Single-cell profiling of human bone marrow progenitors reveals mechanisms of failing erythropoiesis in Diamond-Blackfan anemia

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Abstract

Ribosome dysfunction underlies the pathogenesis of many cancers and heritable ribosomopathies. Here we investigate how mutations in either ribosomal protein large (RPL) or ribosomal protein small (RPS) subunit genes selectively affect erythroid progenitor development and clinical phenotypes in Diamond-Blackfan anemia (DBA), a rare ribosomopathy with limited therapeutic options. Using single-cell assays of patient-derived bone marrow, we delineated two distinct cellular trajectories segregating with ribosomal protein genotypes: almost complete loss of erythroid specification were observed in *RPS*-DBA. In contrast, we observed relative preservation of qualitatively abnormal erythroid progenitors and precursors in *RPL*-DBA. Although both DBA genotypes exhibited a pro-inflammatory bone marrow milieu, *RPS*-DBA was characterized by erythroid differentiation arrest, whereas *RPL*-DBA was characterized by preserved GATA1 expression and activity. Compensatory stress erythropoiesis in *RPL*-DBA exhibited disordered differentiation underpinned by an altered glucocorticoid molecular signature, including reduced *ZFP36L2* expression, leading to milder anemia and improved corticosteroid response. This integrative analysis approach identified distinct pathways of erythroid failure and defined genotype-phenotype correlations in DBA. These findings may help facilitate therapeutic target discovery.

One sentence summary: Genotype-phenotype correlations are revealed by bone marrow single-cell RNA sequencing in Diamond-Blackfan Anemia.

Introduction

Somatic or germline ribosomal protein (RP) gene mutations underpin the pathogenesis of several cancers and inherited bone marrow failure syndromes*(1)*. Diamond-Blackfan anemia (DBA) is a rare heritable ribosomopathy*(2)* characterized by anemia, multisystem congenital abnormalities, and cancer predisposition*(3)*. Corticosteroids are the only widely used class of drugs in DBA*(4)*, but fewer than half of patients respond*(5, 6)*; the remainder require red cell transfusions or allogeneic bone marrow transplantation. Approximately 75% of cases of DBA are caused by heterozygous mutations in individual genes encoding proteins that comprise the large 60S (*RPL*) or small 40S (*RPS*) ribosomal subunit*(6, 7)*. Selective defects in erythropoiesis in DBA are associated with aberrant ribosome biogenesis and activation of p53-dependent apoptotic pathways*(8)*. We previously used samples from individuals with DBA to elucidate the immunophenotypes of early and late erythroid progenitors (EP), which correspond to functionally defined burst-forming unit- (BFU-e) and colony forming unit- (CFU-e) erythroid (E) colonies, respectively*(9, 10)*. Thus, hematopoiesis in DBA is a useful, accessible model for understanding erythropoiesis and the role of the ribosome in cell fate decisions and differentiation dynamics.

Impaired translation or transcription of the master erythroid-megakaryocyte (MK) transcription factor GATA1*(11)* due to defective ribosome biogenesis*(12)* and excess heme toxicity*(13, 14)* have been suggested as unifying mechanisms of erythroid failure across DBA genotypes. However inconsistent findings from human model cellular systems and cell lines*(4, 14–16)* are yet to be reconciled and it remains unclear whether additional mechanisms may contribute to erythroid failure in DBA*(17)*. Furthermore, although the diagnostic criteria of DBA include presentation in infancy with virtually no mature bone marrow erythroblasts (EB)*(3)*, atypical presentations in later life with milder hematological manifestations are not uncommon*(18–22)*. The mechanisms underpinning these heterogeneous clinical phenotypes are yet to be elucidated.

Here we delineate the cellular and molecular landscape of *RPS*- and *RPL*-DBA using primary bone marrow samples from patients. To mitigate potential confounding effects of comparing different cellular differentiation stages in healthy and diseased tissue, and to overcome limited EP numbers in DBA, we employed single-cell transcriptomics (scRNAseq) of hematopoietic stem and progenitor cells (HSPCs)*(23)*. Using this unbiased approach, we specifically aimed to elucidate the phenotypic and functional differences between *RPS*- and *RPL*-DBA erythropoiesis, the mechanisms of erythroid failure, and their relationship with divergent clinical phenotypes.

Results

Severe impairment of erythroid lineage specification is observed in bone marrow from patients with *RPS*- but not *RPL*-DBA.

To define the landscape of DBA hematopoiesis we performed scRNAseq of bone marrow $CD34^+$ lineage $(Lin)^-$ HSPCs using the 10X Genomics chromium platform (**Fig. 1A**). We studied six patients with red cell transfusion-dependent DBA (aged 2 to 19 years) with mutations in three of the four most common DBA genes [*RPS19* (*n=3*), *RPL11* (*n=1*) and *RPL5* (*n=2*)] and three healthy donors (aged 3 to 17 years) (**data file S1**). High quality sequencing data was obtained for all donors and after quality control, 41,415 of 45,888 HSPCs were carried forward for analysis (**table S1**). All cells were integrated and subjected to donor correction by Harmony*(24)*. Unsupervised clustering by the Louvain method identified 19 distinct clusters (**Fig. 1B; fig. S1, A and B**). We used the most highly expressed marker genes (**data file S2**) to assign cell lineage identity to clusters and confirmed their fidelity by projecting marker genes onto multiple published scRNAseq datasets related to hematopoiesis*(25–29)* (**fig. S2A**). Cell type annotation was further verified by calculating lineage gene scores using six gene sets comprising highly lineage-specific canonical markers*(23, 30)*: E and EP; MK and MK progenitors (MKP); myeloid and monocyte/macrophage/neutrophil progenitors (MyP); lymphoid (Ly) and Ly progenitors (LyP); eosinophil, mast cell, and basophil progenitors (EoMBP); and HSC/MPP (stem and multipotent progenitors) (**fig. S3A and data file S3**). Differentiation trajectories were studied by ordering cells in gene expression space using

Force-Directed Graphs (FDG) superimposed with lineage signature gene sets (**Fig. 1C**). This confirmed known lineage branching relationships from immature HSC/MPP to either committed LyP/MyP or EP/MKP/EoMBP*(28, 31)* (**fig. S3B**). Visualization by Uniform Manifold Approximation and Projection (UMAP) colored by donor type and enumeration of transcriptionally defined progenitor populations (**Fig. 1B**) revealed two divergent cellular patterns in DBA that segregated with genotype (**Fig. 1D** and **fig. S3C**): selective loss of EP and MKP in *RPS*-DBA but preservation of these progenitors in *RPL*-DBA, along the same cell state structure as normal bone marrow.

To independently validate the scRNAseq findings, we employed multiparameter flow cytometry of the bone marrow $CD34^+Lin^-$ compartment of 23 normal controls and 25 patients with DBA with six of the most common DBA genotypes*(6, 32)* (**table S2 and data file S1**). This confirmed the marked reduction in immunophenotypic $CD38^+$ and $CD38^-$ EP and MKP, defined as $Lin^-CD34^+CD45RA^-CD71^+$*(4, 23, 33)*, in *RPS*-DBA, whereas EP and MKP were largely preserved in *RPL*-DBA (**Fig. 1E and F**). The frequencies of other transcriptionally defined HSPC subsets (**Fig. 1D**) or immunophenotypically defined GMP, LMPP, or HSC/MPP*(34)* **(fig. S3D**) were not altered and the frequency of $CD34^+$ cells in bone marrow mononuclear cells (BMMNCs) from all DBA genotypes was similar to control pediatric bone marrow (**fig. S3E**). Together these findings suggest that, in *RPS*-DBA, there is depletion of EP and MKP downstream of the EP/MKP versus EoMBP fate decision point, whereas in *RPL*-DBA, EP and MKP are relatively preserved.

EP trajectories are distinct in primary human HSPCs isolated from patients with *RPS*- and *RPL*-DBA.

