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Altered translation of GATA1 in Diamond-Blackfan anemia

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Abstract

Ribosomal protein haploinsufficiency occurs in diverse human diseases including Diamond-Blackfan anemia (DBA),[1,2] congenital asplenia,[3] and T-cell leukemia.[4] Yet how mutations in such ubiquitously expressed proteins result in cell-type and tissue specific defects remains a mystery.[5] Here, we show that *GATA1* mutations that reduce full-length protein levels of this critical

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AUTHOR CONTRIBUTIONS

L.S.L., H.T.G., and V.G.S. conceived the project; L.S.L, H.T.G., J.C.E., S.E., R.G., A.H.B., C.A.S., and V.G.S. performed the research; L.S.L., H.T.G., P.T., H.F.L., E.S.L., and V.G.S. analyzed data; T.I.G. and J.R.G. provided clinical assessments; L.S.L., H.F.L., E.S.L., and V.G.S. wrote the paper with input from all authors.

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hematopoietic transcription factor can cause DBA in rare instances. We show that ribosomal protein haploinsufficiency, the more common cause of DBA, can similarly reduce translation of *GATA1* mRNA - a phenomenon that appears to result from this mRNA having a higher threshold for initiation of translation. In primary hematopoietic cells from patients with *RPS19* mutations, a transcriptional signature of GATA1 target genes is globally and specifically reduced, confirming that the activity, but not the mRNA level, of GATA1 is reduced in DBA patients with ribosomal protein mutations. The defective hematopoiesis observed in DBA patients with ribosomal protein haploinsufficiency can be at least partially overcome by increasing GATA1 protein levels. Our results provide a paradigm by which selective defects in translation due to mutations in ubiquitous ribosomal proteins can result in human disease.

Diamond-Blackfan anemia (DBA, MIM 105650) is characterized by a specific reduction in the production of red blood (erythroid) cells and their precursors without defects in other hematopoietic lineages.[2,6] In more than 50% of cases, DBA is caused by heterozygous loss-of-function mutations (haploinsufficiency) in one of 11 genes encoding ribosomal proteins.[1] 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 leukemia.[3,4] It remains entirely mysterious how mutations that halve the quantity of ubiquitously expressed ribosomal proteins result in such specific human disorders. Numerous theories have been proposed for the pathogenesis underlying these diseases.[7] However, these models are unable to explain the exquisite cell-type specificity of DBA and the other ribosomal disorders. The experimental evidence to support such pathogenic models for DBA is often contradictory.[8] Animal models of DBA do not faithfully mimic the disease and the involvement of different molecular pathways in the hematopoietic defects observed is variable.[9,10]

We reasoned that identifying genetic causes for the remaining 50% of DBA cases might provide insight into the mysterious pathogenesis of this disorder. We recently reported the first non-ribosomal gene mutated in DBA, which we identified by using whole-exome sequencing.[11] Specifically, we reported mutations in the *GATA1* gene, which 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 cells.[11,12] 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 that therefore lacks the N-terminal 83 amino acids.[11,13] The *GATA1* mutations that we previously identified occur in the splice donor site of exon 2; they affect splicing by impairing the production of the mRNA encoding the full-length form.

While intriguing, it remained unclear whether the *GATA1* mutations act through a similar mechanism as the haploinsufficiency for ribosomal proteins or represent a distinct subset of DBA.[7] In an attempt to address this question, we undertook a systematic screening for novel *GATA1* mutations in over 200 additional DBA patients and identified a highly informative mutation in a male patient who had received a clinical diagnosis of DBA (Fig. 1 and Supplementary Fig. 1, Supplementary Table 1). This distinct mutation changed the first translation initiation codon in *GATA1*, ATG, to an ACG (Fig. 1a). Consistent with X-linked

inheritance, the patient's asymptomatic mother was a carrier for this mutation (Fig. 1a). When expressed exogenously in human 293T cells, wild-type *GATA1* cDNA predominantly produced the full-length form of the protein (Fig. 1c). In contrast, the ACG mutant cDNA predominantly expressed the short form of GATA1 lacking the first 83 amino acids, but the mutant cDNA also produced a low-level of full-length GATA1 (Fig. 1c, Supplementary Figure 2), consistent with the observation that mammalian ribosomes are capable of initiating translation at specific non-AUG triplets, including ACG.[14] We ruled out the possibility that the residual full-length GATA1 production resulted from contamination through the use of independent clones and by sequencing (Supplementary Fig. 3). This observation indicates that DBA can be caused by distinct mutations that reduce, but do not entirely abolish, production of full-length GATA1. To gain further insight into the physiological relevance of full-length GATA1 activity, we examined expression of GATA1 proteins during human erythropoiesis and observed that the full-length protein form appears to be specifically upregulated in the course of erythroid differentiation (Fig. 1d).

