Glucocorticoids improve erythroid progenitor maintenance and dampen *Trp53* response in a mouse model of Diamond–Blackfan anaemia

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Summary

Diamond–Blackfan anaemia (DBA) is a rare congenital disease causing severe anaemia and progressive bone marrow failure. The majority of patients carry mutations in ribosomal proteins, which leads to depletion of erythroid progenitors in the bone marrow. As many as 40% of all DBA patients receive glucocorticoids to alleviate their anaemia. However, despite their use in DBA treatment for more than half a century, the therapeutic mechanisms of glucocorticoids remain largely unknown. Therefore we sought to study disease specific effects of glucocorticoid treatment using a ribosomal protein s19 (*Rps19*) deficient mouse model of DBA. This study determines for the first time that a mouse model of DBA can respond to glucocorticoid treatment, similar to DBA patients. Our results demonstrate that glucocorticoid treatment reduces apoptosis, rescues erythroid progenitor depletion and premature differentiation of erythroid cells. Furthermore, glucocorticoids prevent *Trp53* activation in *Rps19*-deficient cells- in a disease-specific manner. Dissecting the therapeutic mechanisms behind glucocorticoid treatment of DBA provides indispensible insight into DBA pathogenesis. Identifying mechanisms important for DBA treatment also enables development of more disease-specific treatments of DBA.

Keywords: Diamond-Blackfan anaemia, erythroid differentiation, *Trp53*, glucocorticoid therapy, erythroid progenitor.

Diamond–Blackfan anaemia (DBA) is a rare early-onset red cell aplasia that is usually manifested during the first year of life (Diamond & Blackfan, 1938; Nathan *et al*, 1978). At diagnosis, patients present macrocytic anaemia, reticulocytopenia and a normocellular bone marrow where erythroid progenitors are scarce or absent (Vlachos & Muir, 2010). The finding of mutations in ribosomal protein s19 (*RPS19*) led to the notion that DBA is a ribosomopathy causing functional haploinsufficiency (Draptchinskaia *et al*, 1999; Farrar & Dahl, 2011). Mutations in *RPS19* are found in 25% of all patients and, together with more than 10 other less frequently affected ribosomal protein genes, they cover 60–70% of all DBA cases (Gazda *et al*, 2004; Landowski *et al*, 2013; Vlachos *et al*, 2013). In 2012 the erythroid specific transcription factor *GATA1* was found to be mutated in a small cohort of DBA patients, challenging the notion that DBA is a disease of exclusive ribosomal origin (Sankaran *et al*, 2012).

Diamond–Blackfan anaemia patients usually require lifelong treatment and apart from chronic blood transfusions, glucocorticoid therapy has been standard for more than half a century to alleviate the anaemia (Gasser, 1951; Vlachos *et al*, 2008). Up to 80% of all DBA patients respond to glucocorticoid therapy and approximately 40% remain steroid responsive over time (Vlachos *et al*, 2008). Despite being the only available pharmacological therapy for DBA, the therapeutic mechanisms behind glucocorticoid treatment remain elusive. It is well established that glucocorticoid receptor activation is essential for erythroid progenitor expansion during stress erythropoiesis that occurs upon

excessive blood loss or chronic anaemia. Mice lacking the ability of glucocorticoid receptor dimerization fail to respond to stress erythropoiesis caused by haemolytic anaemia (Bauer *et al*, 1999) and self-renewing colony assay of erythroid progenitors *ex vivo* requires glucocorticoids (Wessely *et al*, 1997; Leberbauer *et al*, 2005; Dolznig *et al*, 2006; Flygare *et al*, 2011). Multiple glucocorticoid-induced genes are important for erythropoiesis, such as the RNA-binding protein *Zfp36l2*, which has proved crucial for self-renewal of erythroid progenitors (Zhang *et al*, 2013) and the transcription factor *Myb*, which is important for long-term erythroid proliferation (Wessely *et al*, 1997; Lindern von *et al*, 1999). Glucocorticoids also delayed differentiation in both human and avian erythroid cells, which in turn led to increase in the expansion capacity of erythroid progenitors (Golde *et al*, 1976; Wessely *et al*, 1997; Lindern von *et al*, 1999). Thus, glucocorticoids play an important role in the stressed erythroid system and are important in DBA treatment, but less **is** known about their therapeutic mechanisms. In order to elucidate this, more knowledge about disease mechanisms in DBA is crucial.

Elevated *TP53* activity is brought forward as one of the underlying mechanisms behind DBA pathology, and studies in human cells as well as in mice and zebrafish have shown improvement of the DBA phenotype in the absence of *Trp53* (Danilova *et al*, 2008; Dutt *et al*, 2011; Jaako *et al*, 2011; Taylor *et al*, 2012). In the context of intact ribosomal proteins, erythroid cells with a *Trp53* -/- background proliferate better than wild type counterparts, suggesting that *Trp53* has a general inhibitory effect on erythropoiesis (Ganguli *et al*, 2002). It is not fully known how *Trp53* is activated in ribosome protein-deficient erythroid cells, but it is probably explained in part by stress responses due to impaired ribosomal biogenesis, known as ribosomal stress.

The aim of this study was to identify the therapeutic mechanisms of glucocorticoids in DBA by studying the response in an *Rps19*-deficient mouse model and verifying these results in DBA patient cells. Our mouse model proved to be an excellent tool to validate DBA drug treatment and responded to glucocorticoids similarly to DBA patients. We found that glucocorticoid treatment prevented erythroid progenitor depletion and delayed erythroid differentiation, which led to improved expansion of *Rps19*-deficient cells. The increased *Trp53* activity in *Rps19*-deficient cells was reversed by glucocorticoid treatment, indicating a disease specific mechanism of glucocorticoids.

Material and methods

Rps19-deficient mouse strain, transplantation and shRNA induction

We used a doxycycline-inducible *Rps19*-deficient mouse previously developed by us, carrying a reverse transactivator (rtTA)-dependent shRNA against *Rps19* that develops robust anaemia upon doxycycline induction. For all experiments, we used the shRNA-D mice (Jaako *et al*, 2011). The haematopoietic phenotype in these mice is specific to *Rps19* down regulation as it can be cured by enforced expression of *Rps19* (Jaako *et al*, 2014). 8- to 14-week-old WT C57bl/6 males were subjected to lethal irradiation (900 cGy) and transplanted with unfractionated bone marrow from *Rps19*+/+, rtTA/rtTA (carrying transactivator only) or D/+, rtTA/rtTA recipients (carrying transactivator and heterozygous for *Rps19* shRNA). The animals received Ciprofloxacin 125 μg/ml (Arrow Generics, Stevenage, UK) for 2 weeks post-transplantation. Six to nine weeks after transplantation the mice received doxycycline (2 mg/ml) with sucrose (10 mg/ml) in drinking water *ad libitum* for 9 days as well as 100 μg/ml of Prednisolone or corresponding volume of solvent in the drinking water (Sigma-Aldrich, St. Louis, MO, USA). Peripheral blood was analysed fresh on a haematology analyser KX-21N (Sysmex, Kobe, Japan) after 9 days of doxycycline induction.