To refine lineage relationships within EP and MKP in more detail, we performed further Louvain sub-clustering of the 6,380 EP and MKP cells in aggregate (**Fig. 2, A and B**). Inspection of the genes marking four EP and MKP subclusters (**Fig. 2B, data file S4**) showed that subcluster 1 was enriched for genes marking early erythroid development (*CSF2RB*); subcluster 2 for MKP genes;

subcluster 3 for the erythroid differentiation hemoglobin (*HB*) genes and their *AHSP* chaperone*(35)*; and subcluster 4 for erythroid and cell cycle control genes (*AURKB*).

Next we used previously published single-cell transcriptomic and proteomic data of murine and human HSPCs*(31, 36)* to better understand the developmental relationship between the EP and MKP clusters. We focused on expression of key E and MK transcription factors (**Fig. 2C; fig. S4, A and B**), specifically, *GATA2* as an early EP (EEP or BFU-e) marker downregulated at the late EP (LEP or CFU-e) stage*(9, 37)*, *GATA1* upregulated from EEP to LEP and *KLF1* and *FLI1*, antagonizing one another to determine E versus MK cell fates*(36)*. This allowed us to predict that, in normal bone marrow, cluster 1 corresponded to EEP (balanced *GATA1* and *GATA2* expression); cluster 2 to MKP; cluster 3 to LEP (higher expression of *GATA1* and *KLF1* and lower *GATA2*); and cluster 4 to the proliferative EP fraction (Ecycling: higher expression of *AURKB* and *MKI67*), previously shown to precede irrevocable erythroid commitment*(31, 38)*. This was supported by the differential expression patterns of additional genes, such as *CD34* in EEP and Ecycling and *TFR2*, *TFRC (CD71)*, *GYPA*, and *ENG (CD105)* in LEP (**Fig. 2C**). Expression of EP or MKP genes superimposed on FDGs (**fig. S4A**) further validated our subclustering analysis.

Next, we quantified the number of cells in each transcriptional subcluster in DBA bone marrow with reference to total $CD34^+$ cells. All EP and MKP subclusters were depleted in *RPS*-DBA (**Fig. 2D**). By contrast, EP and MKP subcluster frequencies in *RPL*-DBA were similar to normal bone marrow, suggesting distinct erythroid cellular trajectories according to DBA genotype (**Fig. 2D**). To specifically address the transcriptional basis of these differences, we analyzed E and MK transcription factor expression. Compared to controls, *GATA2* (but not *GATA1* or *KLF1*) and *FLI1* expression was increased in all *RPS*-DBA subclusters and in EEP and LEP respectively (**Fig. 2C**), consistent with block in erythroid commitment. In contrast, reduced expression of *FLI1* was identified in *RPL*-DBA EEP, Ecycling and MKP. Exploring this further by charting *FLI1* and *KLF1* co-expression in single EP or MKP cells (**Fig. 2E**) revealed prevailing expression of *KLF1* over

FLI1 in a higher fraction of EP and MKP in *RPL*-DBA, consistent with a predominant, *KLF1*-driven, erythroid program.

We corroborated these findings by measuring the frequency of immunophenotypic EEP, intermediate EP, and LEP*(9, 38)* in additional bone marrow samples. Although there was progressive reduction in all stages of EP development in *RPS*-DBA, these populations were preserved in *RPL*-DBA. This divergence was particularly notable in LEP, which were virtually absent in *RPS*-DBA (**Fig. 2, F and G**). To determine the functional erythroid potential of *RPS*- versus *RPL*-DBA EP, we plated stage-matched single EEP (purified by fluorescence-activated cell sorting, FACS) in erythropoietin (Epo)-supported semisolid erythroid cultures. Although the clonogenic efficiency of DBA samples was normal, there were striking qualitative differences in colonies. In *RPS*-DBA these mainly consisted of small BFU-e forming loose clusters (E clusters) rather than the typical large BFU-E with tight bursts formed by normal control EEP, as previously described*(9)*. By contrast, *RPL*-DBA EEP (**Fig. 2, H and I**) and total $CD34^+$ (**fig. S4C**) generated highly abnormal, small CFU-e-like colonies of less than 100 cells. Giemsa staining of single erythroid colonies confirmed the presence of more mature EB in abnormal DBA colonies versus normal BFU-e (**fig. S4D**), suggesting disordered differentiation. Commensurate with this, expression of the differentiation-associated gene *GYPA* was higher in *RPL*-DBA LEP than in their normal or *RPS*-DBA counterparts **(Fig. 2C)**. Furthermore, in longitudinal Epo-supported liquid cultures, *RPL*-DBA bone marrow HSPCs generated a higher erythroid yield than *RPS*-DBA **(Fig. 2J)** with similar rates of apoptosis (**fig. S4E**), but with a higher fraction of more differentiated mature EB (**Fig. 2, K and L and fig. S4F**) expressing higher amounts of Glycophorin A (GYPA) messenger RNA (mRNA) and protein, compared to control HSPCs **(fig. S4, G and H).**

Furthermore, quantification of cell surface markers and transcription factors using single cell cytometry by time of flight (scCyTOF) in an independent *RPL5*-DBA bone marrow sample revealed lower expression [Log_2 fold change (FC) < -0.4] of CD34 (in MPP2 to PolyEB) and higher expression ($Log_2FC > 0.4$) of CD71 (in EP and ProEB), CD36, and GYPA (in EP, ProEB, and PolyEB), compared with healthy control counterparts (**fig. S3, A to D**). GATA1 and KLF1

expression was not reduced compared with normal (**fig. S3D**), supportive of the scRNAseq data (**Fig. 2C**). In summary, three complementary assays support the presence of distinct, genotype-associated patterns of erythroid failure in DBA from markedly reduced early erythroid specification in *RPS*-DBA to preservation of BFU-e that are functionally impaired with a distinct transition program through the erythroid differentiation hierarchy in *RPL*-DBA.

Phenotypically normal EB are observed in *RPL*-DBA in vivo.

Given that absence of bone marrow EB is one of the required diagnostic criteria of DBA*(3)*, we next investigated whether erythroid differentiation differed between *RPS*- and *RPL*-DBA in vivo. First, we assessed the frequency of $Lin^-CD34^-CD71^+$ EB in fresh DBA bone marrow samples (**Fig. 3A).** EB frequency in *RPS*-DBA bone marrow was lower than both normal controls and *RPL*-DBA. To define the in vivo defect more precisely, we used flow cytometry to measure the frequency of the six previously defined phenotypically distinct stages of EB maturation*(39)* based on CD105 and GYPA expression (**fig. S6, A and B**). Although there were too few EB for analysis in *RPS*-DBA, we identified all the same stages of EB development in *RPL*-DBA as in control bone marrow (**Fig. 3B**), supporting relative preservation of the EP to EB developmental trajectory in *RPL*-DBA. We also compared differential cell counts from 52 bone marrow aspirates from patients with transfusion-dependent DBA (**fig. S6C**) and found that, although EB were reduced in both genotypes compared with controls, there was an approximately three-fold higher erythroid cell frequency and lower myeloid:erythroid ratio in *RPL*- versus *RPS*-DBA bone marrow (**Fig. 3, C and D**). These findings were consistent across six *RPS*- and four *RPL*-DBA genotypes (**fig. S6, D and E; table S2; data file S1**), confirming our finding of preservation of erythroid differentiation beyond the progenitor stage in *RPL*-DBA.

Glucocorticoid pathway-deficient stress erythropoiesis and *ZFP36L2*-mediated inhibition of erythroid differentiation are mediated by glucocorticoids in DBA.

To investigate the molecular basis for these differences in erythroid differentiation, first we confirmed that, in patients with DBA with loss-of-function mutations (**data file S1**), mRNA

expression of the affected RP gene was selectively reduced to approximately 50% of normal (**fig. S7A**). We also found that expression of fetal hemoglobin (*HBG2)* and the fraction of cells expressing *HBG2* were higher in all three EP populations from both DBA sub-types, compared with normal controls, confirming earlier studies*(40)* (**Fig. 4A**). Like *HBG2*, additional markers of stress erythropoiesis, such as *ERFE* and *GDF15(41, 42),* were upregulated in DBA erythropoiesis (**fig. S7B**). Moreover using the Area under the Curve (AUCell) scoring method*(43)* we found enrichment in DBA EP of a set of genes (**data file S3**) upregulated in murine fetal liver, the prototype for stress erythropoiesis*(31)* (**Fig. 4B**). Although stress erythropoiesis often occurs at the expense of the output of other lineages in murine models*(31),* we observed preserved myeloid progenitor frequency and function in *RPL*-DBA (**fig. S3D and fig. S4C**), consistent with our finding that the stress erythropoiesis signature is present in only a proportion of single cells (**Fig. 4A and fig. S7B**) and is insufficient to rescue either EP function or anemia in these patients.

