance of full-length GATA1 activity, we examined expression of GATA1 during human erythropoiesis and observed that expression of the full-length protein appears to be specifically upregulated in the course of erythroid differentiation (Fig. 1d).

These results, obtained from study of the GATA1 initiator codon mutation and from our studies of human erythropoiesis, suggest that the level of GATA1 full-length protein expression is critical for the promotion of normal erythropoiesis. We reasoned that the more commonly observed mutations in ribosomal protein genes in DBA might result in anemia by reducing production of the full-length GATA1 protein, thus connecting these two seemingly disparate sets of molecular lesions. To test this hypothesis, we focused initially on the ribosomal protein gene RPS19, which is mutated in approximately 25% of DBA cases1,2. We used a primary human erythroid culture system to study haploinsufficiency of RPS19 using shRNA-mediated knockdown (Fig. 2a)15–17. Reduced levels of RPS19 protein expression were associated with reduced GATA1 protein expression (both the long and short forms) (Fig. 2a). Although this primary cell system contains mostly erythroid lineage cells, there is the possibility that cells can differentiate into none-erythroid cell types, or that differentiation within the erythroid lineage is impaired, which could confound this analysis16. We therefore turned to the clonal human erythroid K562 cell line to examine whether we could observe similar phenomena in a more homogenous cell population. Upon knockdown of RPS19, we observed reduced GATA1 protein levels (Fig. 2b) without significant effects on the levels of major cellular proteins, as assessed by Coomassie blue staining of cell lysates (Fig. 2c). The levels of other erythroid-important proteins, including transferrin receptor, erythropoietin receptor (EPOR), Janus kinase-2, signal transducer and activator of transcription-5A (STAT5A) and T cell acute lymphocytic leukemia-1 (TAL1), were also unchanged by RPS19 knockdown (Supplementary Fig. 4a). We observed reduced protein levels of both RPS19 and GATA1 within 4 d of infection with shRNA-encoding virus, before major effects on cell growth occurred (Supplementary Fig. 4). Notably, GATA1 mRNA levels were not affected (Fig. 2d), consistent with the notion that an effect on protein translation accounts for the decreased levels of GATA1 protein.

To directly assess whether the decrease in GATA1 protein levels is due to an effect on translation, we performed polysome profiling and fractionation (Fig. 2e). This approach allows for assessment of mRNA abundance in actively translating ribosomes that are present in multiple copies on a single mRNA species, thereby forming polysomes indicative of productive translation initiation18,19. Whereas the level of GATA1 mRNA associated with monosomes was similar or higher in RPS19-knockdown cells as compared with controls (Supplementary Fig. 5), there was a two- to threefold reduction in GATA1 mRNA abundance in polysomes (Fig. 2f). In contrast, all other erythroid-important mRNAs tested showed a different pattern, with similar or increased abundance in larger polysomes following RPS19 knockdown (Fig. 2g). As an alternative approach to demonstrate a selective reduction in GATA1 translation, we labeled cells with the methionine analog L-azidohomoalanine20. When we performed chemical detection of L-azidohomoalanine on immunoprecipitated GATA1, we found a reduced level of newly translated GATA1 in cells with reduced RPS19 levels (Supplementary Fig. 6). Global translation was decreased to ~40% of that in controls (Supplementary Fig. 7), consistent with the slowing of cell growth observed upon RPS19 knockdown and the known global reduction of translation (48–75%) in patients with DBA21,22.

To gain insight into the mechanism by which ribosomal protein haploinsufficiency results in the observed selective defect in translation, we examined the effect of reducing the expression of other DBA-associated ribosomal proteins. When we used shRNA constructs to reduce expression of RPL11, RPL5 or RPS24, which are collectively mutated in ~15% of DBA cases1, we consistently noted decreased protein levels of GATA1 (Fig. 3a–c). The degree of reduction in GATA1 protein levels corresponded well with the extent of knockdown observed for the various ribosomal proteins (Fig. 3a–c and Supplementary Fig. 8). Highlighting the selectivity of this effect, the protein levels of other regulators of erythropoiesis such as EPOR, TAL1, transferrin receptor (CD71) and STAT5A showed no change after ribosomal protein knockdown (Fig. 3a–c), consistent with the results seen with RPS19 knockdown.

