# Mechanisms of failing erythropoiesis in Diamond-Blackfan anemia revealed by single-cell profiling of human bone marrow progenitors

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Genotype-phenotype correlations revealed by bone marrow single-cell RNAseq in Diamond-Blackfan Anemia

## Abstract

Ribosome dysfunction underlies the pathogenesis of many cancers and heritable ribosomopathies. Here we investigate how mutations in either ribosomal protein large subunit (RPL) or small subunit (RPS) genes selectively affect erythroid progenitor (EP) development and clinical phenotypes in Diamond-Blackfan anemia, a rare disease with limited therapeutic options. Using single-cell assays of patient-derived bone marrow (BM), we delineate two distinct cellular trajectories segregating with RP genotype: almost complete loss of erythroid specification (*RPS*-DBA) versus relative preservation of qualitatively abnormal EP/precursors (*RPL*-DBA). Although both DBA genotypes exhibit a pro-inflammatory BM milieu, *RPS*-DBA is characterized by erythroid differentiation arrest while in *RPL*-DBA there is preserved GATA1 expression. Compensatory SE in *RPL*-DBA exhibits disorded differentiation underpinned by an altered glucocorticoid molecular signature, including reduced *ZFP36L2,* leading to milder anemia and improved corticosteroid response in this patient subgroup. Taken together, this integrative analysis approach in primary human hematopoietic cells identifies distinct pathways of erythroid failure, defines genotype-phenotype correlations and facilitates therapeutic target discovery.

## Introduction

Somatic or germline ribosomal protein (RP) gene mutations underpin the pathophysiology 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 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 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 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 and/or transcription of the master erythroid-megakaryocyte (E/MK) transcription factor (TF) GATA1*(11)*, due to defective ribosome biogenesis*(12)* and excess heme toxicity*(13, 14)* respectively, have been suggested as unifying mechanisms of erythroid failure across DBA genotypes. However variable findings from human model cellular systems and cell lines*(4, 14–16)* are yet to be reconciled and it remains unclear whether additional mechanisms contribute to erythroid failure in DBA*(17)*. Furthermore, although the diagnostic criteria of DBA include presentation in infancy with virtually no mature BM 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 BM 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 associated mechanisms of erythroid failure and their relationship with divergent clinical phenotypes.

## Results

### Severe impairment of erythroid lineage specification in *RPS-* but not *RPL*-DBA patient BM

To define the landscape of DBA hematopoiesis we performed scRNAseq of BM CD34+lineage negative (CD34+Lin-) HSPCs using the 10X Genomics chromium platform (**Fig. 1A**). We studied 6 patients with red cell transfusion-dependent DBA (aged 2-19y) with mutations in 3 of the 4 most common DBA genes [*RPS19* (*n=3*), *RPL11* (*n=1*) and *RPL5* (*n=2*)] and 3 healthy donors (aged 3-17y) (**table 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 S2**). 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 top upregulated marker genes (**table S3**) 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. S1C**). Cell type annotation was further verified by calculating lineage gene scores using 6 novel gene sets comprising highly lineage-specific canonical markers*(23, 30)*: erythroid/EP, megakaryocyte/MKP, myeloid/MyP (monocyte/ macrophage/ neutrophil progenitors), lymphoid/LyP, eosinophil/mast cell/basophil (EoMBP) and HSC/MPP (stem and multipotent progenitors) (**fig. S1D and table S4**). Differentiation trajectories were studied by ordering cells in gene expression space using Force-Directed Graphs (FDG), superimposed with lineage signature gene sets (**Fig. 1C and fig. S1E**). This confirmed known lineage branching relationships from immature HSC/MPP to either committed Ly/MyP or EP/MKP/EoMBP*(28, 31)* (**fig. S1F**). 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**): selective loss of EP and MK-progenitors (MKP) in *RPS*-DBA, in contrast to preservation of these progenitors in *RPL*-DBA, along the same cell state structure as normal BM.

To independently validate the scRNA-seq findings, we employed multiparameter flow cytometry of the BM CD34+Lin- compartment of 23 normal controls and 35 DBA patients with 6 of the most common DBA genotypes*(6, 32)* (**tables S1 and S5**). 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 while EP/MKP were largely preserved in *RPL*-DBA(**Fig. 1 E and F)**. The frequencies of other transcriptionally or immunophenotypically defined HSPC subsets (GMP, LMPP, HSC/MPP)*(34)* were not altered (**Fig. 1D and fig. S1G**)and the frequency of CD34+ cells in BM mononuclear cells (BMMNCs) from all DBA genotypes was similar to control pediatric BM (**fig. S1H**). 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 while in *RPL*-DBA EP/MKP are relatively preserved.

### Distinct erythroid progenitor trajectories in *RPS*- and *RPL*-DBA primary human HSPCs

To refine lineage relationships within EP/MKP in more detail, we performed further Louvain sub-clustering of the 6,380 EP/MKP cells in aggregate (**Fig. 2, A and B**). Inspection of the genes marking 4 EP/MKP subclusters (**table S6 and Fig. 2B**) showed that cluster 1 cells expressed genes marking early erythroid development (*CSF2RB*); cluster 2 cells expressed MKP genes; cluster 3 cells enriched for erythroid differentiation genes such as hemoglobin (*HB*) and their *AHSP* chaperone*(35)*; and cluster 4 enriched for erythroid and cell cycle control genes.