To interrogate this further, we considered that exogenous glucocorticoids remain the only medical therapy for DBA. Stress erythropoiesis also requires an endogenous glucocorticoid-dependent transcriptional program that increases erythroid output by favoring EP expansion at the expense of differentiation*(44, 45)*. Consistent with a state of disordered differentiation, and as suggested by our in vitro data (**Fig. 2, K and L; fig. S4F**), we found that expression of erythroid differentiation genes, such as *GYPA, AHSP*, and *HB,* was higher in *RPL*-DBA than control (**Fig. 2C and Fig. 4C**). Since transcription of *HBB* and *AHSP* is repressed by the glucocorticoid receptor in the presence of glucocorticoids*(45),* we hypothesized that the *RPL*-DBA differentiation pattern reflects a failure to appropriately upregulate the endogenous glucocorticoid-dependent program of stress erythropoiesis in DBA. Consistent with this, using a set of genes upregulated by glucocorticoids in murine EP (**data file S3**)*(45, 46),* we found a reduced glucocorticoid response in all DBA EP subclusters from both *RPL*- and *RPS*-DBA, although the reduction was more pronounced in *RPL*-mutated EP (**Fig. 4D**). Notably, expression of *ZFP36L2*, a glucocorticoid-responsive gene critical for glucocorticoid-

mediated differentiation delay and subsequent enhanced erythroid output in murine fetal liver*(46)*, was reduced in primary DBA cells (**Fig. 4E**).

To investigate the role of reduced *ZFP36L2* expression in impaired erythropoiesis, we partially knocked-out *RPL11* (residual *RPL11* mRNA 33-48% of unedited controls; **fig. S7, C and D)** in erythroid K562 cells using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 gene editing. Compared to unedited wild-type clones, *ZFP36L2* expression was reduced in *RPL11*-edited K562 cell clones (**Fig. 4F**) consistent with our data in primary DBA bone marrow cells. Next, as previously identified in murine erythroid cells*(46)*, we found that dexamethasone treatment of *RPL11*-edited and control K562 clones resulted in a time-dependent loss of the cells co-expressing the erythroid differentiation markers GYPA and CD71 (**Fig. 4, G to I**), commensurate with upregulation of *ZFP36L2* expression (**Fig. 4J**). In accordance with this, RNA-seq of ex vivo bone marrow stage-matched EB from a patient with *RPL5*-DBA, harvested before and after successful corticosteroid therapy, showed increased *ZFP36L2* but unchanged *RPL5*, *ADA,* and *HBG2* expression (**Fig. 4K**). Furthermore, lentiviral transduction of *ZFP36L2* cDNA into *RPL11*-edited K562 cells (**fig. S7E**) was sufficient to recapitulate the dexamethasone-induced loss of *GYPA* expression (**fig. S7, F and G**) resulting in a higher relative frequency of $CD71^{+}GYPA^{-}$ versus $CD71^{+}GYPA^{+}$ cells in both wild-type and *RPL11*-edited K562 cells (**Fig. 4, L to N**).

P53 activation and bone marrow inflammatory milieu in DBA

Next, we looked for additional pathways dysregulated in DBA and found negative enrichment of the heme pathway in *RPS*- but not *RPL*- DBA EP and activation of p53 in both genotypes (**Fig. 5, A and B**). Strikingly, we also identified enrichment of inflammatory pathways, including tumor necrosis factor (TNF)-α, interferon (IFN)-α-mediated signaling, and IFN-γ-mediated signaling (**Fig. 5A**), known instigators of stress erythropoiesis*(47, 48)*. Inflammatory responses, IFN-α responses and IFN-γ responses, but not p53 and TNF-α pathways, were particularly enriched in *RPS*- compared with *RPL*-DBA (**Fig. 5B**). Inflammation and cytokine-mediated gene networks were enriched throughout the DBA HSPC compartment (**fig. S8, A and B**), suggesting a generalized

bone marrow pro-inflammatory state linked to RP gene haploinsufficiency. In line with this, we found higher TNF-α and IFN-γ concentrations in DBA than control bone marrow plasma (**Fig. 5C**) as well as increased intracellular expression of both cytokines in DBA versus control $CD3^+$ T cells and $CD3^-CD56^+$ natural killer (NK) cells, but not monocytes, following their in vitro activation (**fig. S8, C and D**).

To provide additional validation of the aberrant erythroid developmental pathways in *RPL*-DBA, we performed bulk RNA sequencing of FACS-purified late basophilic EB from the bone marrow of three additional *RPL*-DBA and three age-matched healthy donors (EB are virtually absent in *RPS*-DBA). As expected, expression of the mutated RP gene was selectively reduced by 50% (**fig. S8E**). Principal component analysis (**Fig. 5D**) showed clear partitioning of control and DBA samples and differential gene expression analysis identified 1709 variable genes (**Fig. 5E**). As in *RPL*-DBA EP, Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis revealed activation of p53, TNF-α, IFN-α and IFN-γ inflammatory pathways in DBA EB (**Fig. 5, F and G**). Immunoproteasome and antigen presentation pathways, consistent with an active IFN-γ pathway, were also enriched, whereas ribosome biogenesis was decreased (**Fig. 5F**). Importantly, expression of the glucocorticoid-responsive gene *ZFP36L2* was decreased in *RPL*-DBA compared with control EB (**Fig. 5H**). Erythroid maturation markers, such as *HBA1*, *HBA2* and *HBB*, were similar due to sorting of stage-matched populations (**fig. S8E**) and *HBG2* was increased (**Fig. 5H**). Therefore, although EB can develop in *RPL*-DBA, they display activation of the same pathological pathways identified in their upstream progenitors. Finally, *eADA*, a purine metabolism enzyme used as a diagnostic biomarker in DBA, was upregulated and highly expressed in *RPL*-DBA EB compared with their normal counterparts, matching the higher serum erythrocyte adenosine deaminase (eADA) activity in patients with *RPL*- versus *RPS*-DBA (**Fig. 5I**)*(6, 32)***.**

Together these findings provide ex vivo evidence of a pro-inflammatory bone marrow milieu in primary human DBA HSPC. Furthermore, although *RPL*-DBA EPs are relatively preserved in

numbers, functionally they appear to correspond to stress erythropoiesis that is deficient in its hallmark endogenous glucocorticoid-regulated program. This aberrant EP function leads to disordered differentiation and, in turn, reduced maintenance of the EP pool.

Preservation of GATA1 and its transcriptional program in *RPL*-DBA progenitors and precursors

GATA1 deficiency, due to reduced transcription*(14)*, translation*(4, 12)* or increased caspase 3-mediated degradation*(16)* are proposed as unifying mechanisms for selective erythroid arrest in DBA. However, such studies were mostly performed in cultured cells, cell lines or whole bone marrow containing both erythroid and non-erythroid cells. We had the opportunity to investigate the role of GATA1 ex vivo at single-cell resolution in *RPS*- and *RPL*-DBA, not confounded by steroid therapy. First we performed GSEA of our transcriptome data against three GATA1 genes sets*(12, 49)*. This identified a depleted transcription factor database (TRANSFAC) or early GATA1 transcriptional signature in *RPS*-DBA EP (**Fig. 6A and fig. S9A**), consistent with the severe, early erythroid specification defect. By contrast, *RPL*-DBA EP and EB were enriched for the GATA1 transcriptional program throughout their developmental trajectory (**Fig. 6A and fig. S9A**). In *RPL*-DBA LEP and EB, the late but not early GATA1 signature was enriched in line with their altered differentiation trajectory (**fig. S9A**). In addition, expression of *GATA1short* and *GATA1full-length* isoforms was unchanged in *RPL*-DBA compared with control EB (**Fig. 6B**). This suggests that DBA caused by RPL haploinsufficiency is unlikely to be underpinned by unbalanced GATA1 isoform abundance, as is the case in patients with DBA-like disease with germline *GATA1* mutations that preserve *GATA1short(50)*.