The similar defect in GATA1 translation resulting from reduction of ribosomal proteins from either the 40S or 60S subunit of the ribosome suggests that an impairment of translation initiation may underlie this observation, which is consistent with the concept that the availability of free ribosomes is the rate-limiting step in translation initiation23. Eukaryotic translation initiation factors (eIFs) play a critical role in translation initiation. This process begins as eIF4E initially interacts with the 5′ terminal cap of mRNAs and then binds eIF4G, which in
turn recruits multiple factors, including eIF4A and eIF3, and allows the ribosome to overcome restrictive motifs in the 5′ untranslated region (5′ UTR) to scan for the appropriate translation initiation AUG codon\textsuperscript{24}. We therefore used a selective inhibitor of the eIF4E-eIF4G interaction (termed 4EGI-1) to examine whether inhibition of this interaction would also selectively affect GATA1 translation\textsuperscript{25–27}. Notably, treatment of K562 cells with 4EGI-1 for 48 h led to a marked decrease in GATA1 protein, whereas other proteins tested were largely unaffected (Fig. 3d). This result shows that GATA1 mRNA has a more stringent requirement for eIF-dependent translation initiation compared to a number of other transcripts. Knockdown of the expression of RPS19, RPL11, RPL5 or GATA1 in primary CD34\textsuperscript{+} cells resulted in a decreased ratio of erythroid to nonerythroid cells (as assessed using the erythroid marker CD235a, also known as glycoporphin A) (Supplementary Figs. 9 and 10), a phenotype characteristic of DBA\textsuperscript{15,16}. Treatment of the primary cell cultures with 4EGI-1 caused a similar and dose-dependent decrease in the relative amount of erythroid cells (Fig. 3e and Supplementary Fig. 11). Notably, this decrease was associated with a reduction in GATA1 protein levels but not with changes in the levels of other tested proteins (Fig. 3f). The decreased erythropoiesis observed with 4EGI-1 treatment could largely be rescued by overexpression of GATA1 (Supplementary Fig. 12), suggesting that GATA1 is a critical downstream target when the eIF4E-eIF4G interaction is inhibited. Collectively, these results show that the reduction in GATA1 translation observed with reduced ribosomal protein levels probably reflects a broad sensitivity of GATA1 mRNA to impairment of translation initiation.

Cells can physiologically regulate gene expression through variation of mRNA translation initiation potential, as different mRNAs can have variable barriers to translation\textsuperscript{23,24}. A number of studies have demonstrated that transcripts with highly structured 5′ UTRs are more poorly translated and have a need for increased initiation potential by the cell\textsuperscript{23,24,28,29}. As the 5′ end of human GATA1 mRNA has never been fully characterized, we performed rapid amplification of the 5′ CDNA ends (5′ RACE) of human GATA1 transcripts from both primary erythroid and K562 cells. This analysis revealed a previously unreported 5′ end of GATA1 mRNA that we found on the majority of clones encoding either the short or the long mRNA isoform (Supplementary Fig. 13a,b). The 5′ end of human GATA1 mRNA was predicted to be highly structured (Supplementary Fig. 13c), and thus translation may be more readily impaired in settings where the translation initiation potential is reduced, such as with ribosomal protein haploinsufficiency\textsuperscript{23,29,30}. In support of this notion, we found using a reporter assay that the GATA1 5′ UTR restricted translation to a greater extent than did other 5′ UTRs of similar length (Supplementary Fig. 13d). Additionally, other transcripts with highly structured 5′ UTRs showed reduced association with larger polysomes (Supplementary Fig. 14), whereas the association of transcripts with unstructured 5′ UTRs with larger polysomes was not affected or was upregulated (Fig. 2g). Therefore, downregulation of translation of GATA1 mRNA—one of a select group of transcripts with a highly structured 5′ UTR—may act as an Achilles’ heel during hematopoietic development to impair erythropoiesis specifically in the context of DBA, consistent with our finding that rare mutations in the GATA1 gene itself are capable of causing DBA. It is possible that other undefined regulatory or structural motifs in the 5′ UTR of GATA1 mRNA could explain the observed effect on translation in the setting of reduced ribosomal protein levels, but we have been
unable to identify any characteristic motifs in this sequence. Mouse Gata1 mRNA has a much shorter and less structured 5' UTR than that of humans, which may explain the failure of ribosomal protein haploinsufficiency to downregulate translation of this gene and cause major impairments of erythropoiesis in mouse models.