Liquid culture and methylcellulose colony assay

Faetal livers from heterozygote D/+ E14·5-15·5 embryos were dissected and enriched for KIT (CD117) positive cells using magnetic beads and LS columns (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers' instructions. The cells were kept in serum-free expansion medium (SFEM; Stem Cell Technologies, Vancouver, Canada) with 2 u/ml erythropoietin (EPO; LEO Pharma, Ballerup, Denmark), 100 ng/ml murine stem cell factor (mSCF) (PeproTech, Rocky Hill, NJ, USA), with or without 100 nmol/l Dexamethasone (Sigma-Aldrich), and/or 0·5-1 μg/ml doxycycline (Sigma-Aldrich) and kept at 37°C with 5% CO_2. Cells were counted manually using trypan blue in a haemocytometer. Colony forming unit-erythroid (CFU-E) assays were performed using serum free semisolid MethoCult #3236 (Stem Cell Technologies) supplemented with 5 u/ml EPO and 50 ng/ml mSCF and were cultured at 37°C with 5% CO_2 and 4%O_2, and the colonies were scored after 4 days.

Real time-polymerase chain reaction (RT-PCR) For real time (RT)-PCR analysis, RNA extraction was performed using RNeasy micro kit (Qiagen, Venlo, Netherlands) according to the manufacturers' instructions and analysis was performed on a 7900 HT fast RT-PCR (Applied biosystems, Waltham, MA). Primers used for RT-PCR were from Life Technologies (Carlsbad, CA, USA): *Hprt* (#01545399_m1), *GAPDH* (#02758991_g1) *Cdkn1a* (#04205640_g1 and 00355782_m1), *Bax* (#00432051_m1 and 00180269_m1), *Ccng1* (#00438084_m1), *Phlda3* (#00449846_m1), *Ptp4a3* (#00477233_m1) and *ZFP36L2* (00272828_m1), and *Zmat3* (#01292424_m1).

Flow cytometry

A modified version of an erythroid differentiation protocol was used for flow cytometric analysis (Liu *et al*, 2013). Antibodies used were phycoerythrin-cyanin 7 (PECy7)-conjugated

TER119 (eBioscience, San Diego, CA, USA), and phycoerythrin (PE)-conjugated CD44 (BioLegend, San Diego, CA, USA). Allophycocyanin (APC)-conjugated B220, CD3 and GR-1 (BioLegend) were used as lineage markers. 7-aminoactinomycin D (7AAD) was used to monitor viability. The plots were gated on live cells of appropriate size, negative for lineage markers. For flow cytometric detection of apoptosis, PE-conjugated AnnexinV apoptosis detection kit (BD biosciences, Franklin Lakes, NJ, USA) was used according to the manufacturer's instructions. Data collection was done on a Canto II flow cytometer (BD Biosciences). For cell cycle analysis, the cells were disrupted in ice-cold ethanol and the DNA was stained with propidium iodide. Cell cycle analysis was done on Aria III flow cytometer (BD Biosciences). All analyses were performed using FlowJo v10 (Tree Star Inc., San Carlos, CA, USA).

Western blot assay

Cells cultured for 72 h were harvested in Laemmli buffer containing 1% mercaptoethanol and proteinase inhibitor cocktail, homogenized and boiled for 5 min. Samples were run on a gradient 4–12% Bis-Tris gel (Life Technologies), and blotted according to manufacturer's instructions. The anti-MYB antibody was from Santa Cruz Biotechnology (Dallas, TX, USA) and anti-Actin antibody from BD Biosciences.

DBA patient cells

A human peripheral blood sample was collected after obtaining informed consent according to ethical guidelines at Universitätsklinikum in Freiburg, Germany. The patient sample was from a 14-year-old transfusion-dependent male DBA patient carrying a heterozygous *RPS19* mutation (c.280C>T, p.Arg94X), and healthy control samples were from one male and one female. The blood was enriched for CD34$^+$ fraction using magnetic MicroBead Kit, according to manufacturer's instructions (Miltenyi Biotech). The cells were subsequently expanded in a 2-phase erythroid culture to promote expansion before being cultured with or without dexamethasone for 24 h. The erythroid expansion protocol was 7 days in serum-free medium containing 20 ng/ml thrombopoietin, 20 ng/ml Flt3-ligand, 100 ng/ml human SCF (hSCF), 10 ng/ml interleukin 3 (Peprotech) and 1 nmol/l dexamethasone, followed by 2 days in SFEM containing 30% serum, 2 u/ml EPO and 50 ng/ml hSCF, prior to washing and stimulation in SFEM with 2 u/ml EPO, 50 ng/ml hSCF with or without 100 nmol/l dexamethasone for 24 h. RNA extraction and RT-PCR analysis were performed as for mouse samples.

Statistical analysis

For statistical analyses the nonparametric unpaired Mann–Whitney U test or paired Wilcoxon test were employed, unless otherwise stated. All statistical calculations were done in the software GraphPad Prism 6·0 (GraphPad Software Inc., San Diego, CA, USA). All graphs display median with interquartile range, unless otherwise stated. *P*-values: *≤0·05, **≤0·01, ***≤0·001, ****≤0·0001.

Microarray statistics

The microarray data were Robust Multiarray Average (RMA) normalized. Differentially expressed genes were identified using Significance Analysis of Microarray (SAM), using a 'multi class' analysis, where a value of delta was chosen so the resulting differentially expressed genes had a false discovery rate (FDR) of 5%. The differentially expressed genes were then clustered into nine partitions using k-means. Gene set enrichment analysis (GSEA) was performed using gene sets defined by the following: *Trp53* genes were taken from KEGG (http://www.genome.jp/kegg/). TER119 and burst forming unit-erythroid (BFU-E) genes were defined from RNA sequencing data http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26086 (Flygare *et al*, 2011). M/A values were calculated where genes with A > 3 were selected first. Of the remaining, the top and bottom 330 genes were taken from a ranked list of M (log ratios) values to define each list. GSEA was then performed using default settings.

Results

Glucocorticoids reverse anaemia in a mouse model of DBA

In our *Rps19*-deficient inducible mouse model 9 days of doxycycline administration caused a robust anaemia with decreased red blood cell (RBC) counts, reduced haematocrit (HCT) and haemoglobin (HGB) levels, as previously reported (Fig 1A–C) (Jaako *et al*, 2011). The anaemia of *Rps19*-deficient mice (D/+) was significantly improved when treated with the glucocorticoid Prednisolone, with a significant increase in RBC counts as well as HGB and HCT compared to untreated *Rps19*-deficient animals. *Rps19*-deficient mice that received Prednisolone also showed significantly lower MCV compared to untreated *Rps19*-deficient mice (Fig 1D). As previously reported, *Rps19*-deficiency led to a decrease in white blood cell counts (WBC) and platelet (PLT) counts (Jaako *et al*, 2011), which were further decreased in prednisolone-treated animals (Fig S1). These results demonstrate that our *Rps19*-deficient mouse model recapitulates the anaemia seen in DBA, and that these mice respond to glucocorticoid treatment similar to DBA patients with improved erythroid blood parameters.