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/MKP clusters. We focused on expression of key E/MK TF(**Fig. 2C; fig. S2, A and B**), specifically, *GATA2* as an early EP (EEP/BFU-e) marker downregulated at the late EP (LEP/CFU-e) stage*(9, 37)*, *GATA1* upregulated from EEP to LEP and *KLF1* and *FLI1*, antagonizing one another to determine E versus MK respective cell fates*(36)*. This allowed us to predict that in normal BM cluster 1 corresponded to EEP (balanced *GATA1/GATA2* expression); cluster 2 to MKP; cluster 3 to LEP (higher expression of *GATA1/KLF1* and lower *GATA2*); and cluster 4 to the proliferative EP fraction (E cycling: higher expression of *AURKB*/*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/Ecycling and *TFR2*, *TFRC (CD71), GYPA* and *ENG (CD105)* in LEP (**Fig. 2C**). The expression of EP/MKP genes superimposed on FDGs (**fig. S2A**)further validated our subclustering analysis.

Next, we quantified the number of cells in each transcriptional subcluster in DBA BM with reference to total CD34+ cells. All EP/MKP subclusters were depleted in *RPS*-DBA (**Fig. 2D**). By contrast, EP/MKP subcluster frequencies in *RPL*-DBA were analogous to normal BM, suggesting distinct erythroid cellular trajectories according to DBA genotype (**Fig. 2D**). To specifically address the transcriptional basis of these differences, we analyzed E/MK TF expression. *GATA2* (but not *GATA1* or *KLF1*)was increased in all *RPS*-DBA subclusters relative to controls and *FLI1* was elevated in EEP and LEP (**Fig. 2C**), consistent with block in erythroid commitment. In contrast, reduced expression of *FLI1* was identified in *RPL*-DBA EEP, E cycling and MKP. Exploring this further by charting *FLI1* and *KLF1* co-expression in single EP/MKP cells (**Fig. 2E**) revealed prevailing expression of *KLF1* over *FLI1* in a higher fraction of EP/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 BM samples. While there was progressive reduction in all stages of EP development in *RPS19/26/24*-DBA, these populations were preserved in *RPL5/11*-DBA. This divergence was particularly notable in LEP which were virtually absent in *RPS-*DBA (**Fig. 2, F and G; table S1**). To determine the functional erythroid potential of *RPS*- vs *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 ofDBA 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. S2c**) generated highly abnormal, small CFU-e-like, colonies of <100 cells. Analysis of single erythroid colonies confirmed the presence of more mature EB in abnormal DBA colonies versus normal BFU-e (**fig. S2D**), 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 BM HSPCs generated a higher erythroid yield than *RPS*-DBA **(Fig. 2J)** with similar rates of apoptosis (**fig. S2E**), but with a higher fraction of more differentiated mature EB (**Fig. 2, K and L and fig. S2F**), expressing higher amounts ofGYPAmRNA and protein*,* compared to control HSPCs **(fig. S2, 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 BM sample revealed lower expression (log2FC< -0.4) of CD34 (in MPP2 to PolyEB) and higher expression (log2FC> 0.4) of CD71 (in EP/ProEB), CD36 and GYPA (in EP/ProEB and PolyEB), compared with healthy control counterparts (**fig. S2, I to L**). GATA1 and KLF1 expression were not reduced compared with normal (**fig. S2L**), 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 phenotypically normal, but functionally severely impaired BFU-e with a distinct transition program through the erythroid differentiation hierarchy in *RPL*-DBA.

### Phenotypically normal EB in *RPL*-DBA *in vivo*

Given that absence/paucity of BM 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 BM samples (**Fig. 3A**)**.** EB frequency in *RPS*-DBA BM was extremely low and significantly 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. S3, 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 BM (**Fig. 3B**), supporting relative preservation of the EP to EB developmental trajectory in *RPL*-DBA. We also compared differential cell counts from 52 BM aspirates from transfusion-dependent DBA patients (**fig. S3C**) and found that, although EB were reduced in both genotypes compared with normals, there was an approximately four-fold higher erythroid cell frequency and lower myeloid: erythroid ratio in *RPL-* versus *RPS*-DBA BM (**Fig. 3, C and D**). These findings were consistent across 6 *RPS*- and 4 *RPL*-DBA genotypes (**fig. S3, D and E; tables S1 and S5**), confirming our unexpected finding of preservation of erythroid differentiation beyond the progenitor stage in *RPL*-DBA.

### Glucocorticoid pathway-deficient stress erythropoiesis (SE) and *ZFP36L2*-mediated erythroid differentiation inhibition by glucocorticoids in DBA

To investigate the molecular basis for these differences in erythroid differentiation, first we confirmed that in DBA patients with loss-of-function mutations (**table S1**) mRNA expression of the affected RP gene was selectively reduced to approximately 50% of normal (**fig. S4A**). We also found that expresssion of fetal hemoglobin (*HBG2)* and the fraction of cellsexpressing *HBG2* were higher in all 3 EP populations from both DBA sub-types, compared with normal controls, confirming earlier studies*(40)* (**Fig. 4A**)*.* Like *HBG2*, additional markers of SE, such as *ERFE* and *GDF15(41, 42)*, were upregulated in DBA erythropoiesis(**fig. S4B**). Moreover using AUCell scoring*(43)* we found enrichment in DBA EP of a set of genes (**table S4**) upregulated in murine fetal liver -the prototype for SE*(31)* (**Fig. 4B**)*.* Alhough SE often occurs at the expense of the output of other lineages in murine models *(31)*, we observed preserved myeloid progenitor phenotype and function in *RPL*-DBA (**fig. S1G and fig. S2C**), consistent with our finding that the SE signature is present in only a proportion of single cells (**Fig. 4A and fig. S4B**) and is insufficient to rescue either EP function or the anemia in these patients.