To test whether our findings are of relevance in patients with DBA, we sought to assess GATA1 in erythroid cells from these patients. Given the difficulty of obtaining a sufficient number of stage-matched erythroid cells from patients with DBA to enable examination of GATA1 protein levels, we used RNA expression data to determine whether there was an alteration in the expression of GATA1 target genes. We performed global gene expression profiling on sorted erythroid progenitors (CD34+CD71highCD45RA− bone marrow mononuclear cells) from three patients with DBA with known RPS19 mutations (Online Methods) and six control subjects. This cell surface phenotype is known to selectively enrich for early erythroid progenitors (erythroid burst-forming and erythroid colony-forming units, or BFU-Es and CFU-Es, respectively); similar colony numbers are obtained from healthy individuals and patients with DBA. After normalization of the gene expression data, we examined both global gene expression and expression of a curated set of GATA1 target genes (Supplementary Fig. 15). The global gene expression profiles showed a high degree of correlation between DBA and control erythroid progenitors (R² = 0.990; Fig. 4a), indicating that we obtained comparable populations of cells from the patients and control subjects. We then applied the gene set enrichment analysis (GSEA) algorithm to assess the expression of GATA1 target genes. Consistent with our hypothesis, we noted significant global down-regulation of GATA1 target genes in erythroid progenitors from patients with DBA compared with controls (Fig. 4c,d, Supplementary Fig. 15 and Supplementary Tables 2–4). Of over 600 target gene sets for other transcription factors (based on the presence of transcription factor binding sites; Online Methods), none demonstrated such a marked and global downregulation, highlighting the selectivity of the effect on GATA1 activity. Notably, the expression of target genes for several other erythroid-important transcription factors and for the tumor suppressor p53 showed no significant changes (Supplementary Fig. 16 and Supplementary Table 5). GATA1 mRNA levels were comparable between DBA and control samples (Fig. 4b), providing support for the idea that altered GATA1 activity in DBA cells is mediated at the translational rather than transcriptional level. These results demonstrate that GATA1 activity is decreased in primary samples from patients with DBA with RPS19 mutations, consistent with the idea that GATA1 dysfunction underlies the erythroid differentiation defect in DBA.

We next examined whether reduced GATA1 levels are sufficient to impair erythropoiesis. When either ribosomal proteins or GATA1 were targeted with shRNAs, we consistently observed increased apoptosis, decreased cell numbers and decreased erythroid differentiation of primary hematopoietic cells (Supplementary Figs. 4, 9 and 10). These findings are consistent with observations made in patients with DBA with mutations in the genes encoding these ribosomal proteins or GATA1. We modified the GATA1 cDNA to contain an optimized 5' UTR lacking known structural barriers to translation, such that it should be effectively translated. Infection with lentivirus harboring this modified GATA1 cDNA rescued the increased apoptosis of erythroid cells with RPS19 knockdown, whereas the mutant GATA1 cDNA that predominantly produces the short form of the protein showed only partial rescue (Supplementary Fig. 17a,b). To assess whether GATA1 could rescue the defect in erythroid differentiation observed in primary hematopoietic cells with reduced ribosomal protein levels, we concomitantly transduced primary cultured CD34+ cells with shRNAs targeting RPL11 or RPL5 and with the modified GATA1 cDNA or appropriate controls. Introduction of GATA1 into these cells improved erythropoiesis (Supplementary Fig. 17c) to an extent comparable to that observed with introduction of the deficient ribosomal protein gene itself, whereas the mutant version of GATA1 had a lower level of rescue activity. Notably, introduction of GATA1 lentivirus into primary bone marrow mononuclear cells from patients with DBA resulted in a two- to fourfold increase in the ratio of CD235a+ erythroid to CD235a− nonerythroid cells (Fig. 4e,f). Not only was the frequency of erythroid cells increased, but there was also an improvement in erythroid differentiation, as indicated by smaller cell size (a reduced cell size characterizes more mature erythroid cells), mature erythroid cell morphology and increased expression of genes critical for terminal erythroid maturation (Fig. 4g–i and Supplementary Fig. 18). These findings show that GATA1, although likely not the only target of dysregulated protein translation in DBA, is a key factor in mediating the erythroid-specific defect observed in this condition.
Given the limitations in comparing erythroid differentiation in samples from patients with DBA, which show delayed and impaired maturation, with erythroid differentiation in cells from normal individuals, the extent of this rescue is difficult to quantitatively assess and is likely to be partial. Nonetheless, these results suggest that modulation of GATA1 protein levels in the context of ribosomal protein haploinsufficiency can improve erythropoiesis, with implications for potential therapeutic approaches to ameliorate DBA and related forms of anaemia, such as that observed in 5q–myelodysplastic syndrome.

