Primary hematopoietic cells from DBA patients with mutations in RPL11 and RPS19 genes exhibit distinct erythroid phenotype in vitro

Diamond-Blackfan anemia (DBA) is caused by aberrant ribosomal biogenesis due to ribosomal protein (RP) gene mutations. To develop mechanistic understanding of DBA pathogenesis, we studied CD34⁺ cells from peripheral blood of DBA patients carrying RPL11 and RPS19 ribosomal gene mutations and determined their ability to undergo erythroid differentiation in vitro. RPS19 mutations induced a decrease in proliferation of progenitor cells, but the terminal erythroid differentiation was normal with little or no apoptosis. This phenotype was related to a G₀/G₁ cell cycle arrest associated with activation of the p53 pathway. In marked contrast, RPL11 mutations led to a dramatic decrease in progenitor cell proliferation and a delayed erythroid differentiation with a marked increase in apoptosis and G₀/G₁ cell cycle arrest with activation of p53. Infection of cord blood CD34⁺ cells with specific short hairpin (sh) RNAs against RPS19 or RPL11 recapitulated the two distinct phenotypes in concordance with findings from primary cells. In both cases, the phenotype has been reverted by shRNA p53 knockdown. These results show that p53 pathway activation has an important role in pathogenesis of DBA and can be independent of the RPL11 pathway. These findings shed new insights into the pathogenesis of DBA.

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Subject Category: Immunity

Diamond-Blackfan anemia (DBA) is a rare congenital disorder characterized by a moderate to severe anemia, in conjunction with erythroblastopenia (absence or <5% of erythroid precursors) in an otherwise normocellular bone marrow. In 40% of DBA cases, various malformations, mostly in the cephalic area, thumbs and upper limbs, are observed. More than 60% of DBA patients respond to steroid treatment. The steroid-resistant DBA patients are treated with regular transfusions in conjunction with iron chelation therapy. The only curative treatment is hematopoietic stem cell transplantation.1–3 Mutations in genes encoding the ribosomal protein S19 gene (RPS19) were the first to be identified in DBA and 25% of DBA patients carry mutations in this gene.4 Recently, mutations in genes encoding other RPs of both the small and the large subunits have been identified in DBA patients: RPS24 (2%),5 RPS17 (<1%),6 RPL5 (7%),7–9 RPL11 (5%),7–9 RPL36 (<1%),8 RPS7 (<1%),8 RPS27a (<1%),6 RPS15 (<1%),8 RPL35a (3%),10 RPS10 (3%)11 and RPS26 (7%).11 More recently, large deletions in RPS19, RPS17, RPS26 and RPS35a genes have been identified in 17% of DBA cases.12 Thus, ≤75% of the DBA patients exhibit a RP gene mutation or deletion. The remaining 25% may carry a mutation in the non-coding region of a RP gene or in other gene(s) yet to be defined. Mutations identified in RPS19, RPS10, RPS24 and RPS26, as well as reduced expression of several RPs by small interfering RNAs, result in defective ribosomal biogenesis at different stages of ribosomal RNA (rRNA) maturation.11,13–16 Defective ribosomal biogenesis appears to be the major cause of DBA.17 Animal models18–23 have begun to provide some mechanistic insights into DBA pathogenesis. Large scale chemical mutagenesis screen for dark skin phenotype identified a mutation in rsps19 or rsp20 genes (Dsk311–12 and Dsk413–15, 16–17.

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Abbreviations: DBA, Diamond-Blackfan anemia; RP, ribosomal protein; rRNA, ribosomal RNA; MDM-2, murine double minute 2, human double minute 2; GFP, green fluorescent protein; MOI, multiplicity of infection; EPO, erythropoietin; FBS, fetal bovine serum; PCR, polymerase chain reaction; ECL, enhanced chemiluminescent; IMDM, Iscove's modified Dulbecco's media; PE, phycoerythrin; APC, allophycocyanin; GPA, glycoporphin A; QRT-PCR, quantitative reverse transcriptase-PCR; HPRT, hypoxanthine guanine phosphoribosyl transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TSST, Tris-buffered saline with Tween 20; PARP, poly (ADP-ribose) polymerase; ECL, enhanced chemiluminescent

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respectively)\(^1\) in association with two clinical features associated with DBA, growth retardation and a modest decrease in red cell count. In this model, p53 expression level was increased and the phenotype was rescued following inhibition of p53. The p53 pathway was also implicated in Zebrafish morpholino models of RPS19 and RPL11, which show a delay in erythroid differentiation.\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\) Furthermore, Fumagalli et al.\(^11\) have shown that depletion of RPS6, RPS23 and RPL7a in hepatocytes leads to the activation of p53 pathway with increased expression of RPL11 suppressing the murine double minute 2 (MDM2) E3 ligase activity against p53.