To interrogate this further we considered that exogenous glucocorticoids remain the only medical therapy for DBA. SE 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. S2F**), we found that expression of erythroid differentiation genes (e.g. *GYPA, AHSP*, *HB* genes) was higher in *RPL*-DBA than control (**Fig. 2C and Fig. 4C**). Since transcription of *HBB* and *AHSP* is repressed by the TF glucocorticoid receptor (GR) 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 SE in DBA. Consistent with this, using a set of genes upregulated by glucocorticoids in murine EP (**table S4**)*(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-*mutatedEP (**Fig. 4D**). We also observed reduced expression of *ZFP36L2*, a glucocorticoid-responsive gene critical for glucocorticoid-mediated differentiation delay and subsequent enhanced erythroid output in murine fetal liver*(46)*, in primary DBA cells(**Fig. 4E**).

To investigate the importance of reduced *ZFP36L2* expression in impaired erythropoiesis, we used

Clustered Regularly Interspaced Short Palindromic Repears (CRISPR)- Cas9 edited *RPL11* kd (knocked down) K562 cells (residual *RPL11* mRNA 33-48% of unedited controls) **(fig. S4, C and D)**. Compared to wt K562 cells, *ZFP36L2* expression was reduced in *RPL11* kd K562 cells consistent with our data in primary DBA BM cells **(Fig. 4F)**. Next, as previously identified in murine erythroid cells*(46)*, we found that dexamethasone treatment of *RPL11* kd and control K562 clones resulted in a time-dependent loss of the cells co-expressing the erythroid differentiation GYPA and CD71 (**Fig. 4, G and H**), commensurate with upregulation of *ZFP36L2* expression (**Fig. 4I**). In accordance with this, RNA-seq of *ex vivo* BM stage-matched erythroblasts from a patient with *RPL5*-DBA, harvested before and after successful steroid therapy, showed increased *ZFP36L2* (**Fig. 4J**) but unchanged *RPL5*, *ADA* and *HBG2* expression*.* Furthermore, lentiviral transduction of *ZFP36L2* cDNA into *RPL11* kd K562 cells (**fig. S4E**) was sufficient to recapitulate the dexamethasone-induced loss of *GYPA* expression(**fig. S4, F and G**) resulting in a higher relative frequency of CD71+GYPA- versus CD71+GYPA+ cells in both wt and *RPL11* kd K562 cells (**Fig. 4, K and L**).

### P53 activation and BM 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 tumour necrosis factor (TNF)α, interferon (IFN)α- and IFNγ-mediated signaling (**Fig. 5A**), known instigators of SE*(47, 48)*. Inflammatory response, IFNα and IFNγm, but not P53 and TNFα 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. S5, A and B**), suggesting a generalized BM pro-inflammatory state linked to RP haploinsufficiency. In line with this, we found higher TNFα and IFNγ concentrations in DBA than control BM plasma (**Fig. 5C**) as well as increased intracellular expression of both cytokines in DBA versus control CD3+ T cells following their *in vitro* activation (**fig. S5, 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 BM of 3 additional *RPL*-DBA and 3 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. S5E**). 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, TNFα, IFNα and IFNγ inflammatory pathways in DBA EB (**Fig. 5, F and G**). Immunoproteasome and antigen presentation pathways, consistent with an active IFN 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, e.g. *HBA1, HBA2* and *HBB* were similar due to sorting of stage-matched populations (**fig. S5E**) and *HBG* 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 eADA in *RPL*- versus *RPS*-DBA patients (**Fig. 5I**)*(6, 32)***.**

Together these findings provide *ex vivo* evidence of a pro-inflammatory BM milieu in primary human DBA HSPC. Furthermore, although *RPL*-DBA erythroid progenitors are relatively preserved in numbers, functionally they appear to correspond to SE that is deficient in its hallmark endogenous glucocorticoid-regulated program, leading to disordered differentiation underpinned by aberrant EP function and 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 BM containing both erythroid and non-erythroid cells. We had the unique 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 3 GATA1 genes sets*(12, 49)*. This identified a depleted TRANSFAC or early GATA1 transcriptional signature in *RPS-*DBA EP (**Fig. 6A and fig. S6A**), 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. S6A**). In *RPL*-DBA LEP and EB, the late but not early G1e GATA1 signature was enriched in line with their altered differentiation trajectory (**fig. S6A**). In addition, expression of *GATA1short* and *GATA1full-length* isoforms was unchanged in *RPL* -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 DBA-like patients with germline *GATA1* mutations that preserve *GATA1short(50)*.