Our finding of impaired translation of GATA1 mRNAs by ribosomes in DBA adds to an increasing appreciation for the role of translational control in mediating cell-type–specific gene expression. We showed that mutations in the GATA1 gene itself can cause DBA by reducing full-length protein levels, and that the more common mutations in genes encoding ribosomal proteins result in impaired erythropoiesis through, at least in part, the same mechanism of decreased GATA1 protein production (Supplementary Fig. 19). Although prior work has implicated p53 in mediating some of the defects observed in DBA and has shown that GATA1 regulates the p53 pathway, the defects we observed in K562 cells upon GATA1 or ribosomal protein knockdown occurred in the absence of p53 (ref. 39). Thus, the cell-type specific nature of the DBA phenotype is probably mediated by multiple pathways, including the p53 pathway, that lie downstream of GATA1. Indeed, we observed that p53 expression is upregulated upon reduction of RPS19 or GATA1 levels in primary hematopoietic cells transduced with GATA1 or control lentivirus. The forward scatter intensity is shown as mean ± s.d. (for three independent samples). ***P < 0.001 using an unpaired two-tailed Student’s t-test.

More generally, our findings suggest that mRNAs that are inefficiently translated by the ribosome experience a further reduction in protein translation under conditions of limited ribosome abundance (or with other impairments in translation initiation), such that synthesis of their encoded proteins would be selectively impaired. Alterations in protein translation have been implicated in diverse human diseases including autism, cancer and other blood disorders. Our finding of selectively impaired protein translation resulting from ribosomal protein haploinsufficiency provides a paradigm for understanding the cell-type–specific defects observed in DBA and conditions such as congenital asplenia and childhood leukemia. In addition, the deeper understanding of DBA pathogenesis that is presented here suggests potential therapeutic avenues that would involve targeting of GATA1 protein production through either gene therapy or small-molecule approaches for amelioration of the anaemia observed in this disease.
METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. The microarray data can be found in the Gene Expression Omnibus with accession number GSE41817.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
L.S.L and V.G.S. conceived the project; L.S.L., H.T.G., J.C.E., S.W.E., R.G., A.H.B., L.S.L., H.T.G., E.S.L. and V.G.S. wrote the paper with input from all authors.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Cell culture. 293T cells (ATCC) were maintained in DMEM with 10% FBS (FBS), 2 mM t-glutamine and 1% penicillin-streptomycin (P/S). K362 cells (ATCC) were maintained at a density between 0.1 × 10^6 and 1 × 10^6 cells per milliliter in RPMI 1640 medium supplemented with 10% FBS, 2 mM t-glutamine and 1% P/S. Culture of primary human cells is described below. Cells were incubated at 37 °C with 5% CO₂. Where indicated, cultures were supplemented with 10–100 μM 4EGI-1 (sc-202997, Santa Cruz Biotechnology) or DMSO.

Culture of human adult peripheral blood–mobilized CD34+ progenitors was performed using a two-stage culture method, as described previously. CD34+ cells were obtained from magnetically sorted mononuclear samples of G-CSF–mobilized peripheral blood from donors and were frozen after isolation. Cells were obtained from the Fred Hutchinson Cancer Research Center, Seattle, USA. Cells were thawed and washed into PBS with 1% FBS, pelleted and then seeded in StemSpan SFEM medium ( StemCell Technologies, Inc.) with 1× CC100 cytokine mix ( StemCell Technologies, Inc.) and 1% P/S. Cells were maintained in this expansion medium at a density between 0.1 × 10^6 and 1 × 10^6 cells per milliliter, with medium changes every other day as necessary. Cells were kept in expansion medium for a total of 5 d. After this expansion phase, the cells were reseeded into StemSpan SFEM medium with 1% P/S, 20 ng/mL SCF (PeproTech, Inc.), 1 U/mL Epo (Amgen), 5 ng/mL IL-3 (PeproTech, Inc.), 2 μM dexamethasone (Sigma-Aldrich) and 1 mM β-estradiol (Sigma-Aldrich). Cells were maintained in differentiation medium, with medium changes every other or every third day as needed. Cells were maintained at a density between 0.1 × 10^6 and 1 × 10^6 cells per milliliter.