Our findings from an informative patient and studies of human hematopoiesis suggest that the level of GATA1 full-length protein expression appears critical for the promotion of normal erythropoiesis. This suggests the hypothesis that the more commonly observed ribosomal protein mutations in DBA may result in anemia by reducing the protein production of GATA1 and thus connect these two seemingly disparate sets of molecular lesions. To test this hypothesis, we initially focused on the ribosomal protein gene, *RPS19*, which is mutated in approximately 25% of DBA cases.[1,2] We utilized a primary human erythroid culture system to study haploinsufficiency of RPS19 using short hairpin RNAs (shRNAs; Fig. 2a).[15–17] We were able to reduce RPS19 protein expression and concomitantly we noted reduced GATA1 protein expression (of both long and short forms) with equal amounts of protein lysate loaded (Fig. 2a). While this primary cell system primarily contains erythroid lineage cells, there is the possibility of differentiation to other lineages and impairments in differentiation within the erythroid lineage, which could therefore confound this analysis.[16] We therefore turned to the clonal human erythroid K562 cell line to examine whether similar phenomena could be observed in a more homogenous cell population. Upon knockdown of RPS19, we observed reduced GATA1 protein levels (Fig. 2b) without significant effects on total cellular proteins, as assessed by Coomassie blue staining of cell lysates (Fig. 2c). The levels of other erythroid-important proteins, including TFRC, EPOR, JAK2, STAT5A, and TAL1, were also unchanged in the RPS19 knockdown (Supplementary Fig. 4a). The reduction of both RPS19 and GATA1 protein levels could be observed within 4 days of infection, before major effects on cell growth occurred (Supplementary Fig. 4). Importantly, total *GATA1* mRNA levels did not appear to be affected (Fig. 2d), consistent with the decreased GATA1 protein levels being due to reduced translation.

To directly assess whether the decrease in GATA1 protein levels was 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 initiation.[18,19] While the levels of *GATA1* mRNA associated with

monosomes was similar or higher in RPS19 knockdown cells as compared with controls (Supplementary Fig. 5), there was a 2–3-fold reduction in *GATA1* mRNA abundance in polysomes (Fig. 2f). In contrast, all erythroid-important mRNAs tested showed a different pattern, with similar or increased abundance in larger polysomes following RPS19 knockdown (Fig. 2g). We also labeled cells with the methionine analog L-azidohomoalanine as an alternative approach to demonstrate a selective reduction in GATA1 translation.[20] When chemical detection of L-azidohomoalanine was performed on immunoprecipitated GATA1, there was a reduction in the level of newly translated GATA1 in cells with reduced RPS19 levels (Supplementary Fig. 6), while global translation was decreased to ~40% of controls (Supplementary Fig. 7), consistent with the slowing of cell growth observed and the known global reduction of translation (48–75%) in DBA patients.[21,22]

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 other DBA-associated ribosomal proteins. Upon reduction of RPL11, RPL5, or RPS24, which are collectively mutated in ~15% of DBA cases,[1] 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; Supplementary Fig. 8). Highlighting the selectivity of this effect, other regulators of erythropoiesis such as EPOR, TAL1, TFRC (CD71), and STAT5A showed no change with these reductions, consistent with the results seen with RPS19 knockdown (Fig. 3a, b). The similar defect in GATA1 translation resulting from reduction of diverse ribosomal proteins from both 40 and 60 S subunits of the ribosome suggests that an impairment of translation initiation may underlie this observation. This observation is consistent with the concept that availability of free ribosomes is the rate-limiting step in translation initiation.[23] 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.[24] We therefore used a selective inhibitor of the eIF4E-eIF4G interaction (termed 4EGI-1) to examine whether this would also selectively affect GATA1 translation.[25–27] Strikingly, when K562 cells were treated with 4EGI-1 for 48 hours, a marked decrease in GATA1 protein was noted, while other proteins remained largely unaffected (Fig. 3d). This shows that *GATA1* mRNA has a more stringent requirement for eIF-dependent translation initiation in comparison with a number of other cellular transcripts. We have observed a decreased ratio of erythroid to non-erythroid (as assessed using the phenotypic marker CD235a/glycophorin A) cells when RPS19, RPL11, RPL5, or GATA1 are reduced in primary CD34+ cell cultures (Supplementary Figs. 9, 10), which is a phenotype characteristic of DBA.[15,16] When 4EGI-1 was added in increasing concentrations to such cultures, a similar and dose-dependent decrease in the relative amount of erythroid cells was observed (Fig. 3e and Supplementary Fig. 11). Importantly, the observed phenotype occurred with a reduction in GATA1 protein levels, but without changes in the levels of a number of ribosomal and other proteins (Fig. 3f). The decreased erythropoiesis with 4EGI-1 treatment could be largely rescued by overexpression of GATA1 itself, suggesting that this is a critical target of this treatment (Supplementary Fig. 12).

Collectively, these results show that the reduction in GATA1 translation observed with reduced ribosomal protein levels likely reflects a broad sensitivity of *GATA1* mRNA to impairments in translation initiation.