To elucidate the effect of *RPS*/*RPL* haploinsufficiency on GATA1 protein expression in primary DBA BM cells, we stained BM sections from 7 *RPL5/11*- and 5 *RPS19/24/26*-DBA patients and 3 healthy controls, with an antibody specific for GATA1*full-length* (**Fig. 6, C to E; fig. S6, B to D**). In *RPS*-DBA, most GATA1+ cells were negative for the erythroid marker 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 scCYTOF (**fig. S6E**) 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 appear to be 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 S5**). In line with our transcriptomic and functional data, patients with *RPL*-DBA presented with anemia at an older age (regardless of gender), and with a higher Hb concentration, than those with *RPS*-DBA (**Fig. 7, A and B; fig. S7, A to C; table S7**). Furthermore, a higher proportion of *RPL*-DBA patients were initially corticosteroid responsive (**Fig. 7C; fig. S7D and table S7),** although long-term steroid dependence was not increased (**Fig. 7D and fig. S7E**) and rates of spontaneous or steroid-induced remission were not significantly different between genotypes (**fig. S7F**). We also confirmed previous observations*(51)* of a higher rate of indel genetic variants (**fig. S7G**) and congenital anomalies(**fig. S7H**) 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. S7I**)*(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. 7E**).

## 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 SE in the pathogenesis of erythroid failure in DBA. DBA EP and EB exhibit many of the molecular hallmarks of SE, including overexpression of *GDF15* and *HBG2,* the orchestrator and signature gene of SE respectively*(40, 41)*, however, the endogenous glucocorticoid-dependent transcriptional signature appears to be defective in DBA-associated SE. These results derived from primary DBA BM erythroid cells mirror those in mice lacking expression of the GR (*NR3C1*)*,* which display anemia associated with accelerated erythroid differentiation under conditions of stress*(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 DBA patients, i.e, 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, i.e., that glucocorticoids improve the quality of SE rather than restore steady state-like erythropoiesis. In line with this, eADA and HbF markers usually remain elevated in steroid-treated DBA (**Fig. 4J**)*(3)*. Conversely, our observation that reduced *ZFP36L2* promotes erythroid differentiation may be exploited in other pathologic states characterized by excessive SE but blocked erythroid differentiation, such as -thalassemia and polycythemia rubra vera, where accelerated maturation (by macrophage depletion for example) ameliorates pathological erythropoiesis and anemia*(54)*.

As well as aberrant SE, in all DBA genotypes we demonstrate activation of P53*(8)*. Although the only other published transcriptomic data from DBA BM did not show P53 pathway enrichment, the threepatients 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 support therapeutic targeting of this pathway in *RPL-*DBA too.

Our work also identifies in both *RPS-* and *RPL*-DBA HSPCs, activation of IFNα, IFNγ and TNFα inflammatory pathways, potential triggers that could incite and sustain SE*(47, 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 BM inflammatory milieu *in vivo*, supported by detection of elevated IFNγ and TNFα in DBA BM plasma. Inflammatory signature imprints were previously reported in DBA patient mature red blood cells*(57)* and in zebrafish *RPL11* morpholinos*(58)* but here we show enrichment in specific hematopoietic lineages and ubiquitously within the BM 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)* and/or non-specifically activated T/ 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 lymphocytes in DBA compared with controls, suggesting that targeted anti-inflammatory agents such as TNF inhibitors, beneficial in anemia associated with chronic inflammation*(60)*, should be investigated in DBA. Reduced expression of *ZFP36L2* might also modulate inflammatory activation of lymphoid and myeloid cells in DBA BM 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 e.g., Shwachman-Diamond syndrome and for acute leukemia, juvenile myelomonocytic leukemia or myelodysplastic syndromes e.g., 5q- MDS associated with somatic *RPS14* haploinsufficiency*(63)*. Like DBA, these disorders are associated with a pro-inflammatory BM 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 DBA patient-derived B cells, profiling of polysome-associated mRNA transcripts showed more severe translation defectsof thelong 5’ UTR*BCAT1*transcript 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, while therapies such as glucocorticoids that delay erythroid differentiation*(52, 53)* and specifically modulate the GC target *ZFP36L2*,are likely to be more effective in *RPL*-DBA.

Finally, data from our large cohort of DBA patients 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 SE 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, DBA patients 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 impacts of ribosome dysfunction*(32, 71)*.

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 BM. Future studies of larger numbers of patients with distinct genotypes within each of the RPS/RPL subgroups are needed to 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 DBA patients. Our data categorize developmental trajectories and in turn, elucidate how these shape clinical phenotypes and therapeutic responses, according to underpinning pathogenic 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 was to integrate clinical, celluar and transcriptomic data from a large cohort of genotypes and phenotyped patients with DBA to elucidate genotype-phenotype correlations. Only patients who met the diagnostic criteria of DBA, 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. Blinding was used to measure GATA1 staining by immunohistochemistry.

### Subject details

Human BM samples (**table S1**) were collected following written informed consent, in accordance with the Declaration of Helsinki under a study approved by the National Research Ethics Service (REC reference 12/LO/0426). Where possible, control and disease samples were age/sex- matched. Healthy donor pediatric BM was collected from sibling donors; samples carrying alpha/beta thalassemia or sickle cell trait were used in selected experiments (excluding RNAseq) given the limited supply of pediatric BM 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

BMMNC were isolated by Ficoll-Hypaque (Sigma-Aldrich, Dorset, UK) 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+ cell selection

CD34+ cells were isolated magnetically from BMMNCs using the MiniMACS Separator kit (Miltenyi Biotech Ltd, Surrey, UK) 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 scCyTOF