Glucocorticoids improve survival and expansion of *Rps19*-deficient erythroid cells

Erythroid cells from patients and DBA cell lines have shown to be pro-apoptotic, which indicates that apoptosis
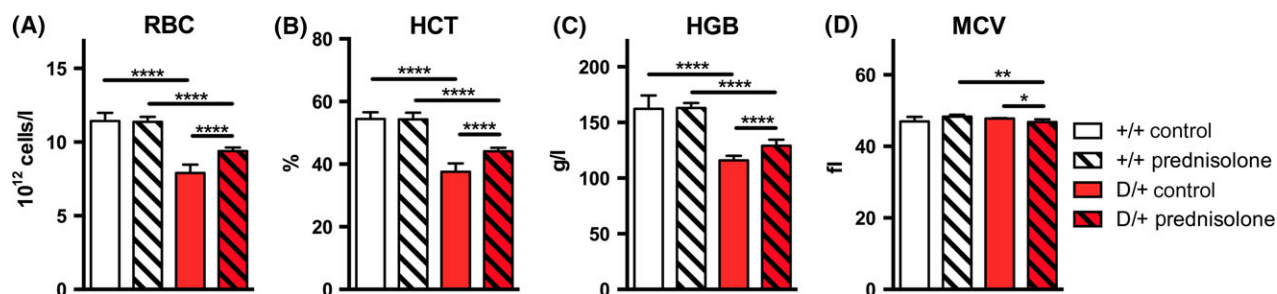
Fig 1. The anaemia of *Rps19*-deficient mice is ameliorated by prednisolone treatment. Blood parameters of mice transplanted with +/+ (no *Rps19* shRNA) or D/+ (heterozygote for *Rps19* shRNA) bone marrow after 9 days of induction with doxycycline and/or prednisolone. A) Red blood cell (RBC) count, B) haemoglobin (HGB) and C) haematocrit (HCT) of D/+ mice receiving prednisolone is significantly improved. D) Mean corpuscular volume (MCV) decreases between *Rps19*-deficient animals receiving Prednisolone compared to *Rps19*-deficient controls. $n = 6$–18 for each treatment group.

plays an important role in DBA pathogenesis (Perdahl *et al*, 1994; Miyake *et al*, 2008). Therefore, we examined apoptosis in *Rps19*-deficient erythroid cells when treated with the synthetic glucocorticoid Dexamethasone. As expected, we observed that *Rps19*-deficient erythroid cells displayed elevated levels of apoptosis *in vitro*, shown by an increase in the AnnexinV$^+$/7AAD$^-$ fraction. Dexamethasone treatment significantly decreased the apoptotic fraction of *Rps19*-deficient cells (Fig 2A), and it also reduced apoptotic levels in healthy cells. This indicates that glucocorticoids have a pro-survival effect on erythroid cells in general, and on *Rps19*-deficient cells in particular. In addition to being more pro-apoptotic, erythroid progenitors from both DBA patients and an *Rps19*-deficient mouse model failed to expand in liquid culture (Hamaguchi *et al*, 2003; Miyake *et al*, 2005; Jaako *et al*, 2011). To examine the effect of glucocorticoids on the expansion capacity of *Rps19*-deficient cells, erythroid expansion cultures were maintained for 7 days. The expansion of *Rps19*-deficient erythroid progenitors was dramatically decreased compared to controls as early as 48 h after doxycycline administration, and did not

recover during the 7-day culture period (Fig 2B). *Rps19*-deficient cells receiving Dexamethasone significantly increased their expansion and allowed the culture to be maintained for the full 7 days. As seen before, healthy control cells expanded better in the presence of Dexamethasone, indicating that glucocorticoids promote expansion of erythroid cells in general. Together, these results show that glucocorticoid treatment decreases apoptosis and promotes expansion of *Rps19*-deficient erythroid cells and that these effects are more pronounced in *Rps19*-deficient cells than in healthy cells.

Glucocorticoids counteract erythroid differentiation and erythroid progenitor depletion caused by Rps19-deficiency

Glucocorticoids improve the phenotype of *Rps19*-deficiency both *in vivo* and *in vitro* and we therefore wanted to identify changes in erythroid gene expression caused by glucocorticoid treatment. We performed a microarray analysis on KIT-enriched faetal liver cells cultured for 24 h in the following conditions: Healthy cells with and without Dexametha-
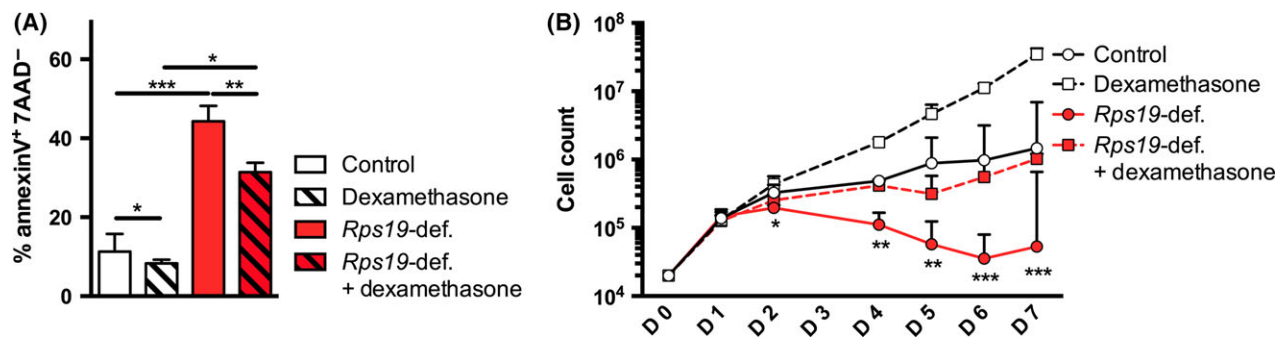

Fig 2. Glucocorticoid treatment improves survival and expansion of *Rps19*-deficient faetal liver cells. (A) *Rps19*-deficient (Rps19-def.) erythroid cells show significant increase in apoptosis after 72 h of culture, and this is alleviated by dexamethasone treatment. The apoptotic fraction (AnnexinV$^+$/7AAD$^-$) is determined by flow cytometry. (B) Expansion of erythroid cells increases following dexamethasone treatment both for healthy and *Rps19*-deficient cells. Cell density never exceeded 1.5×10^6 cells/ml. For clarity, only statistical significance between *Rps19*-deficient and *Rps19*-deficient cells treated with dexamethasone is shown.