Cells can physiologically regulate gene expression through variation of the translation initiation potential, since different mRNAs can have variable barriers to translation.[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.[23,24,28,29] As the 5′ end of human *GATA1* mRNA has never been fully characterized, we performed rapid amplification of the 5′ ends (5′-RACE) of human *GATA1* transcripts from both primary erythroid and K562 cells. This analysis revealed a previously unreported 5′ end of *GATA1* that was found on the majority of clones from both short and long isoform mRNAs (Supplementary Fig. 13a, b). The 5′ end of human *GATA1* mRNA is predicted to be highly structured (Supplementary Fig. 13c) and thus translation may be more readily impaired in settings where the translation initiation potential was reduced, such as with ribosomal protein haploinsufficiency.[23,29,30] In support of this, we found that the *GATA1* 5′ UTR restricted translation to a greater extent than other endogenous 5′ UTRs of similar length using a reporter assay (Supplementary Fig. 13d). Additionally, other transcripts with highly structured 5′ UTRs showed reduced association with larger polysomes (Supplementary Fig. 14), while 5′ UTRs that were unstructured were found in the genes that did not change or were upregulated in these polysomes (Fig. 2g). Therefore, downregulation of *GATA1* mRNA translation - 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 may 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,[31] which may explain the failure of ribosomal protein haploinsufficiency to downregulate translation of this gene and cause major impairments of erythropoiesis in mouse models.[8]

To substantiate whether our findings are of relevance *in vivo* in DBA patients with ribosomal protein gene mutations, we sought to assay GATA1 levels or activity. Given the difficulty of obtaining a sufficient number of stage-matched erythroid cells from DBA patients to examine GATA1 protein levels, we used RNA expression data to examine whether there was an alteration among GATA1 transcriptional target genes. We reasoned that if GATA1 protein levels were specifically reduced in these cells, the transcriptional activity of this key hematopoietic transcription factor should be globally reduced. We performed global gene expression profiling on sorted erythroid progenitors ($CD34^{+}CD71^{high}CD45RA^{-}$ bone marrow mononuclear cells) from 3 DBA patients with known *RPS19* mutations and 6 matched controls. This surface phenotype is known to selectively enrich for early erythroid progenitors (the erythroid burst-forming and colony-forming units, or BFU-Es and CFU-Es, respectively) and results in similar colony numbers from healthy individuals and DBA patients.[32,33] After normalization of the gene expression

data, the expression of both experimentally-derived and curated GATA1 up-regulated target gene datasets was examined (Supplementary Fig. 15). The global gene expression profiles showed a high degree of correlation between the DBA and control erythroid progenitors (R^2 = 0.990; Fig. 4a), supporting the fact that comparable populations of cells were obtained from the patients and controls. We then applied the gene set enrichment analysis (GSEA) algorithm to assess whether the GATA1 target gene sets were either globally up or down regulated in cells from patients.[34] Consistent with our hypothesis, we noted significant global down regulation of GATA1-target gene sets in the erythroid progenitors from DBA patients compared with controls (Fig. 4c, d; Supplementary Fig. 15; Supplementary Tables 2–4). Of over 600 gene sets from other known transcription factor binding sites tested (see Online Methods), none of these other sets demonstrated such a significant and global downregulation, highlighting the selectivity of the effect observed for GATA1 activity. We noted that targets from several other erythroid-important transcription factors and p53 showed no significant change, unlike what was observed for GATA1 targets (Supplementary Fig. 16; Supplementary Table 5). Importantly, *GATA1* mRNA levels were comparable between DBA and control samples (Fig. 4b), providing support for the idea that the altered GATA1 activity is mediated at the translational rather than transcriptional level. These results demonstrate that in primary DBA patient samples with *RPS19* mutations, GATA1 activity is decreased – consistent with it underlying the erythroid differentiation defect in this condition.

We then sought to examine whether the reduced GATA1 levels are sufficient to impair erythropoiesis. When either ribosomal proteins or GATA1 are targeted with shRNAs, we consistently observe increased apoptosis, decreased growth, and decreased erythroid differentiation of primary hematopoietic cells (Supplementary Figs. 4, 9, 10). These findings are entirely consistent with the observations made in DBA patients with mutations in the ribosomal protein or *GATA1* genes.[11] We modified the *GATA1* cDNA to contain an optimized 5′ UTR that lacked known structural barriers and should be effectively translated.[24] When erythroid cells with reduced RPS19 levels were infected with lentiviruses harboring this modified *GATA1* cDNA, we were able to rescue apoptosis to levels close to the baseline observed in these cells, while the mutant form of GATA1 that predominantly produces the short form did not possess such robust activity in this rescue experiment (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 modified *GATA1* cDNA or appropriate controls. We found that introduction of GATA1 into these cells could improve erythropoiesis in the primary hematopoietic cells (Supplementary Figs. 17c) in a comparable manner to the extent of rescue observed with exogenous introduction of the deficient ribosomal protein gene itself,[35] while the mutant version of GATA1 showed a reduced rescue of erythroid differentiation. Importantly, we could show a 2–4-fold increase in the ratio of CD235a+ erythroid to CD235a− non-erythroid cells when GATA1 lentivirus was introduced into primary bone marrow mononuclear cells from DBA patients (Fig. 4e, f). Not only was the frequency of erythroid cells increased, but there was an improvement in erythroid differentiation as indicated by smaller cell size (reduced cell size characterizes more mature erythroid cells),