Cells were suspended in RoboSep, incubated with FcR blocker (Miltneyi Biotech) for 5 minutes at room temperature (RT) then stained with a panel of up to 13 commercial fluorophore-conjugated monoclonal antibodies (**table S8)** 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 BV510 Live/Dead Fixable Stain (BD) for dead cell exclusion, prior to data acquisition on a 4-laser BD LSR Fortessa or BD Fusion. Data were acquired using FACSDIVATM software v8.0.1 and analyzed using FlowJo software (v10.5.3 Tree Star, Ashland, Oregon, USA). 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 RNAseq 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). For intracellular flow cytometry and scCyTOF see **Supplementary methods; tables S8 and S10.**

### Single-cell RNA-sequencing (10x Chromium)

Cells were thawed, stained with FACS antibodies (**table S8**) and sorted as described in the method above. 12-15x103 CD34+Lin- cells were sorted into 2 μL PBS/0.05% Ultrapure 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 or Novaseq S4 to attain >50,000 reads per cell (**table S2**). All scRNAseq analyses were performed using customized pipelines (SingCellaR, in submission,package available from <https://github.com/supatt-lab/SingCellaR>), as previously described*(23)*. 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 <2x106/ml by partial medium changes every 2-3 days. Base medium consisted of stemspan, Penicillin/streptomycin 100units/mL, Stem cell factor 100 ng/mL Interleukin-3 10 ng/mL, Lipids 40μg/mL, L-glutamine 25ng/mL and IL-6 10ng/mL. Epo concentration was increased from 0.5 to 3-4 units/mL on day 7. The cultures were incubated at 37°C with 5% CO2 for up to 14 days.

### Methocult assay

Single EEP or 500 CD34+ -cells were FACS-sorted into 100μl or 1ml 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% CO2 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 erythroblasts in ‘bursts’ were counted on d12-14 as BFU-e while small uni- or bi-centric clusters of erythroblasts 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 2-5x104 cells/200μl. Cells were cytocentrifuged at 400 rpm for 5 min onto Superfrost slides, using a Shandon Cytospin 2 (Fisher Scientific, Loughborough, UK). Slides were air dried, fixed in 100% methanol and stained with working solutions of May-Grünwald Giemsa (MGG; Sigma-Aldrich, Dorset, UK). 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) and 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, Hertfordshire, UK), as per the manufacturer’s version 1.5 protocol (**Supplementary methods**).

### CRISPR/Cas9 genome editing and lentiviral transduction

Ribonucleoprotein comprising Cas9 and single guide RNA (Synthego) was used to knockdown *RPL11* in the K562 cell line. *ZFP36L2* cDNA open reading frame (Genscript) was introduced by lentiviral transduction (**Supplementary methods and table S9**).

### BM plasma

1-2mls BM aspirate was collected in an EDTA BD tube, spun at 1500g at 40C for 12 minutes. The upper plasma layer was aspirated, spun at 40C, and then stored immediately at -800C. Analysis of BM plasma cytokines and chemokines was performed by Eve Technologies using the Human Cytokine Array (HD42) Discovery Assay.

### GATA1 immunohistochemistry

GATA1 immunohistochemistry (IHC) was performed on archived BM paraffin 1mm sections, as previously described*(74)* using an anti-GATA1full length antibody (D52H6, Cell Signaling Technology, Danvers, MA). Staining, imaging and a bespoke imaging analysis protocol are detailed in **Supplementary methods.**

### 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 (2013-2020). Missing data was collected retrospectively. 15/161 patients were excluded from the study as there was insufficient data available to fulfil 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 (**Supplementary methods and table S5**).

### Statistical analyses

Data aggregation and statistical analyses were performed using GraphPad Prism (v8.1.0) and SPSS (Version 26, IBM Corp., Armonk, NY) for experimental and clinical registry data, respectively. Unless otherwise stated, bar charts 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/types of replicates and P value thresholds are described in legends. Differences in continuous variables between groups were assessed using the Students- t test (2 groups; parametric), Mann-Whitney U (2 groups; non-parametric), Kruskal-Wallis test (>2 groups, non-parametric), Wilcoxon rank test (paired, non-parametic) or one-way ANOVA tests (>2 groups, parametric), with Bonferroni, Dunn or Holm-Sidak multiple comparisons tests, respectively. Normality of data was assessed by the Shapiro-Wilk test (Data File S1). 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, related to Figure 1. Single-cell RNA sequencing (scRNAseq) of CD34+Lin- HSPC and cell type annotation.

Fig. S2, related to Figure 2. Characterization of EP phenotype and function.

Fig. S3, related to Figure 3. Characterization of mature erythropoiesis in DBA.

Fig. S4, related to Figure 4. DBA SE and overexpression of *ZFP36L2* in wt and*RPL11*kd K562.

Fig. S5, related to Figure 5. Aberrant molecular signatures in DBA erythropoiesis.

Fig. S6, related to Figure 6. GATA1 transcriptional activity and expression.

Fig. S7, related to Figure 7. Genotype-phenotype correlations in the UK DBA cohort.

Table S1, related to Figures. 1-6 (see excel file). Clinical details for BM samples included in study.

Table S2, related to Fig. 1. Quality control using CellRanger and filtering cut-offs for scRNAseq data.

Table S3, related to Fig. 1 (see excel file). Genes differentially expressed in the 19 K-means clusters for the control and DBA aggregate of CD34+Lin- cells (up to 100 top upregulated genes listed per cluster).

Table S4, related to Figs. 1 and 4 (see excel file). Signature marker genes used to ascribe lineage to scRNAseq clusters, or used for AU Cell scoring.

Table S5, related to Fig. 7. Pathogenic mutations/deletions identified in all patients in DBA UK cohort (pathogenic mutation in RP gene identified in 106 of 161 patients).