sone and *Rps19*-deficient cells with and without Dexamethasone. KIT-enriched faetal liver cell population mostly contains burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) progenitors, as well as some immature erythroblasts not yet expressing TER119. Genes significantly altered by either *Rps19*-deficiency or Dexamethasone treatment were mapped against previously published RNA sequencing data of healthy cells from BFU-E or TER119^{+} cells from murine faetal liver (Flygare *et al*, 2011). In this previously published data set, the BFU-E compartment consisted of faetal liver cells highly purified for the early erythroid progenitor BFU-E by cell sorting. The fraction sorted on positive TER119 expression was composed of more differentiated erythroblasts. In both these fractions, the 330 most highly expressed genes were selected for GSEA (Fig S2A). Genes up regulated by Dexamethasone treatment of healthy cells were much more associated with BFU-Es than with TER119^{+} cells (FDR q = 0·032), indicating that glucocorticoids promote maintenance of erythroid progenitors (Fig 3A). Several genes were up regulated by *Rps19*-deficiency and these were much more associated with TER119^{+} cells (FDR q = 0·011) than with BFU-Es. This was an indication that *Rps19*-deficiency caused increased erythroid differentiation by up regulation of genes associated with the more mature erythroblast TER119^{+} fraction (Fig 3B). Comparison of *Rps19*-deficient cells with or without dexamethasone treatment showed that glucocorticoid treatment caused up regulation of genes highly associated with BFU-Es (FDR q = 0·062) (Fig 3C). Together, this demonstrates that glucocorticoids promote maintenance of erythroid progenitors and delay the increased differentiation and depletion of erythroid progenitors seen in untreated *Rps19*-deficient cells. To further identify dexamethasone-inducible genes that could be responsible for the delayed differentiation and decrease in erythroid progenitor maintenance we compared samples from the microarray analysis cultured under the following three conditions: (i) *Rps19*-deficient cells, (ii) *Rps19*-deficient cells + dexamethasone and (iii) Wild type controls not receiving dexamethasone. Gene clustering was performed on differentially expressed genes in these conditions. We identified two sets of differentially expressed genes that were normalized by dexamethasone (Cluster 2 and 7 in Fig S2B). Among the genes down regulated by *Rps19*-deficiency but reversed by glucocorticoids, we found *Kit* and *Myc*, which both are more highly expressed in erythroid progenitors than mature erythroid cells (Suppl Table I). Another transcription factor important for erythropoiesis induced by glucocorticoids is *Myb*, which is most highly expressed in erythroid progenitors but down regulated with differentiation (Emilia *et al*, 1986). We observed a significant decrease of *Myb* gene expression as well as MYB protein levels in *Rps19*-deficient cells observed previously (Sieff *et al*, 2010). At the gene expression level, glucocorticoid treatment significantly increased *Myb* expression to normal levels (Fig S3). Together, these results indicate that erythroid differentiation is

increased in *Rps19*-deficient cells and that glucocorticoid treatment counteracts this by maintaining the cells in a more immature erythroid progenitor state. These effects were observed as early as after 24 h of glucocorticoid treatment.

Glucocorticoids maintain erythroid progenitors and delay differentiation of Rps19-deficient cells

During the course of *in vitro* erythroid differentiation in liquid culture there is a progressive loss of cells with early erythroid progenitor potential, such as CFU-E cells, and an accumulation of more mature erythroid cells expressing TER119. Our gene expression analysis indicated that glucocorticoid treatment of *Rps19*-deficient cells maintained erythroid progenitors and delayed erythroid differentiation (Fig 3). To validate these findings, we examined CFU-E colony forming potential of KIT-enriched *Rps19*-deficient and healthy faetal liver cells in liquid culture. We hypothesized that CFU-E potential would decrease in *Rps19*-deficient cultures compared to controls over time, and that glucocorticoid treatment could rescue this depletion. As expected, *Rps19*-deficient cells showed decreased CFU-E colony forming potential, starting as early as 24 h after induction with doxycycline (Fig 4A). Dexamethasone treatment significantly retained cells with CFU-E potential in both *Rps19*-deficient and healthy cultures after 72 h. This trend was observed as early as 24 h of culture. CFU-E potential for glucocorticoid treated *Rps19*-deficient cells was equal to that of healthy controls, indicating that glucocorticoids can reverse erythroid progenitor depletion significantly (Fig 4A).

To further examine the maintenance of early erythroid progenitors and identify changes in differentiation between *Rps19*-deficient cells and controls, we employed flow cytometric analysis of the well-established markers of erythroid differentiation CD44 and TER119 (Fig 4B) (Liu *et al*, 2013). *Rps19*-deficient erythroid cells showed increased erythroid differentiation compared to controls, with less cells in the immature CD44^{+}TER119^{−} fraction and more cells in the differentiated CD44^{−}TER119^{+} fraction after 48 h of culture. Dexamethasone treatment significantly reversed this and retained more cells in the immature CD44^{+}TER119^{−} fraction (Fig 4C). These findings were in line with data from the microarray analysis, indicating that increased differentiation of *Rps19*-deficient cells and depletion of erythroid progenitors could be reversed by glucocorticoid treatment.