To elucidate the effect of *RPS* or *RPL* haploinsufficiency on GATA1 protein expression in primary DBA bone marrow cells, we stained bone marrow sections from seven patients with *RPL5/11*-DBA, five patients with *RPS19/24/26*-DBA, and three healthy controls, with an antibody specific for GATA1*full-length* (**Fig. 6, C to E; fig. S9, B to D**). In *RPS*-DBA, most GATA1^{+} cells were negative for the erythroid marker Glycophorin C (GYPC) and morphologically corresponded to non-erythroid precursors (**Fig. 6C**). Overall, GATA1 expression was higher in *RPL*- versus *RPS*-

DBA EB and only marginally lower in *RPL*-DBA compared with control erythroid cells (**Fig. 6E**). Combined cell surface and intracellular staining followed by flow cytometry (**Fig. 6F**) or CyTOF (**fig. S9E**) showed a similar pattern of GATA1 expression in *RPL5*-DBA and control primary EP and EB. Together, these findings show that, in the preserved EP and precursors of *RPL*-DBA, GATA1 expression and its transcriptional program are largely intact.

The distinct clinical phenotype of *RPL*-DBA

Since our data suggested a milder cellular and functional defect in *RPL*- than *RPS*-DBA ex vivo, we next investigated the clinical and hematological correlates of these differences by analyzing the characteristics of the UK DBA registry*(5)* with documented *RPL* (*n=44*) and *RPS* (*n=62*) mutations (**table S2**). In line with our transcriptomic and functional data, patients with *RPL*-DBA presented with anemia at an older age (regardless of sex), and with a higher hemoglobin concentration, than those with *RPS*-DBA (**Fig. 7, A and B; fig. S10, A to C; data file S5**). Furthermore, a higher proportion of patients with *RPL*-DBA were initially corticosteroid responsive (**Fig. 7C; fig. S10D and data file S5),** although long-term steroid dependence was not increased (**Fig. 7D and fig. S10E**) and rates of spontaneous or steroid-induced remission were not different between genotypes (**fig. S10F**). We also confirmed previous observations*(51)* of a higher rate of indel genetic variants (**fig. S10G**) and congenital anomalies (**fig. S10H**) in *RPL*- compared with *RPS*-DBA, as well as associations between *RPL5* and cleft palate and *RPL11* and congenital thumb anomalies (**Fig. 7D and fig. S10I**)*(51)*. Taken together, these genotype-phenotype validate the clinical and biological relevance of the distinct erythroid developmental pathways identified in our transcriptomic and functional studies (**Fig. S11**).

Discussion

Here we applied complementary molecular and functional single-cell analyses to dissect the cellular and molecular mechanisms underlying impaired erythropoiesis in DBA, the prototypic ribosomopathy. We identify two distinct patterns of erythroid failure that segregate with underlying genotype: a severe defect in early erythroid specification in *RPS*-DBA with a consequent almost

complete lack of erythroid precursors, contrasting with relatively preserved erythroid cells throughout their developmental trajectory in *RPL*-DBA, but with disordered EP differentiation.

Our data point to a previously unrecognized role of stress erythropoiesis in the pathogenesis of erythroid failure in DBA. DBA EP and EB exhibit many of the molecular hallmarks of stress erythropoiesis, including overexpression of *GDF15* and *HBG2*, the orchestrator and signature gene of stress erythropoiesis, respectively*(40, 41)*. However, the endogenous glucocorticoid-dependent transcriptional signature appears to be defective in DBA-associated stress erythropoiesis. These results derived from primary DBA bone marrow erythroid cells mirror the anemia associated with accelerated erythroid differentiation under conditions of stress in mice lacking expression of the glucocorticoid receptor (*NR3C1*)*(44)*. Similarly, our transcriptional and cellular data indicate that erythroid differentiation is disordered in *RPL*-DBA, thereby providing a mechanism by which exogenously administered glucocorticoids exert their therapeutic effects in patients with DBA, which is by blocking erythroid differentiation to maintain the EP pool, a cellular mechanism reported for normal erythropoiesis in response to glucocorticoids*(52, 53)*.

A role for the RNA binding protein *ZFP36L2* in mediating glucocorticoid-induced delayed erythroid differentiation has been previously reported*(46)*. In this regard, our finding of reduced *ZFP36L2* expression in DBA erythroid cells and *ZFP36L2*-mediated inhibition of erythroid differentiation in *RPL11*-deficient K562 cells, a surrogate model of human *RPL*- haploinsufficient erythropoiesis, supports a pivotal role for *ZFP36L2* in the pathogenesis of erythroid failure in DBA. We hypothesize that restoration of the ability to upregulate critical glucocorticoid-dependent genes, such as *ZFP36L2*, underpins the therapeutic effect of glucocorticoids in DBA. Thus, glucocorticoids improve the quality of stress erythropoiesis rather than restore steady state-like erythropoiesis. In line with this, eADA and fetal hemoglobin (HbF) markers usually remain elevated in steroid-treated DBA (**Fig. 4K**)*(3)*. Conversely, our observation that reduced *ZFP36L2* promotes erythroid differentiation may be exploited in other pathologic states characterized by

excessive stress erythropoiesis but blocked erythroid differentiation, such as β-thalassemia and polycythemia rubra vera, where accelerated maturation (by macrophage depletion for example) ameliorates pathological erythropoiesis and anemia*(54)*.

As well as aberrant stress erythropoiesis, in all DBA genotypes we demonstrate activation of P53*(8)*. Although the only other published transcriptomic data from DBA bone marrow did not show P53 pathway enrichment, the three patients studied were in clinical remission*(4, 55)*. Inhibition of P53 was recently shown to be beneficial in *RPS* models of DBA erythropoiesis*(8)*. Our data further support therapeutic targeting of this pathway in *RPL*-DBA.

Our work also identifies activation of IFN-α, IFN-γ and TNF-α inflammatory pathways in both *RPS*- and *RPL*-DBA HSPCs, potential triggers that could incite and sustain stress erythropoiesis*(47, 48, 56)*. Although inflammatory signatures are more prominent in *RPS*- than *RPL*-DBA EP, they are pervasive and detected in the progenitors of several blood lineages. This is consistent with a bone marrow inflammatory milieu in vivo, supported by detection of elevated IFN-γ and TNF-α in DBA bone marrow plasma. Inflammatory signature imprints were previously reported in mature red blood cells from patients with DBA*(57)* and in zebrafish *RPL11* morpholinos*(58)*. Here we show enrichment in specific hematopoietic lineages and ubiquitously within the bone marrow environment. Both cell intrinsic and extrinsic defects might trigger inflammatory responses. For instance, RP haploinsufficiency and in turn aberrant rRNA biogenesis may generate rRNA species that trigger cellular RNA sensors and an intrinsic IFN response. Specific for erythropoiesis, association between EB and pro-inflammatory EB island macrophages*(54)* or non-specifically activated T or NK cells might lead to excess inflammatory cytokine production that further impairs already intrinsically compromised DBA erythropoiesis*(59)*. Indeed we identified increased secretion of these cytokines by activated T and NK cells in DBA compared with controls, suggesting that targeted anti-inflammatory agents should be investigated in DBA, including TNF-α inhibitors that are known to be beneficial in anemia associated with chronic inflammation*(60)*.

Reduced expression of *ZFP36L2* might also modulate inflammatory activation of lymphoid and myeloid cells in DBA bone marrow given its known anti-inflammatory effects*(61, 62)*, providing another potential mechanism through which glucocorticoids exert their therapeutic effects in DBA. These findings may also have wider implications for other heritable ribosomopathies, such as Shwachman-Diamond syndrome, and for acute leukemia, juvenile myelomonocytic leukemia or myelodysplastic syndromes (MDS), such as 5q-MDS associated with somatic *RPS14* haploinsufficiency*(63)*. Like DBA, these disorders are associated with a pro-inflammatory bone marrow milieu*(64, 65)* and in turn an increased risk of pre-leukemic and leukemic transformation*(66, 67)*.