To further understand the mechanistic basis of DBA, we explored the ability of primary circulating CD34\(^+\) cells of DBA patients with either RPS19 or RPL11 mutations to undergo *in vitro* erythroid differentiation. In parallel, we studied erythroid differentiation of normal human CD34\(^+\) cells infected with specific short hairpin (sh) RNA against RPS19 and RPL11. We show here that while RPS19 depletion decreases progenitor proliferation without affecting terminal erythroid differentiation, RPL11 depletion affects both progenitor cell proliferation and erythroid differentiation with marked apoptosis. Although the p53 pathway is involved in both situations, its role is more limited in RPS19-deficient samples. Thus, we identified two different erythroid differentiation phenotypes due to different ribosomal mutations that could account for erythroblastopenia, the main characteristic of DBA. Strikingly, p53 pathway was activated without increased RPL11 expression level in RPL11-mutated DBA patients or RPL11-depleted cord blood CD34\(^+\) cells implying that RPL11 is not necessary in p53 activation following RP depletion.

**Results**

**Two different erythroid differentiation phenotypes in DBA depending on the specific RP defect.** We compared the ability of CD34\(^+\) cells isolated from peripheral blood from DBA individuals with RPS19 (*n* = 12) or RPL11 (*n* = 3) mutations and CD34\(^+\) cells from healthy controls (*n* = 15) to undergo terminal erythroid differentiation (Table 1). CD34\(^+\) cells were cultured either in two phases (7 day methylcellulose and then liquid culture for additional 5 days) or in a single phase (liquid culture over 12 days). At day 7 of the culture in methylcellulose, immunoblot analyses confirmed the decreased expression of RPS19 or RPL11 in DBA patients compared with healthy controls (Figure 1a), with RPL11 expression consistently decreasing to a greater extent than RPS19 expression. In liquid culture, we observed a decrease in cell amplification for all DBA patients irrespective of the mutational status. As illustrated in Figure 1b, this decrease was less pronounced from D0 to D7 for samples with RPS19 mutations than for samples with mutations in RPL11. This trend of decreased proliferation continued into later stages of culture (D7 to D10). Cytological examination of erythroid cell precursors generated did not show any significant abnormality when RPS19-mutant CD34\(^+\) cells were cultured (*n* = 12) while immature and some apoptotic or necrotic cells were observed when RPL11-mutant CD34\(^+\) cells were cultured (*n* = 3).

| DBA | Sex | Mode of inheritance | Age at diagnosis (month) | Hb at diagnosis (g/dl) | Mode of treatment at the time of the study | Genotype | Absolute reticulocytes (×10\(^6\) cells/l) | Hb at diagnosis (g/dl) | eADA (nmoles/min/mg) | Congenital malformations | Treatment at the time of the study | Genotype |
|-----|-----|---------------------|-------------------------|-----------------------|-------------------------------------|----------|----------------------------------------|-----------------------|------------------------|-------------------------|-------------------------------------|----------|
| 1   | F   | Sporadic            | 3                       | 3.7                   | NA                                  | RPS19    | 4.9                                    | 0.86                  | 1.61                   | 3                        | T                                   | RPS19    |
| 2   | F   | Sporadic            | 4                       | 5.9                   | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 3   | M   | Sporadic            | 2                       | 3.7                   | NA                                  | RPS19    | 0.86                                   | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 4   | F   | Sporadic            | 3.5                     | 5.9                   | NA                                  | RPS19    | 4.9                                    | 0.86                  | 1.61                   | 3                        | T                                   | RPS19    |
| 5   | M   | Sporadic            | 4.2                     | 5.9                   | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 6   | F   | Sporadic            | 1.5                     | 4.2                   | NA                                  | RPS19    | 4.9                                    | 0.86                  | 1.61                   | 3                        | T                                   | RPS19    |
| 7   | M   | Sporadic            | 1                       | 6.4                   | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 8   | M   | Sporadic            | 4.5                     | 5.9                   | NA                                  | RPS19    | 4.9                                    | 0.86                  | 1.61                   | 3                        | T                                   | RPS19    |
| 9   | F   | Sporadic            | 3                       | 5                     | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 10  | M   | Sporadic            | 2                       | 6.4                   | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 11  | F   | Sporadic            | 3                       | 6.4                   | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 12  | M   | Sporadic            | 1                       | 3                     | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 13  | M   | Sporadic            | 1                       | 3.3                   | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 14  | F   | Sporadic            | 5                       | 3.3                   | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |
| 15  | M   | Sporadic            | 1                       | 6.4                   | NA                                  | RPS19    | 4                                     | 3.6                   | 3.6                    | NA                      | T                                   | RPS19    |