During differentiation, erythroid cells undergo a set of cell divisions before they become mature red cells and exit the cell cycle. Cell cycle analysis of *Rps19*-deficient cells revealed that these cells to a greater extent reside in the G1 phase and less in S and M phase compared to healthy controls (Fig 4D). This cell cycle difference was not significantly reversed by glucocorticoid treatment. Together, these results demonstrate that *Rps19*-deficiency increases erythroid differentiation and depletes erythroid progenitors. Importantly, glucocorticoid administration significantly reverses both these
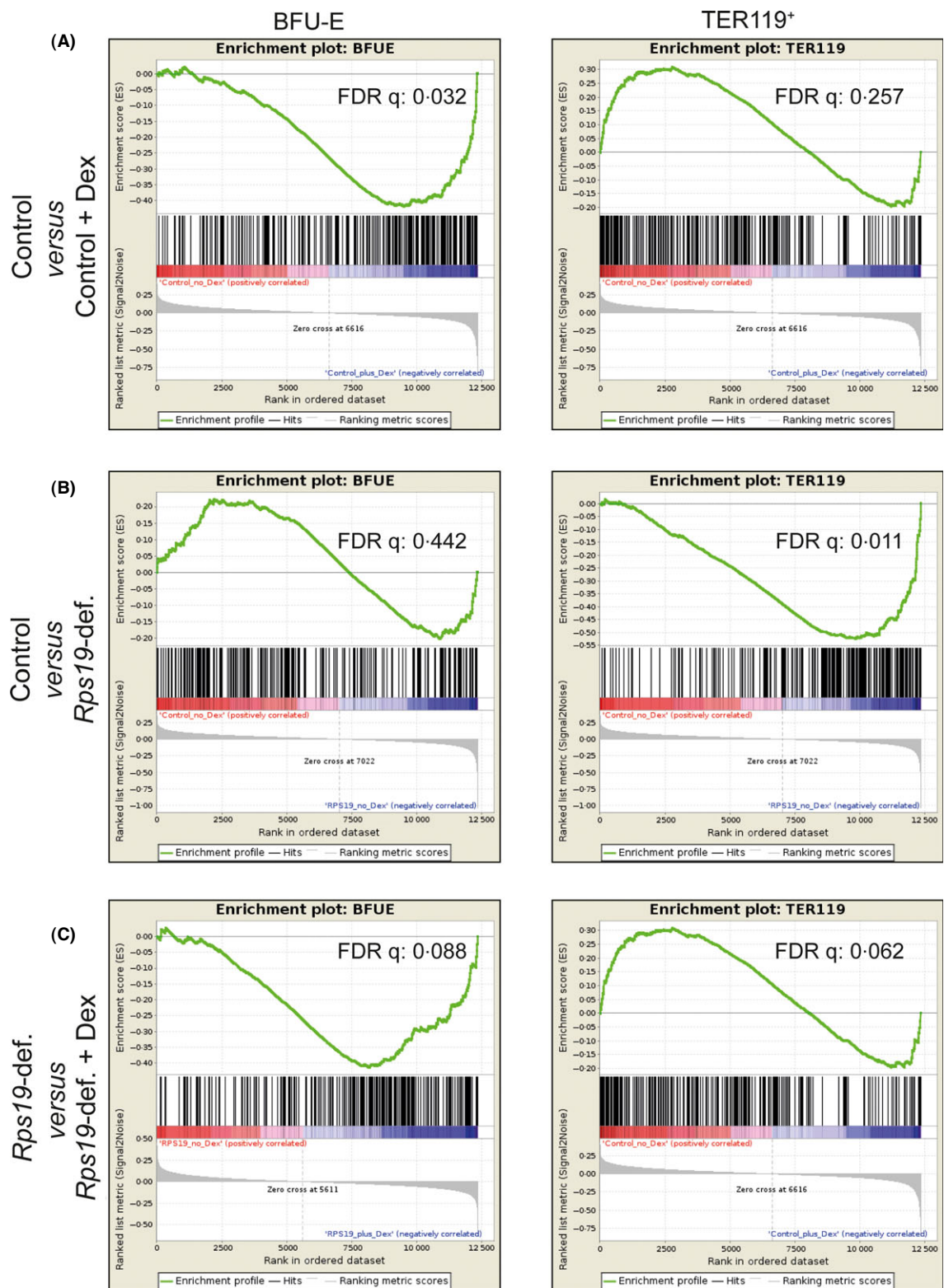
Fig 3. *Rps19*-deficiency increases erythroid differentiation and this is counteracted by dexamethasone (Dex). GSEA was performed on differentially expressed genes from microarray analysis mapped against genes most highly expressed in BFU-Es and TER119^{+} cells from faetal liver (Flygare *et al*, 2011). Enrichment of genes associated with BFU-E or TER119^{+} erythroid fractions between (A) Control and control + dexamethasone samples, (B) Control and *Rps19*-deficient samples, (C) *Rps19*-deficient and *Rps19*-deficient + dexamethasone samples.
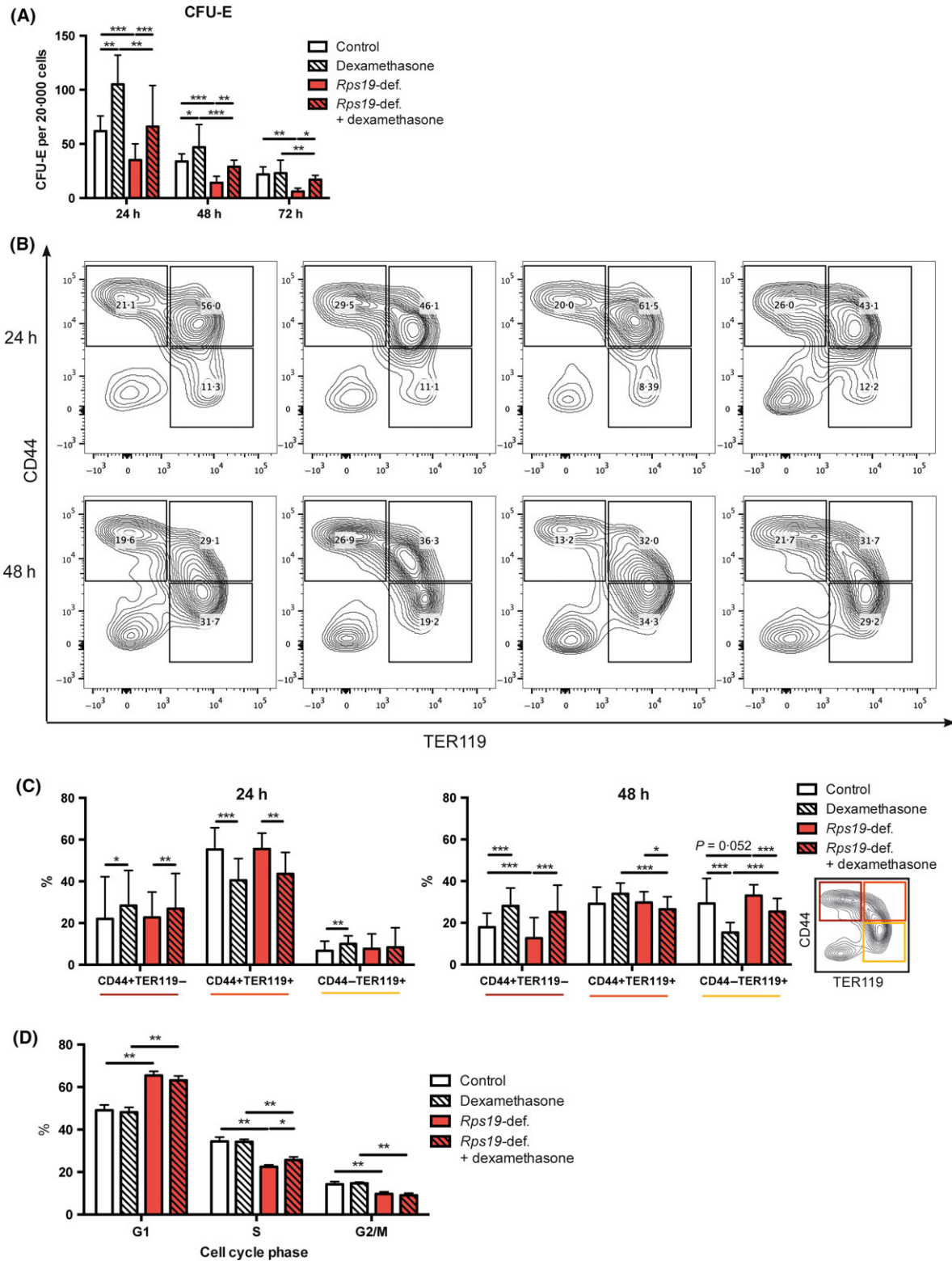
Fig 4. Glucocorticoids prevent erythroid progenitor loss and decrease differentiation in *Rps19*-deficiency. (A) Healthy and *Rps19*-deficient KIT-enriched faetal liver cells cultured with and without dexamethasone for 24, 48 or 72 h and then plated for colony assay, in three separate experiments. (B) Flow cytometric analysis of the erythroid differentiation markers CD44 and TER119 in *Rps19*-deficient and healthy cultures with or without Dexamethasone treatment. Shown here are representative plots for each condition and time point from three separate experiments. (C) Quantification of data from the flow cytometric experiment in (B). (D) Cell cycle distribution analysis of cells cultured for 72 h. Displayed here is mean with standard deviation and paired student's *t*-test was used for statistical analysis.

effects, which allows expansion of erythroid progenitors, despite *Rps19*-deficiency.