GATA1 insufficiency has been suggested as a mechanism of erythroid failure in DBA and the rate of *GATA1* translation is dictated by its engagement with the ribosome*(4)*. In B cells derived from patients with DBA, profiling of polysome-associated mRNA transcripts showed that translation defects of the *BCAT1* transcript, which has a long 5' untranslated region, were more severe in *RPS19* than *RPL11* cells*(68)*. Consistent with this we found reduced GATA1 transcriptional activity in *RPS*-DBA EP, suggestive of GATA1 protein reduction. In *RPL*-DBA however, we show a *GATA1* mRNA and protein expression pattern that is appropriate for the stage of erythroid development as well as an apparently robust GATA1-regulated transcriptional program. Thus, our data suggest that strategies to increase GATA1 translation are more likely to be effective in *RPS*-DBA, whereas therapies such as glucocorticoids that delay erythroid differentiation*(52, 53)* and specifically modulate the glucocorticoid target *ZFP36L2*, are likely to be more effective in *RPL*-DBA.

Finally, data from our large cohort of patients with DBA show that individuals with RPL genotypes are more likely to exhibit a milder hematological phenotype appearing later in life and show improved initial corticosteroid responses. This is consistent with our findings of relative preservation in *RPL*-DBA of the EP populations that are targeted by glucocorticoids*(38, 69)*,

coupled with stress erythropoiesis deficient in the normal endogenous glucocorticoid response pathway. Furthermore, these findings complement the diagnostic value of identified genetic variants and allow more precise prediction of the disease course in patients. Despite the milder hematological phenotype in *RPL*-DBA, some non-hematological manifestations, such as congenital abnormalities, are more severe in *RPL*-DBA and, irrespective of genotype, patients with DBA have a higher risk of malignancy at a younger age than the normal population*(32, 70)*. The paradox of attenuated hematological features but more severe skeletal defects associated with *RPL*-DBA genotypes highlights the diverse biological consequences of ribosome dysfunction*(32, 71)*.

Our study has several limitations. This work is limited to the study of HSPC and erythroid precursors and does not include mature myeloid compartments or stromal cells, also potential sources of inflammatory cytokines in DBA bone marrow. Future scRNAseq and functional studies could address for example, the potential role of erythroblastic island macrophages in erythroid failure in the two DBA subgroups. Investigation of larger numbers of patients with distinct genotypes within each of the *RPS*-DBA and *RPL*-DBA subgroups are needed to further refine genotype-phenotype correlations. Finally, the precise mechanisms by which glucocorticoid response pathways are impaired in the face of RP gene haploinsufficiency remains to be addressed.

In summary, we present unbiased charting at single-cell resolution of erythropoiesis in patients with DBA. Our data delineate developmental trajectories and, in turn, elucidate how these shape clinical phenotypes and therapeutic responses, according to genotypes. Furthermore, we provide access to a unique single-cell transcriptomic dataset from pediatric HSPCs in a ribosomopathy, providing cell intrinsic and extrinsic pathogenetic insights, including candidate therapeutic targets for failing erythropoiesis, such as P53 and *ZFP36L2*. Finally, our study is a paradigm of the power of single cell-analysis in deciphering phenotypes and cellular and molecular mechanisms, paving the way for precision-based approaches in rare heritable diseases.

Materials and Methods

Study design

The overall objective of this study was to integrate clinical, cellular, and transcriptomic data from a large cohort of patients with DBA to elucidate genotype-phenotype correlations. Only patients who met the diagnostic criteria of DBA*(3)* with confirmed pathogenic RP gene mutations were included. Number of biological replicates were determined by primary sample availability and are specified in figure legends. Outliers are included.

Patient details

Human bone marrow samples (**data file S1**) were collected following written informed consent, in accordance with the Declaration of Helsinki under a study approved by the National Research Ethics Committee (REC reference 12/LO/0426). Where possible, control and disease samples were age and sex-matched. Healthy donor pediatric bone marrow was collected from sibling donors; samples carrying α or β thalassemia or sickle cell trait were used in selected experiments (excluding RNAseq) given the limited supply of pediatric bone marrow and the importance of age matching*(72)*. At least one true hematologically normal individual was included in the control group in each experiment.

BMMNC isolation and $CD34^+$ cell selection

BMMNC were isolated by Ficoll-Hypaque (Sigma-Aldrich) density centrifugation as per the manufacturer's instructions and used for flow cytometric analysis, subjected to $CD34^+$ selection or cryopreserved, as determined by downstream experiments. $CD34^+$ cells were isolated magnetically from BMMNCs using the MiniMACS Separator kit (Miltenyi Biotech Ltd) as per the manufacturer's instructions. The typical purity and yield of the selected population was >95% following two column passages.

Flow cytometry, FACS, and CyTOF

Cells were suspended in RoboSep, incubated with FcR blocker (Miltenyi Biotech) for 5 minutes at room temperature then stained with a panel of up to 13 commercial fluorophore-conjugated monoclonal antibodies (**table S3)** for 20 minutes at 4°C. After washing, cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich; 1µl/100µl of 5ng/ml stock) or Brilliant Violet (BV) 510 Live/Dead Fixable Stain (BD Biosciences) for dead cell exclusion, prior to data acquisition on a 4-laser BD LSR Fortessa or BD Fusion flow cytometer. Data were acquired using FACSDIVA software v8.0.1 and analyzed using FlowJo software (v10.5.3 Tree Star). Cell doublets and non-viable cells were excluded. Gates were set with fluorescence-minus-one plus immunoglobulin isotype controls or, where cell numbers were limiting, with unstained controls. For cell sorting, cells were passed through a 70µm mesh cell strainer prior to sorting on a BD Aria III (scRNAseq), BD Fusion (bulk RNA-seq and cell culture) or Sony MA900 (K562). The sort purity was assessed by recovery of sorted cells and was consistently >95%. To measure apoptosis, cells were stained with an antibody against Annexin V in Annexin V Binding Buffer (BioLegend), as per the manufacturer's instructions. For intracellular flow cytometry and scCyTOF see **supplementary methods and tables S3 and S4.**

Single-cell RNA-sequencing (10x Chromium)

Cells were thawed, stained with flow antibodies (**table S3**) and sorted as described in the method above. 12-15x10^3 $CD34^+Lin^-$ cells were sorted into 2 µL phosphate buffered saline (PBS) plus 0.05% Ultrapure bovine serum albumin (BSA) followed by adjustment of the cell number/volume to the target for loading onto the 10x Chromium Controller. Processing was performed as per the Chromium Single Cell 3' library and Gel Bead Kits (10x Genomics) v2 or v3. Pre-amplified cDNA was subjected to library preparation and multiplexing then sequenced on a HiSeq 2500, NextSeq 550 or Novaseq 6000 (**table S1**). All scRNAseq analyses were performed using customized pipelines [SingCellaR, package available from https://github.com/supatt-lab/SingCellaR, as

previously described*(23)*; code deposited on Zenodo (10.5281/zenodo.5167626)]. Analyses are detailed in **supplementary methods.**

In vitro liquid erythroid culture

Total $CD34^+Lin^-$ or FACS-isolated EEP subpopulations were cultured in 96 well round- or flat-bottom plates and concentration was maintained at less than $2x10^6$/ml by partial medium changes every 2 to 3 days. Base medium consisted of stemspan (Stem Cell Technologies), penicillin/streptomycin (100units/mL; Sigma-Aldrich), stem cell factor (100 ng/mL; PeproTech), interleukin (IL)-3 (10 ng/mL; PeproTech), lipids (40 μg/mL; Sigma-Aldrich), L-glutamine (25 ng/mL; Sigma-Aldrich), and IL-6 (10 ng/mL; PeproTech). The concentration of Epo (Bio-Techne) was increased from 0.5 to 3-4 units/mL on day 7. The cultures were incubated at 37°C with 5% CO_2 for up to 14 days.