mature morphology, and increased expression of genes critical for terminal erythroid maturation (Fig. 4g, h, i; Supplementary Fig. 18).[36] These findings show that GATA1, while not the only target of translation dysregulation in DBA, is an important factor in mediating the erythroid-specific defect observed in this condition. Given the limitations in comparing differentiation of DBA patient samples, which normally show delayed and impaired maturation, with cells from normal individuals,[37] the extent of this rescue is likely to be partial and is difficult to quantitatively assess. Nonetheless, these results suggest that modulation of GATA1 protein levels in the context of ribosomal protein haploinsufficiency can improve erythropoiesis and this may lead to potential therapeutic approaches to ameliorate DBA and related forms of anemia, such as that observed in 5q- myelodysplastic syndrome.[35]

Our finding of impaired translation of *GATA1* mRNAs by the ribosome in DBA adds to our increasing knowledge of translational control in mediating cell-type specific gene expression.[5] We have shown that mutations in the *GATA1* gene itself can cause DBA by reducing full-length protein levels, while the more common ribosomal protein mutations in this disease result in impaired erythropoiesis at least in part through the same mechanism of decreased GATA1 protein production (Supplementary Fig. 19). While prior work has implicated p53 as mediating some of the phenotypes in DBA and this pathway is regulated by GATA1,[7,15,38] we would note that the phenotypes that we observe in K562 cells occur in the absence of p53,[39] suggesting that cell-type specificity in DBA is likely mediated by multiple pathways, including p53, that lie downstream of GATA1. Indeed, we observe that p53 is upregulated upon reduction of RPS19 or GATA1 in primary hematopoietic cells, suggesting that these factors act through a common pathway (Supplementary Fig. 20). More generally, our findings suggest that 5′ UTRs of mRNAs with low affinity that are inefficiently translated by the ribosome, would have further reduced translation under conditions of limited ribosome abundance (or with impairments in translation initiation) and thus would be selected against, such that synthesis of their encoded proteins would be more selectively impaired.[23,40] Altered translation has been implicated in diverse human diseases including autism, cancer, and other blood disorders.[26,27] Our finding of selectively impaired translation resulting from ribosomal protein haploinsufficiency provides a paradigm for understanding the previously mysterious cell-type specific defects observed in DBA and conditions such as congenital asplenia,[2,3] as well as childhood leukemia.[4] 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 anemia observed in this disease.

ONLINE METHODS

Cell culture

293T cells were maintained in DMEM with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, and 1% penicillin/streptomycin (P/S). K562 cells 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 L-glutamine, and 1% P/S. Culture of primary human cells is described

below. Cells were incubated at 37°C with 5% CO_2. Where indicated, cultures were supplemented with 10–100 μM 4EGI-1 (sc-202597, Santa Cruz Biotechnology) or DMSO.

Culture of human adult peripheral blood-mobilized CD34+ progenitors was performed using a two-stage culture method, as has been described previously.[41,42] 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 1x CC100 cytokine mix (Stem Cell Technologies, Inc.) and 1% P/S. Cells were maintained in this expansion medium at a density of 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 days. 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 of 0.1×10^6 and 1×10^6 cells per milliliter.

Bone marrow mononuclear cells from Diamond-Blackfan anemia patients and healthy controls were isolated from human bone marrow aspirates using Ficoll-Paque Plus (17-1440-02, GE Healthcare) density gradient centrifugation. Mononuclear cells were cultured in IMDM with 3% human AB plasma, 2% human AB serum, 1% P/S, 3 U/ml Heparin, 200μg/ml Holo-Transferrin (Sigma-Aldrich), 10 μg/ml Insulin, 10ng/ml SCF (PeproTech, Inc.), 1ng/ml IL-3 (PeproTech, Inc.), 2 μM dexamethasone (Sigma-Aldrich) and 3 U/ml Epo (Amgen) similar to recently described protocols for culturing human erythroid cells.[43] Cells were cultured for 1–2 days before infection as described below and analyzed 4–5 days after infection.

Bone marrow mononuclear cells from DBA patients were collected after appropriate informed consent was obtained. Patient 1 is 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 adenosine deaminase level was elevated. Patients 2 and 3 were both male and in their 40s 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 since only select records were available for review. Both Patient 2 and 3 were steroid-dependent to prevent the need for transfusions. Neither individual had any physical anomalies and erythrocyte adenosine deaminase levels were reportedly elevated. *RPS19* mutations in these two individuals were c.3G>A Met1Ile and c.185G>A Arg62Gln, respectively.

Lentiviral vectors and infection

The shRNA constructs targeting human RPS19 (sh913 and sh916, RefSeqID NM_001022), human GATA1 (sh19-23, RefSeq ID NM_002049), 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.1s (the empty pLKO.1 vector with a 1.2 kb stuffer element) were used. For rescue experiments, erythroid cells were co-transduced with shRNAs targeting ribosomal proteins with either the HMD control, HMD-GATA1, or HMD-GATA1 mutant, which contain the respective cDNAs. Double transduced cells were identified after puromycin selection and GFP expression driven by an IRES-GFP from the HMD vector.