Table S6, related to Fig. 2 (see excel file). Genes differentially expressed in the 4 K-means clusters for the control and DBA aggregate of EP\_MKP cells (up to 100 top upregulated genes listed per cluster).

Table S7, related to Fig. 7 (see excel file). Statistical analyses of DBA clinical registry.

Table S8, related to Figs. 1, 3 and 5. Antibodies used for flow cytometry.

Table S9, TaqMan assays used for RT-PCR.

Table S10, Antibodies used for scCyTOF.

Data File S1. Individual level data for all figures where n<20/group.

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## Figure Legends

### Fig. 1. Preservation of erythroid lineage specification in RPL but not RPS-DBA patient BM.

1. Study design for single cell RNA-sequencing (scRNAseq) experiments, showing source of DBA and healthy BM from allogeneic BM transplant (BMT) donors. Erythroid hierarchy depicted as follows: hematopoietic stem (HSC) and multipotent (MPP) cells, within the CD34+CD38- BM subfraction, mature into committed EEP (BFU-e) and LEP (CFU-e), within the CD34+CD38+ BM subfraction. These then differentiate into CD34- EB, which enucleate to form reticulocytes that egress into the peripheral blood and enucleate to form red blood cells (RBC).
2. 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 also shown.
3. Force-directed graph (FDG) embedding of 6 major hematopoietic cell types in control and DBA cells, colored by key marker gene sets (**table S4 and fig. S1D**). Grey cells represent uncommitted cell types or cells expressing >1 lineage gene set. Abbreviations for progenitors: HSC/MPP: hematopoietic stem/multipotent progenitor cells; EP: erythroid; LyP: lymphoid; MKP: megakaryocyte; MyP: myeloid; EoMBP: eosinophil, mast cell, basophil.
4. *Left panel:* Circus plots and *right panel*: bar blot depiciting proportions of cells in each of 6 cell types identified by scRNASeq among total CD34+Lin- cells, by donor type.
5. Flow cytometry gating strategy used to identify CD71+CD45RA- EP/MKP cells in the CD38- immature and CD38+ mature sub-compartments of BM CD34+ enriched cells. Frequencies are shown as % of CD34+Lin- cells.
6. Cumulative data showing frequency of BFU-e and MKP within CD34+Lin-CD71+CD38- BM subfraction and of BFU-e, CFU-e and MKP within CD34+Lin-CD71+CD38+ BM subfraction (*n=23* control, *20 RPS*-DBA and *9* *RPL*-DBA BM).

Bars show mean ± SEM of biological replicates.\*\*\*\*P<0.0001; \*\*P<0.01; \*P<0.05, *n.s*: not significant. Groups were compared by one-way ANOVA with Holm-Sidak’s (**1D**) or Kruskal-Wallis with Dunn’s multiple comparisons test, as appropriate (**1F**). See also **fig. S1; tables S1, 2, 3, 4 and 8.**

### Fig. 2. Distinct features of the erythroid progenitor trajectory in *RPS-* and *RPL*-DBA primary human HSPCs.

1. UMAP aggregate of all control and DBA EP/MKP cells (*n=6380*) depicting 4 distinct subclusters.
2. Heatmap showing 10 top differentially expressed genes (DGE: row labels, *right*) for each of the four EP/MKP sub-clusters (color-coded columns). Labels *top* denote definition of clusters by their marker genes into the following groups: early erythroid progenitors (EEP), cycling EP (Ecycling), late erythroid progenitors (LEP) and megakaryocyte progenitors (MKP).
3. Bubble plot showing the average level of expression of individual genes (depicted by color) and the fraction of cells expressing that gene (depicted by bubble size), within EP/MKP subclusters from control, *RPS*-DBA or *RPL*-DBA BM. Boxes highlight DGE with log2FC >0.4 and adjusted p < 0.05.
4. Bar plot depicting proportion of cells in each of 4 EP/MKP clusters among total CD34+Lin- cells, by donor type.
5. Single cell co-expression of *KLF1* and *FLI1* TFs in control*, RPS*- and *RPL*-DBA EP/MKP cells. Dashed line shows where cells that express equal expression of both genes reside. Fraction of cells above and below line shown.
6. Representative flow cytometry plots showing 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 % of total CD34+ cells.
7. Frequency of EP subsets, expressed as % of CD34+ BM 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) BM. Green symbols depict two BMsamples from same patient, two yrs apart.
8. Morphology of colonies generated on day 12-14 in methylcellulose medium from single-cell EEP FACS-purified *ex vivo* from control, *RPS*- and *RPL*-DBA BM. Images are representative of three independent experiments.
9. Frequency and type of hematopoietic colonies generated in methylcellulose from single-cell EEP FACS-purified *ex vivo* from control, *RPS*- and *RPL*- DBA BM (*n=3*). Significant differences in colony types shown.
10. Erythroid yield (total cell number x % CD71+CD14/16/61- cells) in a longitudinal serum-free erythroid liquid culture of FACS-purified CD34+Lin- HSPCs from control, *RPS*- and *RPL*-DBA BM (*n=2*).
11. Flow cytometric analysis of erythroid differentiation stage (characterized by Lin, CD71 and GYPA markers) of erythroblasts (EB) on day 13 of culture of control, *RPS-* and *RPL-*DBA BM HSPCs.
12. Cumulative data from two independent experiments showing fraction of early and late EB generated multiplied by erythroid yield from control, *RPS*- and *RPL*-DBA BM HSPCs (*n=2*).