Bone marrow mononuclear cells from patients with DBA and healthy controls were isolated from human bone marrow aspirates using Ficol-Paque Plus (17-1440-02, GE Healthcare) density gradient centrifugation. Mononuclear cells were cultured in IMDM with 3% human type AB plasma, 2% human AB serum, 1% P/S, 5 U/mL heparin, 200 μg/mL Holo-Transferin (Sigma-Aldrich), 10 μg/mL insulin, 10 ng/mL SCF (PeproTech, Inc.), 1 ng/mL IL-3 (PeproTech, Inc.), 2 μM dexamethasone (Sigma-Aldrich) and 1 U/mL Epo (Amgen), similarly to recently described protocols for culturing human erythroid cells. Cells were cultured for 1–2 d before infection as described below and analyzed 4–5 d after infection.

Bone marrow mononuclear cells from patients with DBA or healthy donors were collected after appropriate informed consent was obtained. Patient 1 was male and was 6 months old at the time of bone marrow collection. The patient had been noted to have fussiness and pallor at 4 months of age, and a complete blood count performed at that time showed a hemoglobin level of 4.3 g/dL. The patient remained on monthly transfusions to avoid symptomatic anemia. The patient had no physical anomalies. The erythrocyte adenine deaminase level was elevated at 1.83 IU per gram of hemoglobin. All 11 known ribosomal protein genes, as well as GATA1, were sequenced in this patient, and no definitive pathogenic mutations were found. Patients 2 and 3 were both male and were 34 and 27 years old, respectively, at the time of bone marrow collection. These patients both had a macrocytic anemia that was diagnosed in infancy. Both individuals had required intermittent transfusions, but details of their medical history were limited, as only select records were available for review. Both patient 2 and patient 3 were dependent on steroid therapy to prevent the need for transfusions. Neither individual had any physical anomalies, and erythrocyte adenine deaminase levels were elevated at 0.99 and 1.92 IU per gram of hemoglobin (normal range 0.33–0.96 IU per gram of hemoglobin), respectively. Patients 2 and 3 had the RPS19 mutations c.3G>A Met1Ile and c.185G>A Arg62Gln, respectively.

Study approval. All patients or their families provided written informed consent to participate in this study. The use of human cells was approved by the Institutional Biosafety Committee and Institutional Review Board of Boston Children’s Hospital and the Committee on the Use of Humans as Experimental Subjects at the Massachusetts Institute of Technology.

Lentiviral vectors and infection. The shRNA constructs targeting human RPS19 (sh1913 and sh916, RefSeqID NM_0010202), human GATA1 (sh19-23, RefSeq ID NM_0020499), human RPS24 (sh1-2, RefSeq ID NM_001026), human RPL5 (sh1-5 RefSeq ID NM_000969) and human RPL11 (sh1-5 RefSeq ID NM_000975) were obtained from the Mission shRNA collection ( Sigma-Aldrich). The constructs were in the pLKO.1-puro lentiviral vector. The sequences of the shRNAs used in this study are listed in Supplementary Table 6.

As controls, the lentiviral vectors pLKO-GFP and pLKO.I (the empty pLKO.1 vector with a 1.2 kb stuffer element) were used (The RNAi Consortium of the Broad Institute of MIT and Harvard). For rescue experiments, erythroid cells were cotransduced with shRNAs targeting ribosomal proteins with either the HMD control, HMD-GATA1 or HMD-GATA1 mutant, which contain the respective cDNAs. The HMD-GATA1 construct was made by subcloning the GATA1 cDNA into the HMD lentiviral vector using the EcoRI and XhoI restriction sites in this vector. The HMD-GATA1 T-C mutant construct was made by performing site-directed mutagenesis of the original HMD-GATA1 construct using the QuickChange II site-directed mutagenesis kit (Agilent). Double-transduced cells were identified by puromycin selection and GFP expression driven by an IRES-GFP in the HMD vector.