Glucocorticoids reverse up regulation of Trp53 targets caused by Rps19-deficiency

The tumour suppressor *Trp53* is known to be highly involved in the DBA phenotype, as shown in animal models of DBA (Danilova *et al*, 2008; Taylor *et al*, 2012). In our mouse model, *Rps19*-deficient erythroid cells demonstrated a dramatic up regulation of *Trp53* downstream target genes, including *Cdkn1a* (*p21*) and *Bax* that regulate cell cycle and apoptosis (Jaako *et al*, 2011). Activation of *Trp53* in the *Rps19*-deficient mice involves 5S ribonucleoprotein particle (5S RNP)-mediated inhibition of MDM2. Deletion of the *Trp53* gene or disruption of the 5S RNP-MDM2 interaction in the *Rps19*-deficient DBA mouse ameliorates the anaemia *in vivo* (Jaako *et al*, 2015). We therefore wanted to examine the effect of glucocorticoid treatment on *Trp53* activity in *Rps19*-deficient erythroid cells. The expression of multiple *Trp53* target genes was significantly elevated in *Rps19*-deficient erythroid cells, shown by GSEA of microarray data mapped against the *Trp53* KEGG pathway (Fig 5A). The same thing was seen for multiple *Trp53* target genes including *Cdkn1a* and *Bax* by RT-PCR (Fig 5B–C). Dexamethasone treatment significantly blocked the up regulation of multiple *Trp53* target genes, demonstrating that glucocorticoids have a potent dampening effect on *Trp53* activity in *Rps19*-deficient cells (Fig 5C). Glucocorticoids clearly displayed this effect after 72 h, but a trend was also seen after 24 h of treatment (compare Fig 5B and 5C). Importantly, glucocorticoids did not down regulate expression of the *Trp53* target genes in healthy cells, indicating that glucocorticoids had this effect specifically in cells with elevated *Trp53* activity. Both *Rps19*-deficient and control cells responded to glucocorticoid stimulation by up regulation of the glucocorticoid receptor target gene *Per1* at both 24 and 72 h (Fig S4A–B).

In order to determine if glucocorticoid receptor activation reduces nuclear TP53 levels we performed a mini screen with selected steroid-associated compounds including 30 glucocorticoid receptor agonists. The readout of the screen was average nuclear TP53 staining intensity in *RPS19*-deficient human A549 (TP53 wild-type) cells (Fig S5). While *RPS19*-deficiency caused a dramatic increase in nuclear TP53 protein levels, this increase was moderately but significantly reduced when cells were treated with the group of glucocorticoid compounds. Neither androgens nor oestrogens had this effect. The results suggest that glucocorticoids are able to reduce nuclear TP53 protein accumulation caused by ribosomal stress.

To validate the clinical relevance of this effect, we examined gene expression of *Cdkn1a* in erythroid cells from one DBA patient carrying an *RPS19* mutation. Indeed, the expression of *CDKN1A* was highly elevated in this patient compared to healthy donors, and *CDKN1A* expression was dampened after only 24 h of glucocorticoid treatment (Fig 5D). The patient cells were responsive to glucocorticoids, shown by up regulation of *ZFP36L2*, a direct target of the glucocorticoid receptor important for erythroid progenitor self-renewal (Fig S4C). Overall, these results demonstrate that glucocorticoids counteract up regulation of multiple *Trp53* target genes in *Rps19*-deficient erythroid cells. This effect is not seen in healthy controls, which indicates that it is specific to DBA pathology.

Discussion

Glucocorticoids are often chosen as first line treatment for DBA, and they have now been used to treat DBA patients for more than half a century (Gasser, 1951). It is well known that glucocorticoids are crucial for stress erythropoiesis and self-renewal of erythroid progenitors *in vitro*, but little is known about their therapeutic mechanisms in DBA treatment. One previous attempt has been made to study glucocorticoid responsiveness in a mouse model of *Rps6*-deficiency, but these mice did not respond to glucocorticoid treatment (Keel *et al*, 2012). Here, for the first time, we show glucocorticoid responsiveness in a mouse model of DBA, demonstrating that it is a relevant and useful model to study therapeutic mechanisms of DBA treatment. We also demonstrate that glucocorticoids prevent the depletion of erythroid progenitors and the increase in erythroid differentiation observed in *Rps19*-deficient cells. Furthermore, we demonstrate that glucocorticoids inhibit up regulation of multiple *Trp53* target genes, an effect not seen in healthy cells and thus specific to the DBA phenotype.