Charts show mean ± SEM of biological replicates.\*\*P<0.01; \*P<0.05, *n.s*: not significant. Groups were compared by Fisher’s exact test (I) or one-way ANOVA with Holm-Sidak’s (D) or Kruskal-Wallis with Dunn’s multiple comparisons test (G). See also **fig. S2; tables S3, 6 and 8.**

### Fig. 3. Phenotypically normal mature EB in *RPL*-DBA *in vivo.*

1. *Left panel:*Representative flow cytometry plots showing frequency of Lin-CD34-CD71+ EB within total BMMNCs. *Right panel:*Cumulative data of EB frequencies within total BMMNCs in control (*n=10*), *RPS-* (*n=17*) and *RPL*-DBA (*n=8*) BM. Green symbols depict two samples from the same patient, two years apart.
2. **b)** Frequencies of EB subpopulations as measured by flow cytometry
3. in total BMMNC derived from control (*n=8*) and *RPL*-DBA (*n=7*) BM.
4. Representative morphological appearances of BMA (stained with hematoxylin and eosin) in control and patients with a *RPS19* and a *RPL5* mutation.
5. EB frequencies in total nucleated cells and myeloid to erythroid lineage cell ratio in BM aspirate slide preparations from normal pediatric BM controls (*n=15*) and patients with *RPS-DBA* (*n=32; 34* for M:E) and *RPL-*DBA (*n=17; 18* for M:E).

Charts show mean ± SEM of biological replicates.\*\*\*\*P<0.0001; \*\*P<0.01; \*P<0.05; *n.s*: not significant. Groups were compared by Kruskal-Wallis with Dunn’s multiple comparisons test. See also **fig. S3.**

### Fig. 4. Glucocorticoid pathway-deficient SE and *ZFP36L2*-mediated erythroid differentiation inhibition by glucocorticoids in DBA.

1. Violin plots depicting the mean expression (yellow dot) and distribution (minimum to maximum) of *HBG2,* a SE gene in control*, RPS*-DBA and *RPL-*DBA EP subclusters (EEP, Ecycling, LEP). Fraction of cells expressing the gene is shown on x axis.
2. Mean expression (yellow dot) and distribution of AUcell score of SE gene set in control*, RPS*-DBA and *RPL-*DBA EP subclusters.
3. Bubble plot showing expression of erythroid differentiation genes in control*, RPS*-DBA and *RPL-*DBA EP subclusters.
4. Violin plots depicting expression of *ZFP36L2* in control*, RPS*-DBA and *RPL-*DBA EP subclusters.
5. AUcell score of GC response genes in control*, RPS*-DBA and *RPL-*DBA EP subclusters.
6. *ZFP36L2* measured by RT-PCR in wt (*n=6*) and *RPL11* kd (*n=4*) K562clones, normalized to wt and *GAPDH*. Data points represent RNA extracted at independent time points.
7. Erythroid differentiation, represented by CD71 and GYPA expression (as measured by flow cytometry) of un-and Dex-treated wt and *RPL11* kd K562 clones (representative of 4 independent experiments).
8. Relative frequencies of early and late EB as measured by flow cytometry, in Dex- treated wt and *RPL11* kd K562 clones*,* normalized to un-treated *(n=4*). [e.g. for early EB (%CD71+GYPA-Dex - %CD71+GYPA- untreated)/ %CD71+GYPA- untreated]
9. *ZFP36L2* measured by RT-PCR in Dex- and un-treated wt and *RPL11* kd K562 clones (*n=4*), normalized to untreated cells and *GAPDH.*
10. *ZFP36L2, RPL5, ADA* and *HBG2,* measured by RNA-seq of EB FACS-isolated from BM of a patient with *RPL5-*DBA 1 month pre- and 16 months post- steroid therapy. Hemoglobin concentration at time of BM sampling also shown.
11. Lentiviral transduction efficiency (% Green Fluorescent Protein+) and erythroid differentiation (represented by CD71 and GYPA expression) of wt and *RPL11* kd K562clones,2 days after transduction with mock (empty vector) *or ZFP36L2.* Plots are representative of 3 independent experiments.
12. Relative frequencies of early and late erythroid in wtand *RPL11* kd (calculated as in 4H)*,* 2-4 days after lentivirus transduction with mock or *ZFP36L2 [n=3 (D2) or n=2 (D3 and 4)].*

Charts 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 Wilcoxon rank sum test (5A,B,D and E), unpaired Student’s t test (5F) or paired Student’s t test (4I). See also **fig. S4 and tables S4, S8 and S9**.