For production of lentiviruses, 293T cells were transfected with the appropriate viral packaging and genomic vectors (pVSVG-G and pDelta8.9) using FuGene 6 reagent (Promega) according to the manufacturer’s protocol. The medium was changed the day after transfection to RPMI 1640 or StemSpan SFEM medium. After 24 h, this medium was collected and filtered using an 0.45-μm filter immediately before infection of primary hematopoietic or K362 cells. The cells were mixed with viral supernatant in the presence of 8 μg/mL polybrene (Millipore) in a 6-well plate at a density of 250,000–500,000 cells per well. The cells were spun at 2,000 r.p.m. for 90 min at 22 °C and left in viral supernatant overnight. The medium was replaced the morning after infection. Selection of infected cells was started 24 h after infection with 1 μg/mL or 2 μg/mL puromycin for primary hematopoietic cells and K362 cells, respectively. The infection efficiency for pLKO-GFP–infected cells was assessed by measuring the frequency of GFP+ cells by flow cytometry 48 h post infection. Typically, the frequency of GFP+ cells was between 30–60% and >95% for primary hematopoietic cells and K362 cells, respectively.

Quantitative RT-PCR. Isolation of RNA was performed using the miRNeasy Mini Kit (Qiagen). An on-column DNase (Qiagen) digestion was performed according to the manufacturer’s instructions. RNA was quantified by a NanoDrop spectrophotometer (Thermo Scientific). Reverse transcription was carried out using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using the ABI 7900 Machine Real-Time PCR system and SYBR green PCR Master Mix (Applied Biosystems). Quantification was performed using the ∆∆Ct method. Normalization was performed using β-actin mRNA as a standard, unless otherwise indicated. The primers used for quantitative RT-PCR are listed in Supplementary Table 7.

5′ RACE. 5′ RACE was conducted using total RNA from K362 cells and in vitro cultured primary erythroid cells using the FirstChoice RLM-RACE Kit (AM1700, Life Technologies) according to the manufacturer’s instructions. RACE PCR products were subcloned using the TOPO TA cloning kit (450641, Invitrogen) followed by Sanger sequencing. The primers used for 5′ RACE are listed in Supplementary Table 7.

Western blotting. Cells were harvested at indicated time points, washed twice in PBS, resuspended in RIPA lysis buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate, 1 mM DTT) supplemented with 1× Complete Protease Inhibitor Cocktail (Roche) and incubated for 30 min on ice. After centrifugation at 14,000 r.p.m. for 10 min at 4 °C to remove cellular debris, the remaining supernatant was transferred to a new tube, supplemented with sample buffer and incubated for 10 min at 70 °C. Equal amounts of proteins were separated by SDS gel electrophoresis using the NuPAGE Bis-Tris gel system (Invitrogen) and MOPS running buffer. Subsequently, proteins were transferred onto a PVDF membrane using NuPAGE transfer buffer (Invitrogen). Membranes were blocked with 3% BSA-PBST for 1 h and probed with GATA1 goat polyclonal antibody (M-20, sc-1234, Santa Cruz Biotechnology) at a 1:500 dilution, RPS19 mouse monoclonal antibody (WW-4, sc-100836, Santa Cruz Biotechnology) at a 1:500 dilution, RPL5 goat polyclonal (D-20, sc-103865, Santa Cruz Biotechnology) at a 1:500 dilution, RPL11 goat polyclonal (N-17, sc-25931, Santa Cruz Biotechnology) at a 1:500 dilution, RPLS goat polyclonal (D-20, sc-103865, Santa Cruz Biotechnology) at a 1:500 dilution.
Biotechnology) at a 1:500 dilution, RPS20 goat polyclonal (G-15, sc-53035, Santa Cruz Biotechnology) at a 1:500 dilution, RPS24 rabbit polyclonal (ab102986, Abcam) at a 1:1,000 dilution, EPOR rabbit polyclonal (M-20, sc-697, Santa Cruz Biotechnology) at a 1:500 dilution, CD71 rabbit polyclonal (H-300, sc-9099, Santa Cruz Biotechnology) at a 1:500 dilution, JAK2 rabbit polyclonal (Clone 06–255, Millipore) at a 1:750 dilution, STAT5A rabbit polyclonal (C-17, sc-435, Santa Cruz Biotechnology) at a 1:500 dilution, TAL1 goat polyclonal (C-21; sc-12984, Santa Cruz Biotechnology) at a 1:500 dilution, p53 rabbit monoclonal (7F5, 2527, Cell Signaling Technology) at a 1:1000 dilution, β-actin mouse monoclonal (AC-15, Sigma) at a 1:2,500 dilution or GAPDH mouse monoclonal antibody (GC5; sc-32233, Santa Cruz Biotechnology) at a 1:1,000 dilution in 3% BSA-PBST for 1 h at room temperature or overnight at 4 °C. Membranes were washed four times with PBST, incubated with donkey anti-mouse, anti-goat or anti-rabbit peroxidase-coupled secondary antibodies (715-035-150, 705-035-147 or 711-035-152, respectively; Jackson ImmunoResearch) at a 1:5,000 to 1:10,000 dilution in 3% BSA-PBST for 1 h at room temperature, washed three times with PBST and incubated for 1 min with Western Lightning Plus-ECL substrate (PerkinElmer). Proteins were visualized by exposure to scientific imaging film (Kodak).