Abbreviations: C, corticosteroids; eADA, erythrocyte adenosine deaminase; F, female; IT, treatment independence; M, male; NA, not available; T, transfusions.

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Accordingly, a slight decrease in the percentage of maturing cells (CD36$^+$/GPA$^+$ (glycophorin A)) was observed in cultures of RPL11-mutated cells ($n=3$) compared with RPL11-mutated ($n=2$) and control ($n=8$) cells (Figure 2b), and the percentage of CD34$^+$ erythroid cells was higher in RPL11-mutated cell cultures (Figure 2c). In addition, the percentage of apoptotic cells was much higher in RPL11-mutated cell cultures, as assessed by the quantitation of DAPI$^+$/Annexin-V$^+$ cells (Figure 3a), by the TUNEL assay (data not shown), and by documentation of increased level of cleaved caspase-3 (Figure 3b).

Knockdown of RPS19 and RPL11 reduces the proliferation of erythroid precursors. As we are unable to obtain enough primary CD34$^+$ cells from DBA patients to perform all the necessary studies, we used primary normal human CD34$^+$ cells and shRNA-encoding lentiviruses to specifically inhibit RPL11 and RPS19 mRNA (Supplementary
Depletion of RPS19 or RPL11 induces a decrease in cell growth. (a) Cell growth curves during erythroid differentiation following infection with shRPS19 and shRPL11 compared with the non-infected cells or infected with the shSCR (three independent experiments). (b) Number of cell divisions calculated at several time points according to the formula: number of cell division = log(extent of amplification)/log2. Data from three independent experiments.

Figure 3: Degree of apoptosis in the DBA patients during erythroid differentiation. (a) Percentage of Annexin-V-positive cells during terminal erythroid differentiation in DBA patients, who carried mutation in RPS19 (RPS19<sup>+</sup>/Mut) (n = 2) (dark grey plot), in RPL11 (RPL11<sup>+</sup>/Mut) (n = 3) (light grey plot) compared with the controls (n = 8) (black plot). The differences were statistically significant between controls and RPS11<sup>+</sup>/Mut at days 7, 10 and 12 (P < 0.001). Only two RPS19<sup>+</sup>/Mut samples were studied for Annexin-V and statistical significance was not studied. However, nine other samples were studied by the TUNEL assay. If we pool the results from these two techniques investigating apoptosis, a much less statistical difference has been observed between controls and the RPS19<sup>+</sup>/Mut samples (P < 0.05) than for RPS11<sup>+</sup>/Mut. (b) Representative western blot of cleaved caspase 3, protein involved in apoptotic pathway during terminal erythroid differentiation in one RPS19- and one RPL11-mutated DBA patients compared with their controls.