### Figure 5. P53 activation and BM inflammatory milieu in DBA.

1. Bubble plot showing normalized enrichment score (NES) and false discovery rate (-Log10FDR) of significantly enriched (FDR q value <0.25) pathways of interest on gene set enrichment analysis (GSEA) of differentially expressed genes (DGE) between DBA and control EP subclusters. Pathways are from the Hallmark or KEGG gene sets of Molecular Signatures Database.
2. Bubble plot showing enriched pathways between *RPS-* and *RPL-*DBA EP subclusters.
3. Concentration of TNFα, IFNα and IFNγ in control, *RPS*- and *RPL*-DBA BM plasma (*n=5* for con and RPS-DBA, *n=6* for RPL-DBA) as assessed by luminometry.
4. Principal component analysis (PCA) depiction of bulk RNAseq data from stage-matched EB derived from three healthy pediatric controls and three DBA patients (*n=1* *RPL11* and *n=2 RPL5*), separated by PC1, PC2 and PC3.
5. Volcano plot highlighting 1709 DGE, of which 1101 are unregulated. Thresholds (dotted lines) are adjusted P value < 0.05, and Log2 fold change ±1. Names of top 50 DGE are shown.
6. GSEA showing enrichment of Hallmark/ KEGG gene sets in pre-ranked DGE 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.
7. Ingenuity Pathway Analysis of DGE between *RPL*-DBA and control EB, showing top four upstream regulators of the DBA transcriptome.
8. Bubble plot depicting Log2 fold change and -Log10 adjusted P value of *ZFP36L2* and 3 SE genes, quantified by bulk RNA-seq in *RPL*-DBA (*n=3*) and control (*n=3*) EB. All genes have Log2FC > 0.6 and adjusted P values < 0.05.
9. *Left panel:* *eADA* mRNA, quantified by bulk RNA-seq of FACS-purified EB from control and *RPL*-DBA (*n=3*) BM. Adjusted p value is shown. *Right panel*: peripheral blood red blood cell eADA activity (nm/mg Hb/hour), off blood transfusions in patients with *RPS-*DBA (*n=14*) and *RPL-*DBA (*n=11*).

Charts show mean ± SEM of biological replicates.\*\*\*\*P<0.0001; \*\*P<0.01, \*P<0.05, *n.s*: not significant. Groups were compared by Kruskal-Wallis test (5C) or Mann-Whitney U test (5I). See also **fig. S5.**

### Fig. 6. Preservation of GATA1 and its transcriptional program in *RPL*-DBA.

1. GSEA against a TRANSFAC-derived GATA-1 gene set *(12)* of DGE between control and DBA subgroups at different erythroid stages (scRNAseq for EP and bulk RNAseq for EB). Significant FDR q values (<0.25) highlighted in bold face.
2. Full length and short *GATA1* transcript isoform abundance as determined by transcript analysis of bulk RNAseq of stage-matched EB from healthy control and *RPL*-DBA BM (*n=3*). *TPM*: transcripts per million.
3. *Top panel:* Representative image of GATA1 expression assessed by immunohistochemistry of healthy control, *RPS*- and *RPL*-DBA BM sections. GATA1 expression shown by brown staining in erythroblasts (EB; identified by their unilobar round nuclei), megakaryocytes (MK; large multilobe nuclei) and eosinophil/ mast cell/basophils (EoMB; bilobed/horseshoe nuclei and granular cytoplasm). Expression is weaker in EoMB compared with EB/MK cells, and decreases in late EB relative to early/intermediate EB. *Lower panel:* Co-staining for GATA1 and the erythroid-specific marker GYPcophorin C (GYPC) to distinguish EB (GYPC+ red membrane/cytoplasmic rim) from EoMBP/MK (GYPC-).
4. Correlation between GATA1 expression and nuclear diameter in control BM.
5. Violin plot showing single cell GATA1 expression, measured in BM EB from control (*n=1781 cells*; 3 donors), *RPS*-DBA (*n=286*; 4 donors) and *RPL*- DBA (*n=1179*; 7 donors). Distribution is from lower adjacent value: 5th centile to upper adjacent value: 95th centile). Dotted line shows mean expression of all samples and y axis shows number of SDs that each data point differs from mean (Z-score).
6. Representative plots showing GATA1 and GATA2 expression measured by intracellular flow cytometry in control and *RPL5*-DBA BM erythroid progenitors (CD71hiGYPA-) and EB (CD71+GYPA+). Quadrants in GATA1/GATA2 plot were set using fluorescence minus one plus isotype controls.

Groups were compared by Kruskal-Wallis with Dunn’s multiple comparisons test (**B** and **E**). \*\*\*\*P<0.0001; \*\*P<0.01. *n.s*- not significant. See also **fig. S6 and table S10.**

### Fig. 7. The distinct clinical phenotype of *RPL*-DBA.

1. Age at presentation with anemia according to *RPS*-DBA (*n=62*) or *RPL*-DBA (*n=44*) genotype. Dots depict data points for individual patients.
2. Haemoglobin level (g/L) at presentation, according to *RPS* (*n=59*) or *RPL* (*n=38*) genotype.
3. Fraction of steroid-responsive (SR) i.e., transfusion-independent for ≥ 6 months versus steroid unresponsive (SUR) according to *RPS* (*n=59*) or *RPL-*DBA (*n=37*) genotypes. Numbers of cases are shown in the bars.
4. Odds ratio and 95% confidence intervals of specific clinical features and outcomes in *RPL*- versus *RPS*-DBA. P values are shown on the right.
5. A proposed model of *RPS*- vs *RPL*-DBA erythropoiesis summarizing the findings of this work.

Upper and lower horizontal lines of box plot represent 75th and 25th percentile respectively. Whiskers represent maximum and minimum values. Continuous variables were compared across genotype using the Mann-Whitney U test (**A** and **B**). Frequencies of cases were compared across genotypes using the Pearson chi-square test (**C**). All variables significant on univariate analysis were tested by binary logistic or multiple linear regression, as appropriate. P value shown refer to regression analyses. \*\*P<0.01; \*P<0.05. See also **fig. S7; tables S5 and S7.**