Flow cytometry analysis and apoptosis. For flow cytometry analysis, in vitro cultured erythroid cells were washed in PBS and stained with propidium iodide (PI), 1:60 APC-conjugated CD235a (glycophorin A, clone HIR2, 17-9978-47, eBioscience), 1:60 FITC-conjugated CD71 (OKT9, 1-0719-42, eBioscience), 1:60 PE-conjugated CD41a (HIP12, 12-0419-42, eBioscience) and 1:60 PE-conjugated CD11b (ICRF44, 12-0118-42, eBioscience). For apoptosis analysis, the Annexin V-APC staining kit was used according to the manufacturer’s instructions (550474, BD Pharmingen). FACS analysis was conducted on a BD Bioscience LSR II flow cytometer. Data were analyzed using Flowjo 8.6.9 (TreeStar).

Flow cytometry–activated cell sorting of human bone marrow populations. Mononuclear cells from human bone marrow aspirates were isolated by Ficoll-Paque PLUS (17-1440-02, GE Healthcare) density gradient centrifugation. Cells were preincubated with human Fc receptor binding inhibitor (14-9161, eBioscience) and stained with propidium iodide (PI) and antibodies against CD235a, CD41a and CD71 as described above. Cell sorting was conducted on a BD Bioscience Aria I.

Untranslated region reporter constructs and luciferase reporter assay. For generation of the luciferase reporter constructs, the 5’ UTR of the luciferase gene in the pGL3-Promoter Vector (Promega) was replaced by the 5’ UTRs of GAPDH, ACTB or GATA1. The 5’ UTRs were inserted 14 bp downstream of the major transcription initiation site of the SV40 promoter. 293T cells were seeded in a 24-well plate at a density of 50,000 cells per well. For transfection of 293T cells, 350 ng of each of the luciferase reporter vectors were cotransfected with 35 ng per well of the pRL-SV40 vector (Promega) using the FuGene 6 reagent according to the manufacturer’s protocol (Promega). Cells were incubated at 37 °C with 5% CO2 until analysis at 48 h. For measuring luciferase reporter activity, the Dual-Glo Luciferase assay system (E2920, Promega) was used according to the manufacturer’s protocol. Briefly, cells were resuspended in Dual-Glo Luciferase assay reagent and incubated at room temperature for 10–30 min, followed by measurement of firefly luminescence on a Safire 2 microplate reader (Tecan). Subsequently, Dual-Glo Stop and Glo reagent was added to the suspension and incubated for 10–30 min at room temperature, followed by measurement of Renilla luciferase activity. For each sample, the ratio of firefly/Renilla luminescence was calculated and normalized to the signal of the GAPDH-5’ UTR construct.

Polyosome profiling. K562 cells were incubated with 100 µg/ml of cycloheximide for 10 min at 37 °C, washed twice with ice-cold PBS containing 100 µg/ml of cycloheximide and lysed in 10 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 100 mM KCl, 2 mM DTT, 100 µg/ml cycloheximide, 500 µM 2’-O-MeRNA (Promega) and 1× Complete Protease Inhibitor, EDTA-free (Roche) by passing the lysate through a 26-gauge needle 4 times. Polysomes were separated on a 10–50% linear sucrose gradient containing 20 mM HEPES-KOH (pH 7.4), 5 mM MgCl2, 100 mM KCl, 2 mM DTT and 100 µg/ml cycloheximide and centrifuged at 36,000 r.p.m. for 2 h in a SW41 rotor in an XE-90 ultracentrifuge (Beckman Coulter). Gradients were fractionated using a Biocomp Gradient Station fractionator. Absorbance at 254 nm was used to visualize the gradients using an Econon UV monitor (Bio-Rad). Fractions were collected and phenol-chloroform extraction was performed to isolate RNA. β-actin primers were used for normalization of abundance of the mRNA of interest in monosome and polysome gradient fractions, similarly to what has been previously described43.