Erythropoiesis requires a tight regulation between self-renewal and differentiation of erythroid progenitors to generate sufficient numbers of mature red blood cells. Steady state erythropoiesis involves a set number of differentiation divisions and is regulated by EPO and SCF (Muta *et al*, 1995). Glucocorticoids are not required for steady state erythropoiesis, but are vital for situations of stress erythropoiesis, such as excessive bleeding or severe anaemia. This is best illustrated by lack of recovery from haemolytic anaemia of mice with disrupted glucocorticoid receptor function (Bauer *et al*, 1999). In steady state erythropoiesis, cultures treated with glucocorticoids give rise to more BFU-E colonies than untreated controls, indicating that they are important for erythroid progenitor self-renewal and maintenance (Flygare *et al*, 2011; Narla *et al*, 2011). We observe that *Rps19*-deficient erythroid cells have decreased capacity of CFU-E formation, which leads to decreased erythroid output and thus a decreased number of cells able to reach erythroid maturation. This is in line with other studies showing decreased CFU-E and BFU-E colony-forming abilities in erythroid cells from DBA patients (Nathan *et al*, 1978; Lipton *et al*, 1986; Giri *et al*, 2000). We show that glucocorticoid-treated *Rps19*-deficient cultures retain more CFU-E colony-forming capacity compared to their untreated counterparts, indicating that
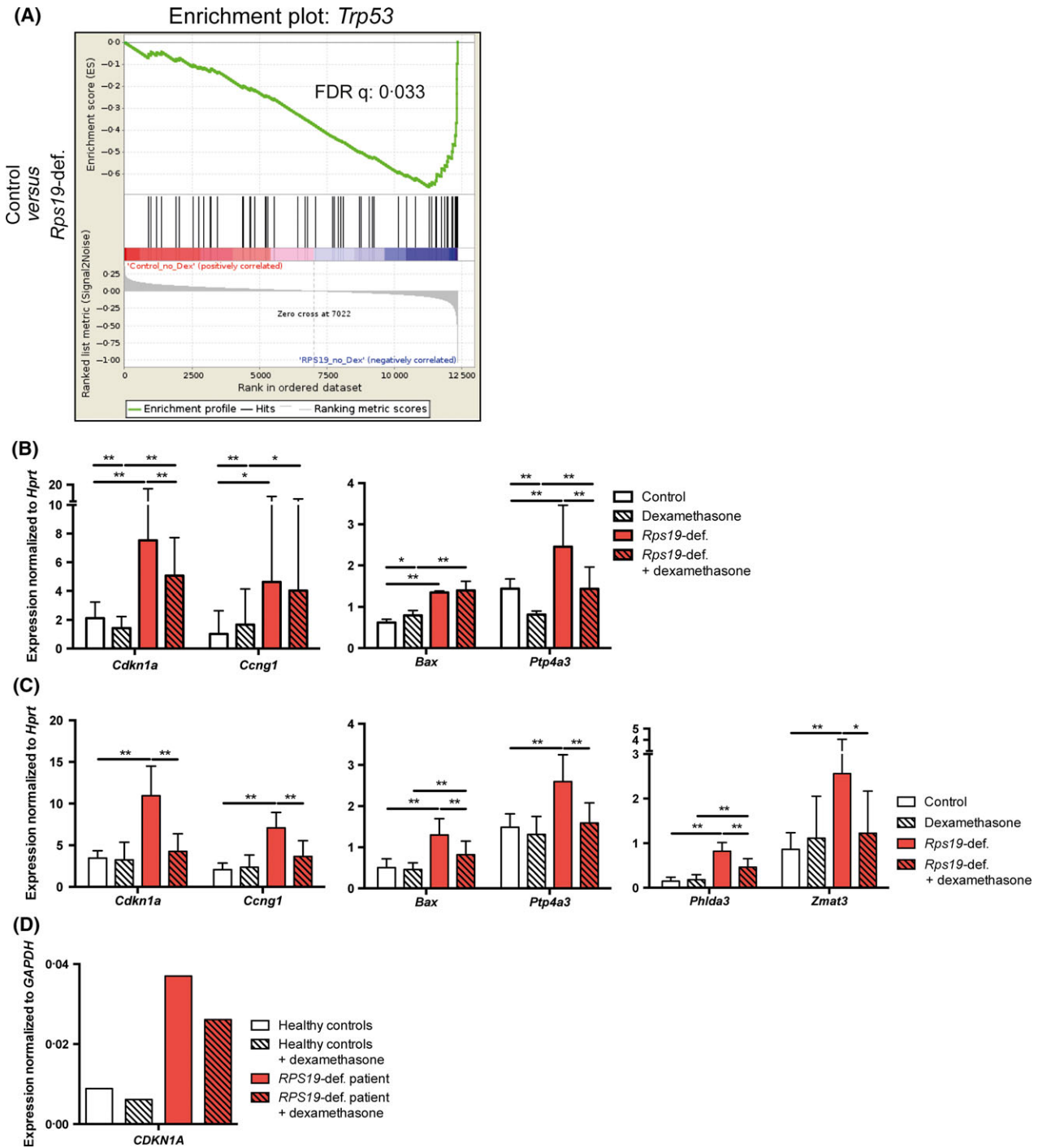
Fig 5. *Trp53* target genes are up regulated in *Rps19*-deficient cells, but not in the presence glucocorticoids. (A) GSEA of genes significantly up regulated by *Rps19*-deficiency mapped to the *Trp53* KEGG pathway. (B) *Trp53* responsive gene expression after dexamethasone and doxycycline treatment for 24 h and (C) 72 h. Results from three separate experiments. (D) Gene expression of *CDKN1A* in blood cells from peripheral blood of one DBA patient carrying an *RPS19* mutation and two healthy control subjects.

glucocorticoid treatment can significantly rescue erythroid progenitor depletion. This is in line with a study observing an increase in the erythroid progenitor population of steroid responsive DBA patients compared to transfusion-dependent patients (Iskander *et al*, 2015). In human bone marrow the

erythroid output was decreased when *RPS19* was knocked down and, similar to our findings, this was rescued by gluco-corticoids (Ebert *et al*, 2005).

In our study, gene expression analysis demonstrated a sig-nature of increased differentiation of *Rps19*-deficient cells.
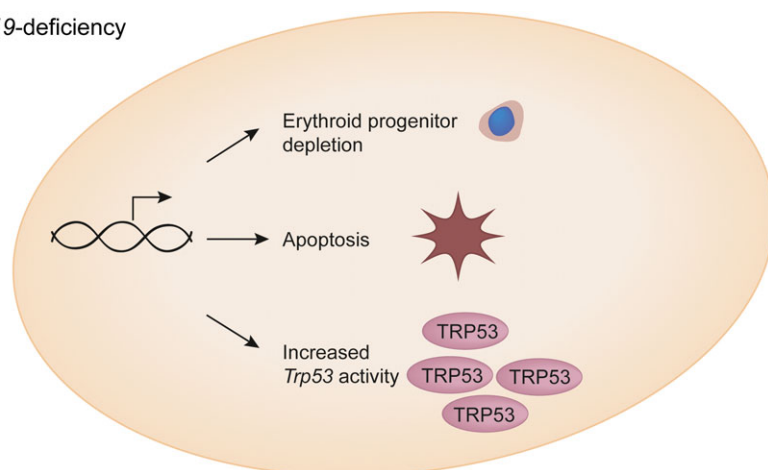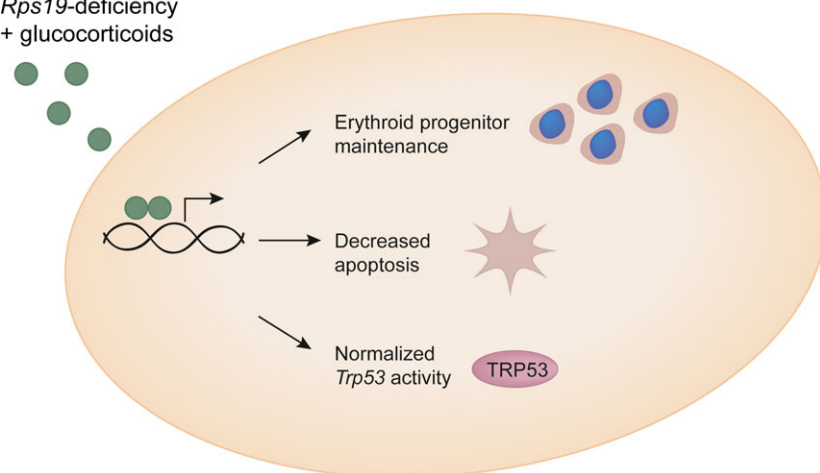
Rps19-deficiency

Rps19-deficiency
+ glucocorticoids

Fig 6. Glucocorticoids act by rescuing depletion of erythroid progenitors and dampen *Trp53* activity. Glucocorticoid treatment thereby allows expansion and replenishment of the *Rps19*-deficient erythroid system.