For production of lentiviruses, 293T cells were transfected with the appropriate viral packaging and genomic vectors 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 at 0.45 μm immediately prior to infection of primary hematopoietic or K562 cells. The cells were mixed with viral supernatant in the presence of 8 μg/ml polybrene in a 6-well plate at a density of 250,000–500,000 cells per well. The cells were spun at 2000 rpm 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 hours after infection with 1 μg/ml or 2 μg/ml puromycin for primary hematopoietic cells and K562 cells, respectively. The infection efficiency for pLKO-GFP infected cells was assessed by measuring the frequency of GFP+ cells by flow cytometry 48 hours post infection. Typically, the frequency of GFP+ cells for primary hematopoietic cells and K562 cells were between 30–60% and >95%, 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 ΔΔCt 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′ Rapid Amplification of cDNA ends (RACE) was conducted using total RNA from K562 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 1x Complete Protease Inhibitor Cocktail (Roche), and incubated for 30 min on ice. After centrifugation at 14,000 rpm 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, RPS20 goat polyclonal (G-15, sc-55035, Santa Cruz Biotechnology) at a 1:500 dilution, RPS24 rabbit polyclonal (ab102986, Abcam) at a 1:1000 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-835, Santa Cruz Biotechnology) at a 1:500 dilution, TAL1 goal 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 1:2500 dilution, or GAPDH mouse monoclonal antibody (6C5; sc-32233, Santa Cruz Biotechnology) at a 1:1000 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, and 711-035-152; Jackson ImmunoResearch) at a 1:5000 to 1:10000 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 (Perkin Elmer). 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 Glycophorin A/CD235a (HIR2, 17-9987-42, eBioscience), 1:60 FITC-conjugated CD71 (OKT9, 11-0719-42, eBioscience), 1:60 PE-conjugated CD41a (HIP8, 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.

Cloning of UTR reporter constructs and luciferase reporter assay

For cloning of the luciferase reporter constructs the endogenous 5′ untranslated region of the luciferase gene of the pGL3-Promoter vector (Promega) was replaced by the respective 5′UTRs of GAPDH, ACTB and 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, 350ng of the respective luciferase reporter vectors were cotransfected with 35 ng per well of the pRL-SV40 vector using the FuGene 6 reagent according to the manufacturer's protocol (Promega). Cells were incubated at 37°C with 5% CO_2 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 until 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 until measurement of Renilla luciferase activity. For each sample, the ratio of Firefly:Renilla luminescence was calculated and normalized to the signal of the GAPDH-UTR construct.

Polysome profiling

K562 cells were incubated with 100 μg/ml of cycloheximide for 10 minutes 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), 5mM MgCl2, 100 mM KCL, 2 mM DTT, 100 μg/ml cycloheximide, 500 U/ml RNasin (Promega) and 1x 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 $MgCl_2$, 100 mM KCl, 2 mM DTT and 100 μg/ml cycloheximide and centrifuged at 36,000 rpm for 2 hours in a SW41 rotor in the XE-90 ultracentrifuge (Beckman Coulter). Gradients were fractionated using a Bicomp Gradient Station fractionator with absorbance at 254 nm used to visualize the gradients using the Econo UV monitor (BioRad). Fractions were collected and phenol-chloroform extraction was performed to isolate RNA. β-Actin primers were used for normalization of abundance from monosome and polysome gradient fractions, similar to what has previously been described.[44]

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 media (R7513, Sigma Aldrich) with 10% FBS and 2 mM L-Glutamine for 1 h at 37°C, 5% CO_2 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% CO_2. 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 1x Complete Protease Inhibitor Cocktail (Roche), and incubated for 30 min on ice. After centrifugation at 14,000 rpm 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 (Amersham Biosciences) using 532 nm excitation and 580 nm longpass emission. After TAMRA imaging, gels were post-stained with SYPRO Ruby Protein Gel Stain (S12001, Invitrogen) and imaged on Typhoon 9200 imager using 473 nm excitation and 610 nm long-pass emission.

Immunoprecipiation 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, 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 hour 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 40μl 2xLDS buffer incubated at 70°C for 10min. 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 described.[45]

Microarray gene expression analysis

Bone marrow mononuclear cell samples were obtained from three DBA patients with *RPS19* mutations (including a codon 84 frameshift, Arg62Gln, and Leu131Arg mutations), as well as 6 control samples from healthy individuals. $CD71^{high}CD34^{+}CD45^{-}$ populations from the mononuclear cells were sorted as described[46] 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 was normalized using the RMA normalization method as implemented in the Bioconductor package in R (http://www.bioconductor.org/). This data is deposited under accession number GSE41817 in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/).