Protein labeling and detection. Click-IT chemistry was conducted for metabolic labeling of proteins. Briefly, K562 cells were washed with warm PBS and incubated in methionine-free RPMI medium (R7513, Sigma-Aldrich) with 10% FBS and 2 mM l-glutamine for 1 h at 37 °C, 5% CO2 to deplete methionine reserves. For labeling, Click-IT AHA (l-azidohomoalanine, C10102, Life Technologies) was added at a final concentration of 50 µM for 4 h at 37 °C, 5% CO2. Cells were harvested, washed twice in PBS, resuspended in RIPA lysis buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate) supplemented with 1× Complete Protease Inhibitor Cocktail (Roche) and incubated for 30 min on ice. After centrifugation at 14,000 r.p.m. for 10 min at 4 °C to remove cellular debris, the remaining supernatant was transferred to a new tube. 50 µg of protein lysate was used for the Click reaction with tetramethylrhodamine alkyne (TAMRA, T10183, Life Technologies) using the Click-IT Protein Reaction Buffer Kit (C10276, Invitrogen) in a total volume of 200 µl according to the manufacturer’s instructions. Total cell proteins were separated by SDS-gel electrophoresis, and TAMRA signal was detected on a Typhoon 9200 imager (Amer sham Biosciences) using 532 nm excitation and 580 nm long-pass emission. After TAMRA imaging, gels were post-stained with SYPRO Ruby Protein Gel Stain (S12001, Invitrogen) and imaged on a Typhoon 9200 imager using 473 nm excitation and 610 nm long-pass emission.

Immunoprecipitation coupled to Click-IT on beads labeling. Whole cell lysate was used for immunoprecipitation conducted with a goat polyclonal antibody against GATA1 (M-20, sc-1234, Santa Cruz Biotechnology) bound to Dynabeads Protein G (10003D, Life Technologies) for 3 h with rotation at 4 °C. The Dynabeads-antibody-antigen complex was washed three times with RIPA buffer and resuspended in 50 µl RIPA buffer, and the Click-IT reaction was performed using TAMRA alkyne and the Click-IT Protein Reaction Buffer Kit (C10276, Invitrogen) for 1 h at 4 °C in a total reaction volume of 200 µl. The immunoprecipitate was then washed once in RIPA buffer and then bound proteins were eluted in 40 µl 2× LDS buffer incubated at 70 °C for 10 min. Proteins were separated by SDS-gel electrophoresis followed by TAMRA detection as described above or western blot analysis using antibodies against GATA1.

Sanger sequencing and analysis. Sanger sequencing of patient samples was performed using standard PCR-based methods and analyzed as has been described43.

Microarray gene expression analysis. Bone marrow mononuclear cell samples were obtained from three patients with DBA with RPS19 mutations (a frameshift mutation at codon 84, Arg62Gln, and Leu131Arg), as well as six control samples from healthy individuals. CD71highCD34+CD45− cells populations from mononuclear cells were sorted as described43, and RNA was isolated from these samples. The RNA was hybridized to Affymetrix HG-133A microarrays, and the arrays were scanned for further analysis. Subsequently, the array data were normalized using the RMA normalization method as implemented in the Bioconductor package in R (http://www.bioconductor.org/). These data are deposited under accession number GSE41817 in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). GATA1 target genes were derived from the GSE628 data set by comparing either the 21 h vs. 0 h time points (the majority of known GATA1 upregulated targets have been shown to be upregulated at the 21 h timepoint) or the 30 h vs. 0 h time points (ref. 42). Genes with a log2 change of >1.0 and with a P value of <0.05 at the two sets of time points were used to derive gene sets43, which are lists of genes that were used for the enrichment analysis discussed below. In addition, GATA1 targets were derived from the TRANSFAC database (http://www.gene-regulation.com/pub/databases.html)43. These gene sets were
used to run the gene set enrichment analysis (GSEA) algorithm, as described\textsuperscript{34}. As controls, we used gene sets that contain genes that share a transcription factor binding site defined in the TRANSFAC using the C3 transcription factor target set from the MSigDB database (http://www.broadinstitute.org/gsea/msigdb/index.jsp).

**Statistical analyses.** All pairwise comparisons were assessed using an unpaired two-tailed Student’s $t$-test, unless otherwise indicated in the main text. Results were considered significant if the $P$ value was <0.05.

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