Methocult assay

Single EEP or 500 $CD34^+$ cells were FACS-sorted into 100 μl or 1 ml of H4034 medium (Stem Cell Technologies) in each well of a flat-bottomed 96-well plate or a 24-well plate, respectively. Cultures were incubated at 37°C with 5% CO_2 for 14 days. Colonies were photographed using an inverted microscope (Evos x1 Core) and scored by morphological assessment according to established criteria*(73)*. Specifically, dense colonies of EB in 'bursts' were counted on days 12 to 14 as BFU-e whereas small uni- or bi-centric clusters of EB were counted on day 7 as 'CFU-e-like'. BFU-e colonies with abnormal morphology or color were termed 'erythroid clusters'*(9)*. On day 14, selected colonies were plucked and cytospun to allow morphological examination of their cellular composition.

Microscopy using cytospins

FACS-sorted or cultured cells were suspended in RoboSep, at a concentration of $2\text{-}5x10^4$ cells per 200 μl. Cells were cytocentrifuged at 400 rpm for 5 minutes onto Superfrost slides, using a Shandon Cytospin 2 (Thermo Fisher Scientific). Slides were air dried, fixed in 100% methanol and stained with working solutions of May-Grünwald Giemsa (MGG; Sigma-Aldrich). Cytospins were

photographed using a Nikon eclipse E400 inverted microscope and camera.

Bulk RNA-sequencing

RNA was extracted using the NucleoSpin RNA XS kit (Macherey-Nagel). Directional mRNA libraries were prepared using the NEBNext Poly(A) mRNA Magnetic Isolation Module, NEBNext Directional RNA First and Second Strand Synthesis Modules and the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs), as per the manufacturer's version 1.5 protocol. For further details, see the **supplementary methods**.

Bone marrow plasma

One-two ml of bone marrow aspirate was collected in an EDTA tube (BD Biosciences) and spun at 1500g at 4°C for 12 minutes. The upper plasma layer was aspirated, spun at 4°C, and then stored immediately at -80^{0}C. Analysis of bone marrow plasma cytokines and chemokines was performed by Eve Technologies using the Human Cytokine Array (HD42) Discovery Assay.

Clinical registry data

Clinical and laboratory data were collected prospectively and uniformly from 161 patients with presumed DBA notified to St Mary's Hospital, Imperial College Healthcare Trust, London, UK over a 7-year period (2013 to 2020). Missing data were collected retrospectively. 15 of 161 patients were excluded from the study as there was insufficient data available to fulfill the diagnostic criteria for DBA*(3)*. Targeted next generation sequencing*(7)* or whole exome sequencing was used to screen for RP gene and *GATA1* mutations (**table S2**).

Statistical analyses

Data aggregation and statistical analyses were performed using GraphPad Prism (v8.1.0) and SPSS (Version 26, IBM Corp.) for experimental and clinical registry data, respectively. Unless otherwise stated, bar plots show mean ± standard error of the mean and a two-tailed p value of less than 0.05 was considered significant. Statistical tests used, numbers and types of replicates, and P value thresholds are described in legends. Differences in continuous variables between two groups were

assessed using the Student's t test (parametric), Mann-Whitney U test (non-parametric) or Wilcoxon rank test (non-parametric) and comparisons between more than 2 groups were assessed using the Kruskal-Wallis test (non-parametric) or a one-way ANOVA test (parametric), with Dunn or Holm-Sidak multiple comparisons tests, respectively. Normality of data was assessed by the Shapiro-Wilk test (**data file S6**). Fisher's exact or Pearson chi-square test was used to compare proportions of categorical variables. For clinical registry data, all variables found to be significant in univariate analyses were included in a multivariate stepwise logistic or linear regression analysis for binary and continuous variables, respectively.

List of Supplementary Materials

Materials and Methods

Fig. S1 to S11.

Table S1 to S5.

Data File S1 to S6.

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flow cytometry, GATA1 immunohistchemistry and CRISPR/Cas9); performed bioinformatic analyses (scRNAseq and bulk RNAseq); collected and analyzed clinical registry data; and wrote the manuscript. GW and EH developed and performed bioinformatic analyses. MR and NT contributed to bioinformatic analysis of bulk RNAseq data and ZM analyzed CyTOF data. CC performed and analyzed experiments (sample processing, cell culture, flow cytometry, GATA1 immunohistochemistry and CRISPR/Cas9) and contributed to preparation of manuscript figures. BP, KP, HR, CP, QA, MZ and Ak performed experiments as follows: scRNAseq (BP); CRISPR/Cas9 (KP, HR, QA); CyTOF (CP); flow cytometry (MZ) and sample processing (Ak). PT and KN performed immunohistochemistry. RS contributed to statistical analysis. AJM, MB, JDLF and DB provided resources and expertise. VC, KP and AR provided methodology and expertise. AC and ST designed bioinformatic pipelines. JDLF and LK contributed clinical samples. IR and DMB helped to design the project, interpreted data, and edited the manuscript. AK conceived the project, designed experiments, interpreted data, and wrote the manuscript. All authors read and approved the submitted manuscript. **Competing interests:** AK provides consulting for Gilead Greece; JDLF is on the advisory board of bluebird bio, CRISPR Therapeutics/VERTEX, Novartis, Beam Therapeutics and Jazz, has received speaker fees from Jazz and has received a research grant from bluebird bio; AM provides consulting for Novartis, Celgene/BMS, Abbvie, CTI and Gilead, has received speaker fees from Novartis and has received research funding from Novartis and Celgene/BMS; BP provides consulting for Constellation and has received speaker fees from Novartis. All other authors have no conflicts of interests to declare.

Data and materials availability: All data associated with this study are in the paper or supplementary materials. The raw RNA-seq datasets generated during the current study are available at the NCBI GEO DataSets under the accession codes (GSE156441 and GSEXX). CyTOF data are available at flow repository under accession code FR-FCM-Z3QN and code is available at Zenodo (10.5281/zenodo.5167626) and GitHub (https://github.com/guanlinW/Iskander_et-al_DBA_2021).

Figure Legends

Figure 1. Erythroid lineage specification is preserved in bone marrow isolated from patients with *RPL*-DBA but not *RPS*-DBA.

A) Study design for single cell RNA-sequencing (scRNAseq) experiments showing source of DBA and healthy bone marrow from allogeneic bone marrow transplant (BMT) donors. Erythroid hierarchy depicted as follows: hematopoietic stem (HSC) and multipotent (MPP) cells, within the $CD34^{+}CD38^{-}$ bone marrow subfraction, mature into committed EEP (BFU-e) and LEP (CFU-e), within the $CD34^{+}CD38^{+}$ bone marrow subfraction. These then differentiate into $CD34^{-}$ EB, which enucleate to form reticulocytes that egress into the peripheral blood and form red blood cells (RBC).

B) Uniform Manifold Approximation and Projection (UMAP) embedding of nineteen cell clusters generated by Louvain clustering of 41,415 $CD34^{+}$Lineage $(Lin)^{-}$ HSPCs from 9 donors in aggregate: control (15,434 cells; 3 donors) and DBA (25,981 cells; 6 donors, 3 *RPS*-DBA, 3 *RPL*-DBA). Cell type annotation of each cluster are also shown.

C) Force-directed graph (FDG) embedding of 6 major hematopoietic cell types in control and DBA cells is shown, colored by key marker gene sets (**data file S3 and fig. S3A**). Gray cells represent uncommitted cell types or cells expressing greater than 1 lineage gene set.

D) Circos plots (left) and a bar plot (right) depict proportions of cells in each of 6 cell types identified by scRNAseq among total $CD34^{+}Lin^{-}$ cells by donor type.

E) The flow cytometry gating strategy used to identify $CD71^{+}CD45RA^{-}$ EP and MKP cells in the $CD38^{-}$ immature and $CD38^{+}$ mature sub-compartments of bone marrow $CD34^{+}$ cells is shown. Frequencies are shown as percent of $CD34^{+}Lin^{-}$ cells. AF700, alexa fluor 700; PeCy7, phycoerythrin cyanine7; FITC, fluorescein isothiocyanate; APCy7, allophycocyanin cyanine7.