GATA1 up-regulated target genes were derived from the GSE628 dataset using either the 21 hour time point vs. 0 hours (the majority of known GATA1 up-regulated targets have been

shown to be up-regulated at this time point) or 30 hours vs. 0 hours.[47] Genes with $\log_2$ change of > 1.0 with a p-value of < 0.05 at the two sets of time points were used to derive gene sets.[42] In addition, GATA1 targets were derived from the TRANSFAC database (http://www.gene-regulation.com/).[48] These gene sets were used to run the gene set enrichment analysis (GSEA) algorithm, as has been described.[49] As controls, we utilized gene sets that contain genes that share a transcription factor binding site defined in the TRANSFAC (version 7.4, http://www.gene-regulation.com/) database[48] using the C3 transcription factor target set from the MSigDB (http://www.broadinstitute.org/gsea/msigdb/index.jsp).

Statistics

All pairwise comparisons were assessed using an unpaired two-tailed Student t-test, unless otherwise discussed in the main text. Results were considered significant if the p-value was < 0.05.

Study Approval

All patients or their families had provided written informed consent to participate in this study. The institutional review boards at Boston Children's Hospital and Massachusetts Institute of Technology approved the study protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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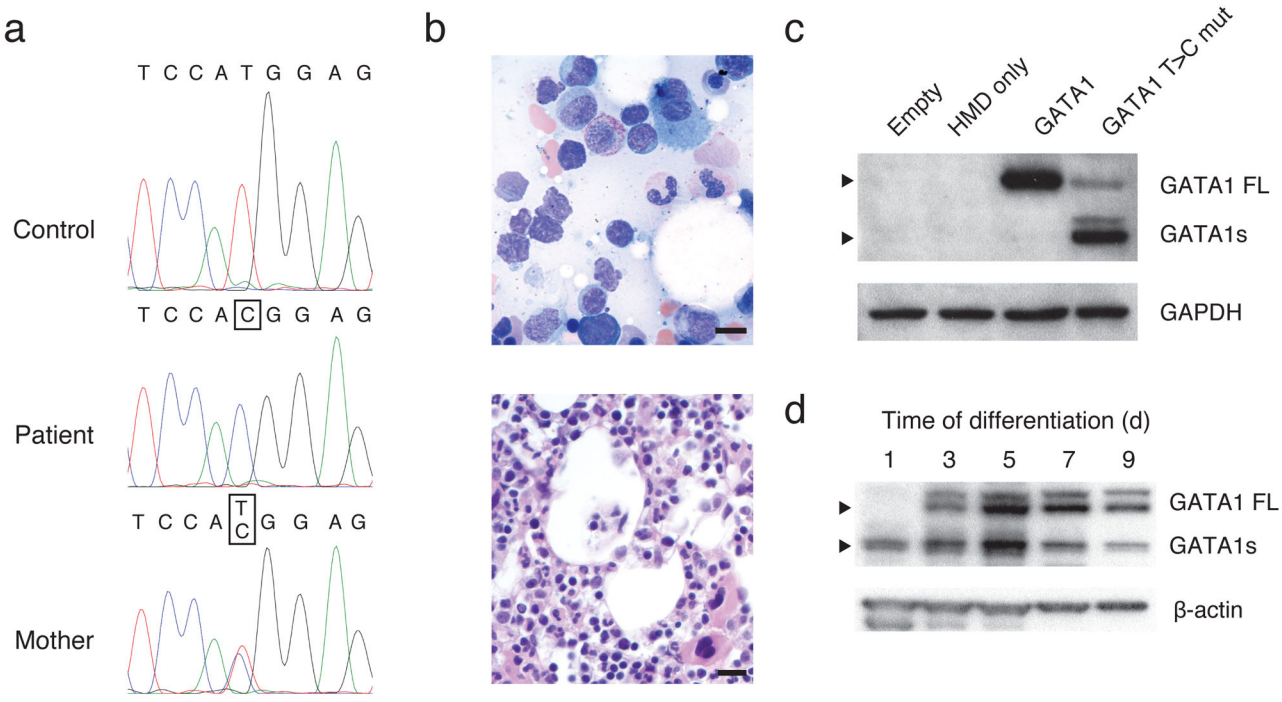

Figure 1. GATA1 full-length protein production is necessary for human erythropoiesis and its disruption results in DBA

(**a**) Sanger sequencing of the first initiator codon region from exon 2 of *GATA1* in a male DBA patient, the patient's mother, and a healthy control.

(**b**) Bone marrow aspirate (above at 100X objective magnification with scale bar at bottom right showing 10 μM distance) and biopsy (below at 50X objective magnification with scale bar showing 30 μM distance) sections from marrow of the DBA patient.

(**c**) The production of full-length and short protein forms of GATA1 from the full-length mRNA with exogenous expression of either no vector, HMD lentiviral vector, HMD-GATA1 wildtype, or the HMD-GATA1 mutant vectors in 293T cells. Arrowheads indicate the major protein forms of GATA1 full length and GATA1 short.

(**d**) Western blots against GATA1 (C-terminal antibody) with a β-actin loading control. Arrowheads indicate the major protein forms of GATA1 full length and GATA1 short.