The gene expression results further suggested that glucocorticoid treatment of *Rps19*-deficient cells decreased differentiation and increased maintenance of erythroid progenitors. This explanation to the therapeutic effect of glucocorticoids was confirmed by colony forming, cell expansion and flow cytometry analyses, demonstrating that glucocorticoid treatment reversed both increase in differentiation and depletion of erythroid progenitors in *Rps19*-deficient cells. It could seem counterproductive for glucocorticoids to delay differentiation in an *Rps19*-deficient erythroid system, but we postulate that this, together with increased maintenance of erythroid progenitors, prevents the system from being prematurely differentiated, which would result in exhaustion of self-renewing cells. Counteracting this exhaustion could allow erythroid expansion to occur despite *Rps19*-deficiency. This would be in line with the significant improvement of anaemia we observed in our mouse model of DBA.

TP53 has been identified a key player in the pathogenesis of DBA in both fish and human model systems (Danilova *et al*, 2008; Dutt *et al*, 2011; Taylor *et al*, 2012). Similarly to others, we have also shown that the anaemia of our *Rps19*-deficient mouse model could be significantly improved in a *Trp53* null background (Jaako *et al*, 2011). Likewise, the phenotype of a mouse model of the ribosomal disease Treacher Collins syndrome is reversed in a *Trp53* null background, demonstrating an important role for *Trp53* in the pathology of ribosomopathies (Jones *et al*, 2008). The mechanism behind *Trp53* activation in DBA is not fully understood, but probably involves activation of ribosomal stress pathways. In situations where one ribosomal component is not available in sufficient amounts, ribosome biogenesis fails and the ribosomal proteins RPL5, RPL11 and 5S rRNA diffuse from the nucleolus and act as sensors of ribosomal stress by forming a complex that inhibits MDM2. As MDM2 is a E3 ubiquitin ligase that promotes degradation of TRP53, formation of this complex leads to TRP53 stabilization (Lohrum *et al*, 2003; Zhang *et al*, 2003; Dai & Lu, 2004; Donati *et al*, 2013). Some studies have reported that erythroid defects in mouse cells and zebrafish models of ribosomal deficiency are not always corrected by *Trp53* depletion (Torihara *et al*, 2011; Jia *et al*, 2013; Singh *et al*, 2014). Although *TP53* seems to be important for the disease, it is plausible that there is more to the pathology of DBA than increased *TP53* activity alone. Interestingly, both RPL5 and RPL11 are found mutated in DBA, but in these cases it is

unclear if *TP53* activation has a role in the pathogenic mechanism (Singh *et al*, 2014) and more studies are needed to elucidate if ribosomal stress and *TP53* activation is a central pathogenic mechanism in DBA. In this study, we found both a significant up regulation of TP53 protein and multiple *TP53* responsive genes in both human and murine *Rps19*-deficient cells. Also, our microarray analysis reveals a strong *Trp53* activation signature in *Rps19*-deficient erythroid cells. Glucocorticoids reverse the increased expression of multiple *Trp53* target genes involved in both cell cycle (*Cdkn1a* and *Ccng1*) and apoptosis (*Bax*). Interestingly, no change in *Trp53* target gene expression was seen in healthy cells regardless of glucocorticoid administration, indicating that this mechanism is specific to *Rps19*-deficient cells and thus disease-specific for DBA. Neither treatment with androgen- nor oestrogen-associated compounds resulted in any decrease of TP53 protein levels, indicating that this effect is specific to glucocorticoids. This effect was seen with treatment of multiple glucocorticoids, leading us to conclude that glucocorticoid receptor activation can have a *TP53* dampening effect. One previous study reported negative cross talk between *TP53* and the glucocorticoid receptor in a neuroblastoma cell line (Sengupta *et al*, 2000), but because glucocorticoids have very cell type-specific effects, it remained highly unclear if this was also true in a DBA setting. Here, we show, for the first time, that glucocorticoids dampen the elevated *Trp53* activity seen in DBA, in a disease-specific manner.

Together, the results from this study demonstrate that glucocorticoid treatment dampens the elevated *Trp53* activity seen in erythroid cells from DBA patients and murine cells, by decreasing the expression of multiple *Trp53* responsive genes, coupled with decreased levels of apoptosis in these cells. To our knowledge, this is the first time it has been shown that glucocorticoid treatment reverses *Trp53* activity and affects erythroid maintenance in the context of DBA. This study also demonstrates that glucocorticoid treatment of *Rps19*-deficient cells reverse the depletion of erythroid progenitors as well as counteracts the increase in erythroid differentiation, leading to increased expansion and CFU-E colony forming capacity (Fig 6).

Glucocorticoid treatment of DBA patients has been frequently used for decades, but comes at the cost of severe side effects. In the effort to develop more disease-specific treatments for DBA it is important to identify the specific mechanisms responsible for increased erythroid output of glucocorticoid treatment. Based on our results, it is likely that other glucocorticoid receptor ligands with dissociated functions have a more potent effect on the mechanisms relevant for DBA therapy and at the same time induce fewer side effects. This has been seen in other diseases of chronic glucocorticoid treatment, such as Duchenne muscular dystrophy (Biggar *et al*, 2006). The present study identifies the desired effects for a better DBA drug and is therefore a step forward toward development of more potent and safer glucocorticoid receptor agonists for DBA therapy.

Author contributions

SES and JF designed the research study, SES, KS and MW performed the research, SES analysed the data and performed statistical analyses, SS performed statistical analysis of the microarray data, MW provided DBA patient samples, AJG and RDH performed research on the human cell line, PJ and SK gave intellectual input to the study and provided the mouse model, and SES and JF wrote the manuscript.

Competing interests

The authors have no competing interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.

Fig S1. Blood parameters of *Rps19*-deficient and healthy mice.

Fig S2. Gene expression analyses of microarray and RNA sequencing data.

Fig S3. Decreased *Myb* expression is normalized by glucocorticoids in *Rps19*-deficient cells.

Fig S4. Murine and patient cells respond to glucocorticoid stimulation.

Fig S5. TP53 protein is less upregulated in *RPS19*-deficient human cells treated with glucocorticoids.

Table SI. List of genes significantly altered by Dexamethasone treatment of *Rps19*-deficient cells. Log$_2$ gene expression values of genes from cluster 2 and 7 from Fig S2.

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