F) Cumulative data shows the frequency of BFU-e and MKP within $CD34^{+}Lin^{-}CD71^{+}CD38^{-}$ bone marrow subfraction and of BFU-e, CFU-e and MKP within $CD34^{+}Lin^{-}CD71^{+}CD38^{+}$ bone marrow subfraction (*n=23* control, *20 RPS*-DBA and *9 RPL*-DBA bone marrow

samples). Colored symbols depict two bone marrow samples from the same patient collected at least one year apart.

Bars show mean ± SEM of biological replicates. ****P<0.0001; **P<0.01; *P<0.05; *n.s.*: not significant. Groups were compared by a one-way ANOVA with Holm-Sidak's correction (**E**) or a Kruskal-Wallis with Dunn's multiple comparisons test (**F**).

Figure 2. Features of the erythroid progenitor trajectory differentiate *RPS*-DBA and *RPL*-DBA primary human HSPCs.

A) A UMAP aggregate is shown of all control and DBA EP and MKP cells (*n=6380*) depicting four distinct subclusters.

B) A heatmap is shown for the 15 top differentially expressed genes (row labels, right) for each of the four EP and MKP sub-clusters (color-coded columns).Labels across the top indicate cluster assignment according to their marker genes: early erythroid progenitors (EEP), cycling EP (Ecycling), late erythroid progenitors (LEP) and megakaryocyte progenitors (MKP).

C) A bubble plot shows the average expression of individual genes (depicted by color) and the fraction of cells expressing that gene (depicted by bubble size) within EP and MKP subclusters from control, *RPS*-DBA, or *RPL*-DBA bone marrow. Boxes highlight differentially expressed genes with $Log_2FC > 0.4$ and adjusted P value < 0.05. TF, transcription factor; UMI, unique molecular identifier.

D) A bar plot depicting proportion of cells in each of four EP and MKP clusters among total $CD34^+Lin^-$ cells by donor type is shown.

E) Single cell co-expression of *KLF1* and *FLI1* is shown for control, *RPS*-DBA and *RPL*-DBA EP and MKP cells. The diagonal marks cells with equal expression of both genes. The fraction of cells above and below the diagonal line is shown.

F) Representative flow cytometry plots show the gating strategy for $CD71^+CD41a^-$ erythroid progenitors (EP): $CD71^+CD36^-CD105^-$ early (EEP), $CD71^+CD36^-CD105^+$ intermediate (IntEP) and $CD71^+CD105^+CD36^+$ late (LEP) erythroid progenitors. Frequencies are shown as percent of total $CD34^+$ cells.

G) The frequency of EP subsets is shown as percent of $CD34^+$ bone marrow in control (*n=11* for EEP, *n=8* for Ecycling and LEP), *RPS*-DBA (*n=6* for EEP, *n= 5* for Ecycling and LEP) and *RPL*-DBA (*n=7* for EEP, *n=5* for Ecycling and LEP) bone marrow. Purple symbols depict two bone marrow samples from the same patient collected at least one year apart.

H) Morphology of colonies generated on days 12 to 14 in methylcellulose medium from single-cell EEP FACS-purified ex vivo from control, *RPS*- and *RPL*-DBA bone marrow is shown. Images are representative of three independent experiments. Scale bars, 100 µm.

I) The frequency and type of hematopoietic colonies generated in methylcellulose from single-cell EEP FACS-purified ex vivo from control, *RPS*-DBA and *RPL*-DBA bone marrow (*n=3*) are shown. Significant differences in colony types are indicated.

J) Erythroid yield (total cell number multiplied by percent $CD71^{+}CD14/16/61^{-}$ cells) is shown from a longitudinal serum-free erythroid liquid culture of FACS-purified $CD34^{+}Lin^{-}$ HSPCs from control, *RPS*-DBA and *RPL*-DBA bone marrow (*n=2*).

K) Flow cytometry analysis of erythroid differentiation stage (characterized by Lin, CD71 and GYPA markers) is shown for EB on day 13 of culture of control, *RPS*-DBA and *RPL*-DBA bone marrow HSPCs.

L) Cumulative data from two independent experiments show the fraction of early and late EB generated multiplied by erythroid yield from control, *RPS*-DBA and *RPL*-DBA bone marrow HSPCs (*n=2*).

Plots show mean ± SEM of biological replicates. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.05; *n.s.*: not significant. Groups were compared by a one-way ANOVA with Holm-Sidak's (**D**), a Kruskal-Wallis with Dunn's multiple comparisons test (**G**) or a Fisher's exact test (**I**).

Figure 3. Phenotypically normal mature EB are observed in *RPL*-DBA in vivo.

A) Representative flow cytometry plots (left) show the frequency of $Lin^-CD34^-CD71^+$ EB within total BMMNCs. Cumulative data of EB frequencies within total BMMNCs is shown (right) in control (*n=10*), *RPS*-DBA (*n=17*) and *RPL*-DBA (*n=8*) bone marrow. Purple symbols depict two samples from the same patient collected two years apart. APC, allophycocyanin; SSC, side scatter.

B) Frequencies of EB subpopulations were measured by flow cytometry in total MNC derived from control (*n=8*) and *RPL*-DBA (*n=7*) bone marrow.

C) Representative morphological appearances are shown for bone marrow aspirates (stained with hematoxylin and eosin) from controls and patients with DBA with a *RPS19* and a *RPL5* mutation. Scale bars, 50 µm.

D) EB frequencies in total nucleated cells and myeloid to erythroid lineage cell ratio (M:E ratio) were measured in bone marrow aspirate slide preparations from normal pediatric bone marrow controls (*n=15*) and patients with *RPS*-DBA (*n=32; 34* for M:E) and *RPL*-DBA (*n=17; 18* for M:E).

Plots show mean ± SEM of biological replicates. ****P<0.0001; ***P<0.001; *P<0.05; *n.s.*: not significant. Groups were compared by a Kruskal-Wallis with Dunn's multiple comparisons test.

Figure 4. Glucocorticoid pathway-deficient stress erythropoiesis and *ZFP36L2*-mediated erythroid differentiation inhibition by glucocorticoids are observed in DBA.

A) Violin plots depicting the mean expression (yellow dot) and distribution (minimum to maximum) of *HBG2*, a stress erythropoiesis gene in control, *RPS*-DBA and *RPL*-DBA EP subclusters (EEP, Ecycling and LEP). The fraction of cells expressing *HBG2* is shown on the x axis.

B) Mean expression (yellow dot) and distribution of AUCell score of stress erythropoiesis (SE) gene set is shown for control, *RPS*-DBA and *RPL*-DBA EP subclusters.

C) A bubble plot shows the expression of erythroid differentiation genes in control, *RPS*-DBA and *RPL*-DBA EP subclusters.

D) The AUCell score of glucocorticoid response genes in control, *RPS*-DBA and *RPL*-DBA EP subclusters is shown by violin plots.

E) Violin plots depict the expression of *ZFP36L2* in control, *RPS*-DBA and *RPL*-DBA EP subclusters. The fraction of cells expressing *ZFP36L2* is shown on the x axis.

F) *ZFP36L2* was measured by RT-PCR in wild-type (wt, *n=6*) and *RPL11* knockdown (kd, *n=4*) K562 clones, normalized to wt and *GAPDH*. Data points represent RNA extracted at independent time points.

G) Erythroid differentiation of K652 cells was assessed by CD71 and GYPA expression by flow cytometry of untreated and dexamethasone (Dex)-treated wt and *RPL11* kd K562 clones. A representative of 4 independent experiments is shown. Numbers in the flow plots indicate the percent of live single cells.

H) Fold change of early ($CD71^+$ $GYPA^-$) and late ($CD71^+$ $GYPA^+$) EB markers in Dex-treated wt K562 clones, normalized to untreated *(n=4*). For early EB, the fold change was calculated as (% $CD71^+GYPA^-$ of Dex-treated – % $CD71^+GYPA^-$ of untreated) / % $CD71^+GYPA^-$ of untreated.

I) Fold change of early ($CD71^+GYPA^-$) and late ($CD71^+GYPA^+$) EB markers in Dex-treated *RPL11* kd K562 clones, normalized to untreated *(n=4*).