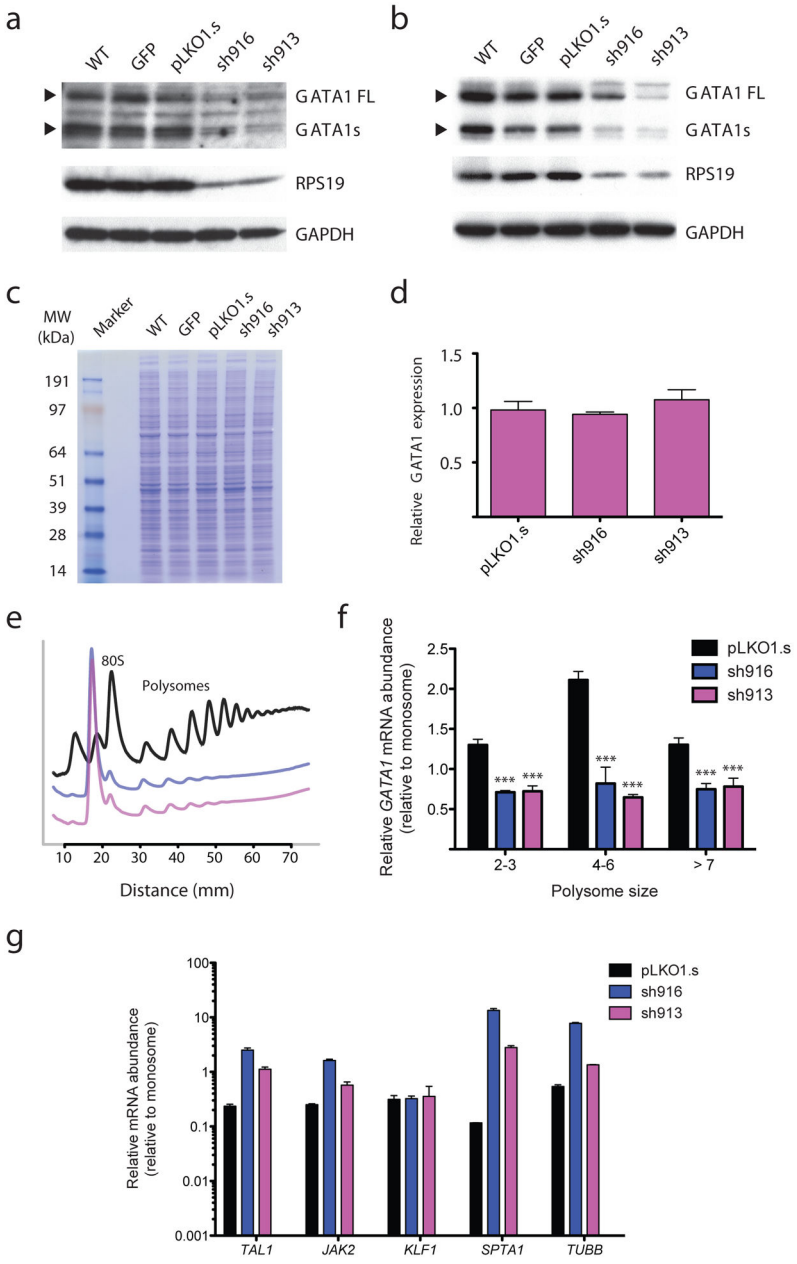

Figure 2. Ribosomal protein haploinsufficiency results in reduced translation of GATA1

(**a**) Protein lysates from CD34+ derived erythroid progenitors at the CFU-E to proerythroblast stage (day 4 of differentiation) from cells infected with RPS19 shRNA vectors, sh916 and sh913. Arrowheads indicate the major protein forms of GATA1 full length and GATA1 short.

(**b**) Protein lysates from K562 cells infected with RPS19 shRNA vectors, sh916 and sh913. Arrowheads indicate the major protein forms of GATA1 full length and GATA1 short.

(**c**) Total protein lysates from control infected or RPS19 shRNA infected cells. Molecular weight (MW) markers are shown on the left side of the gel.

(**d**) *GATA1* mRNA levels were measured from RPS19 shRNA infected cells (normalized to β-actin; here quantitative RT-PCR results from exons 5–6 are shown with similar results obtained for other exons; $n \geq 3$ per group).

(**e**) Polysome profiles from control or RPS19 shRNA treated cells 4 days following infection. The 80S and polysomes are labeled. All graphs are separated on the arbitrary y-axis (derived from relative absorbance at 254 nm) for ease of visualizing the data with the x-axis showing distance along the sucrose gradient.

(**f**) Relative abundance of *GATA1* mRNA in polysome fractions as assessed by RT-PCR (normalized to β-actin; *** $p < 0.0001$). All data are shown as the mean ± the standard deviation ($n \geq 3$ per group).

(**g**) Relative mRNA abundance in larger polysomes (>4 ribosomes) compared to monosomes. Data are normalized to β-actin. Data are plotted on a $\log_{10}$ scale for ease of viewing the range of relative abundance of various erythroid-important mRNAs. The data are shown as the mean ± the standard deviation ($n \geq 3$ per group).