J) *ZFP36L2* was measured by RT-PCR in Dex-treated and untreated wt and *RPL11* kd K562 clones (*n=4*), normalized to untreated and *GAPDH*.

K) *ZFP36L2*, *RPL5*, *ADA*, and *HBG2* expression was measured by RNA-seq of EB isolated from bone marrow of a patient with *RPL5*-DBA and purified by FACS one month pre- and 16 months post-steroid therapy. Red line shows hemoglobin (Hb) concentration at time of bone marrow samplings.

L) Lentiviral transduction efficiency (% GFP^+) 2 days after lentiviral transduction with mock (empty vector) *or ZFP36L2* cDNA. Plots are representative of three independent experiments. Numbers in the flow plots indicate the percent of live single cells.

M) Erythroid differentiation (represented by CD71 and GYPA expression) of wt and *RPL11* kd K562 clones is shown from 2 to 4 days after lentiviral transduction with mock (empty vector) *or ZFP36L2* cDNA. Plots are representative of three independent experiments. Numbers in the flow plots indicate the percent of live single cells.

N) Fold change of *GYPA* in wt and *RPL11* kd K562 clones was quantified 2 to 4 days after lentiviral transduction with mock or *ZFP36L2* cDNA (*n=3* at day 2 and *n=2* at day 3 and 4).

Plots show mean ± SEM of replicates. ****P<0.0001; ***P<0.001; **P<0.01, *P<0.05; *n.s.*: not significant. Groups were compared by a Wilcoxon rank-sum test (**A, B, D and E**), an unpaired Student's t test (**F**) or a paired Student's t test (**I**).

Figure 5. P53 activation and bone marrow inflammatory milieu are increased in DBA.

A) The bubble plot shows normalized enrichment score (NES) and false discovery rate (-Log_{10}FDR) of significantly enriched (FDR q value <0.25) pathways of interest on GSEA of differentially expressed genes (DEGs) between DBA and control EP subclusters. Pathways are from the Hallmark or KEGG gene sets of Molecular Signatures Database.

B) The bubble plot shows enriched pathways between *RPS*-DBA and *RPL*-DBA EP subclusters.

C) The concentration of TNF-α, IFN-α and IFN-γ in control, *RPS*-DBA, and *RPL*-DBA bone marrow plasma (*n=5* for control and *RPS*-DBA, *n=6* for *RPL*-DBA) was assessed by luminometry.

D) A principal component analysis (PCA) depiction of bulk RNA-seq data from stage-matched EB derived from three healthy pediatric controls and three patients with DBA is shown (*n=1 RPL11* and *n=2 RPL5*), separated by principal component (PC) 1, PC2, and PC3.

E) A volcano plot is shown, highlighting 1709 DEGs, of which 1101 are upregulated. Thresholds (dotted lines) are adjusted P value < 0.05, and $Log_2FC \pm 1$. Names of top 50 DEGs are shown.

F) GSEA plots show enrichment of Hallmark and KEGG gene sets in pre-ranked DEGs between control (*n=3*) and *RPL*-DBA (*n=3*) EB. Each bar represents a gene. The curve peak shows the normalized enrichment score (NES), which reflects the degree to which a gene set is overrepresented at the top or bottom of the ranked list.

G) Ingenuity Pathway Analysis of DEG between *RPL*-DBA and control EB is shown highlighting the top four upstream regulators of the DBA transcriptome, p53, IFN-α2, IFN-γ, and TNF-α.

H) The bubble plot depicts the Log_2FC and $-Log_{10}$ adjusted P value of *ZFP36L2* and 3 stress erythropoiesis genes, quantified by bulk RNA-seq in *RPL*-DBA (*n=3*) and control (*n=3*) EB. All genes have $Log_2FC > 0.6$ and adjusted P values < 0.05.

I) *eADA* mRNA was quantified by bulk RNA-seq of FACS-purified EB from control and *RPL*-DBA (*n=3*) bone marrow (left). Adjusted P value is shown. Peripheral blood red blood cell eADA activity (nm/mg Hb/hour) is shown (right), in patients with *RPS*-DBA (*n=14*) and *RPL*-DBA (*n=11*) who are treatment-independent or steroid-treated but are not receiving blood transfusions. UL normal, upper limit of normal.

Plots show mean ± SEM of biological replicates. ****P<0.0001; **P<0.01, *P<0.05; *n.s.*: not significant. Groups were compared by a Kruskal-Wallis test (**C**) or a Mann-Whitney U test (**J, right panel**).

Figure 6. GATA1 and its transcriptional program is preserved in *RPL*-DBA.

A) GSEA is shown against a TRANSFAC-derived GATA-1 gene set *(12)* of DEG between control and DBA subgroups at different erythroid stages (scRNAseq for EP and bulk RNA-seq for EB). Significant FDR of <0.25 are highlighted in bold face.

B) Full length and short *GATA1* transcript isoform abundance were determined by transcript analysis of bulk RNA-seq of stage-matched EB from healthy control and *RPL*-DBA bone marrow (*n=3*). TPM, transcripts per million.

C) Top: Representative images show GATA1 expression assessed by immunohistochemistry of healthy control, *RPS*- and *RPL*-DBA bone marrow sections. GATA1 expression is shown by brown staining in EB (identified by their unilobar round nuclei), MK (large multilobe nuclei) and EoMB (bilobed/horseshoe nuclei and granular cytoplasm). Expression is weaker in EoMB compared with EB or MK cells, and decreases in late EB relative to early/intermediate EB. Bottom: Co-staining for GATA1 and the erythroid-specific marker Glycophorin C (GYPC) to distinguish EB ($GYPC^+$ red membrane or cytoplasmic rim) from EoMBP or MK ($GYPC^-$). Scale bars, 10 μm.

D) The correlation between GATA1 expression and nuclear diameter in control bone marrow is shown.

E) Violin plots show single cell GATA1 expression, measured in bone marrow EB from control (*n=1781 cells*; 3 donors), *RPS*-DBA (*n=286*; 4 donors) and *RPL*- DBA (*n=1179*; 7 donors). Distribution is from the 5^{th} percentile to the 95^{th} percentile. Dotted line shows mean expression of all samples and the y axis shows number of SDs that each data point differs from mean (Z-score).

F) Representative plots show GATA1 and GATA2 expression measured by intracellular flow cytometry in control and *RPL5*-DBA bone marrow EP ($CD71^{hi}GYPA^-$) and EB (CD71+GYPA+). Quadrants in GATA1 versus GATA2 plot were set using fluorescence minus one plus isotype controls. AF647, alexa fluor 647; BV421, brilliant violet 421 ; PE, phycoerythrin.

Groups were compared by a Mann-Whitney U test (**B**) or a Kruskal-Wallis with Dunn's multiple comparisons test (**E**). ****P<0.0001; **P<0.01; *n.s.*, not significant.

Figure 7. *RPL*-DBA has a distinct clinical phenotype from *RPS*-DBA.

A) Age at presentation with anemia is plotted according to *RPS*-DBA (*n=62*) or *RPL*-DBA (*n=44*) genotype. Dots depict data points for individual patients.

B) Haemoglobin concentration (g/L) at presentation is shown, according to *RPS* (*n=59*) or *RPL* (*n=38*) genotype.

C) The fraction of steroid-responsive (SR) cases (transfusion-independent for greater than 6 months) versus steroid unresponsive (SUR) cases is shown according to *RPS* (*n=59*) or *RPL*-DBA (*n=37*) genotypes. Numbers of cases are shown in the bars.

D) Odds ratio and 95% confidence intervals of specific clinical features and outcomes in *RPL*- versus *RPS*-DBA are shown. P values are shown on the right.

Upper and lower horizontal lines of box plot represent 75^{th} and 25^{th} percentile, respectively. Whiskers represent maximum and minimum values. Continuous variables were compared across genotypes using a Mann-Whitney U test (**A** and **B**). Frequencies of cases were compared across genotypes using a Pearson chi-square test (**C**). All variables significant on univariate analysis were tested by binary logistic or multiple linear regression, as appropriate. P values shown refer to regression analyses. **P<0.01; *P<0.05.