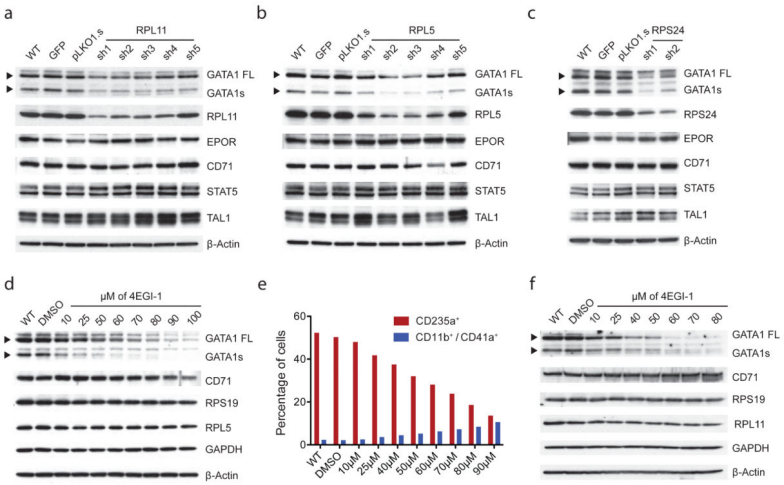

Figure 3. Defects in diverse ribosomal proteins and eukaryotic initiation factors impair GATA1 translation

(**a, b, c**) Western blots are shown from cells obtained 5 days following infection with shRNAs targeting RPL11, RPL5, and RPS24. Arrowheads indicate the major protein forms of GATA1 full length and GATA1 short.

(**d**) Protein levels after increased concentrations of eIF4E-eIF4G interaction inhibitor 4EGI-1 are used to treat K562 erythroid cells (48 hours of treatment). Arrowheads indicate the major protein forms of GATA1 full length and GATA1 short.

(**e**) Erythropoiesis (as measured by CD235a+ cells) and myeloid cell production (as measured by CD11b+ or CD41a+ cells) following treatment of primary hematopoietic cells with 4EGI-1 (48 hours of treatment).

(**f**) Protein levels after increased concentrations of 4EGI-1 are used to treat CD34-derived primary erythroid cells (48 hours of treatment). Arrowheads indicate the major protein forms of GATA1 full length and GATA1 short.

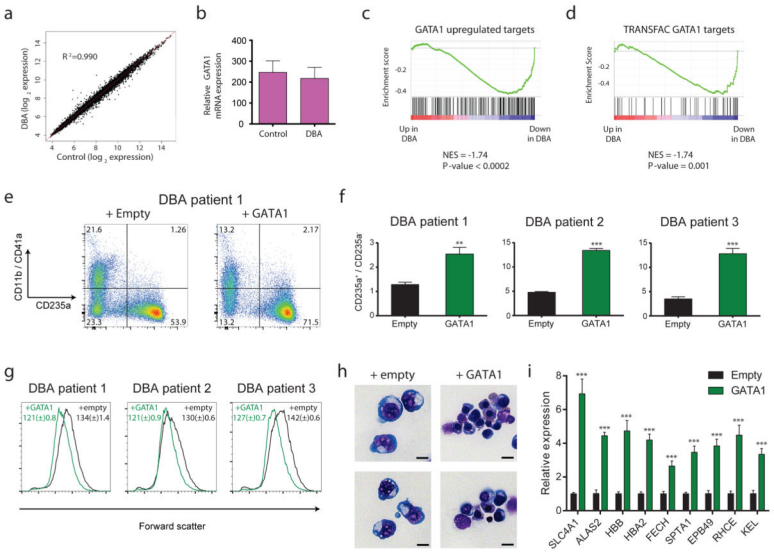

Figure 4. Global disruption of GATA1 transcriptional activity in DBA patients and rescue of ribosomal protein haploinsufficiency with exogenous GATA1

(**a**) Mean gene expression values of DBA and control patient samples are plotted on a scatter plot with a linear regression shown in red. The coefficient of determination is shown.

(**b**) Relative *GATA1* mRNA levels are plotted from DBA and control samples.

(**c, d**) Enrichment profiles from GSEA comparing the relative expression of genes in DBA patients versus controls and examining the distribution of GATA1 target genes in this data (shown as black bars).

(**e**) Representative FACS plots on transduction of bone marrow mononuclear cells from a DBA patient with GATA1 or HMD (empty) control lentiviruses.

(**f**) The ratio of erythroid ($CD235a^+$) to non-erythroid ($CD235a^-$) -cells is shown after transduction of GATA1 for three independent DBA patients. The data are shown as the mean ± the standard deviation (n = 3). (** $p < 0.01$; *** $p < 0.001$).

(**g**) Representative FACS forward scatter histogram plots (measuring cell size) of cultured DBA patient cells transduced with control or GATA1 lentivirus. The mean forward scatter intensity is shown ± the standard deviation (n = 3).

(**h**) Representative cytospin images of cultured DBA patient cells transduced with control or GATA1 lentivirus. Transduced cells were sorted based on GFP expression and stained with May-Grünwald-Giemsa staining. Scale bar = 10 μm.

(**i**) Gene expression analysis by RT-qPCR in DBA patient cells transduced with GATA1 relative to the empty vector control. The data are shown as the mean ± the standard deviation (for 3 independent samples). (Normalized to β-actin; *** $p < 0.001$)