Depletion of RPL11 specifically delays erythroid differentiation with increased apoptosis. Flow cytometry using the differentiation antigens CD34, CD36, CD71 and GPA showed a delay in erythroid differentiation following RPL11 depletion but not following RPS19 depletion. Indeed, the same percentages of CD36<sup>+</sup>/GPA<sup>+</sup> cells (50 ± 9%) and CD71<sup>+</sup> (79 ± 6%) were present after shRPS19 treatment as in controls (42 ± 9% and 53 ± 13% of CD36<sup>+</sup>/GPA<sup>+</sup>, 78 ± 1% and 85 ± 4% of CD71<sup>+</sup> in non-infected and shSCR-infected cells, respectively; Figures 5a and b). In contrast, less CD36<sup>+</sup>/GPA<sup>+</sup> (20 ± 9%) and CD71<sup>+</sup> (31 ± 15%) cells were found following infection with shRPL11 (Figures 5a and b). This was related to a blockage or delay in erythroid differentiation as more residual CD34<sup>+</sup>-positive cells were detected in RPL11 (24 ± 10%)-depleted cell cultures than in control (8 ± 1% and 4 ± 1.5%) and RPS19 (7 ± 3%)-depleted cell cultures (Figure 5b).

Cytological examination showed that RPL11-depletion induced the appearance of apoptotic cells at day 7, which was not seen in controls and RPS19-depleted cells (data not shown). Flow cytometry analyses confirmed that RPL11 depletion induced a striking increase in the percentage of Annexin-V-positive cells at D7 (Figure 5c), and immunoblot experiments detected cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP) in RPL11-depleted cells.
which was not observed in controls and in RPS19-depleted cells, in which Bcl-Xₙ was increased (Figure 5d). These findings are consistent with the higher degree of apoptosis observed in primary CD34⁺ cells with reduced expression of RPL11 due to gene mutations.

**p53 pathway is activated in primary CD34⁺ cells from DBA patients and following RPS19 or RPL11 depletion in normal CD34⁺ cells.** We analyzed the p53 pathway in erythroid cells from six DBA patients – three with mutant RPS19 (RPS19⁺/mut) and three with mutant RPL11 (RPL11⁺/mut). Increased level of p53 was noted in all DBA patients, suggesting stabilization of the protein (Figures 6a and b). Furthermore, the transcript of p21, a primary target of p53, was markedly increased in erythroid cells with mutant RPS19 (32.3-fold, and 7.4-fold at D7 and D10, respectively) and with mutant RPL11 (17-fold and 49.4-fold at D7 and D10, respectively) confirming activation of p53 (Figure 6a). An increase in p53 and phospho-p53 protein expression levels was also observed in the DBA patients and in RPS19- and RPL11-depleted CD34⁺ cells (Figures 6b and d, Supplementary File 3), with RPL11 depletion increasing the protein levels to a higher extent than RPS19 depletion. To further confirm the activation of p53, we tested the transcript levels of three p53 targets (p21, Bax and Noxa). Following depletion of RPL11, significant increase in transcripts of all three targets was noted with the most notable increase (16.2-fold) seen for p21. The effect of RPS19 depletion was less pronounced with a slight increase in Bax (twofold) and a larger increase in p21 (fivefold), with no increase in Noxa mRNA (Figure 6c). Although an increase in p21 protein was observed with both RPS19 and RPL11 shRNAs, the increase in Bax protein expression level was most pronounced following depletion of RPL11 (Figure 6d). Taken together, these results show that depletion of RPS19 and that of RPL11 lead to differential activation of the p53 pathway in erythroid cells.

As the documented decrease in cell proliferation cannot be totally explained by apoptosis, especially after RPS19 depletion, we studied cell cycle progression of erythroid cells derived from primary CD34⁺ cells of two RPL11⁺/mut DBA patients and following RPS19 and RPL11 depletion in normal CD34⁺ cells (Figure 7). An increased number of cells in G₀/G₁ phase was observed in both patients carrying RPL11 mutation, which was more significant at D13 than at D10 (Figure 7a). A small increase in cells in G₀/G₁ phase was also noted following infection with the shRPS19 (49 ± 0.6%) compared with either non-infected cells (43 ± 0.6%) or cells infected with shSCR (42 ± 0.4%) (Figure 7b top and bottom panels). Depletion of RPL11 induced a slightly larger increase in the number of cells in G₀/G₁ phase (59 ± 0.5%). In parallel, a decrease in percentage of cells in S and in G₂/M phases was noted (Figure 7b). Moreover, cells in subG₁, which represent apoptotic cells, were present following infection with the shRPL11 (8 ± 0.8%), confirming that increased apoptosis is a feature of RPL11 depletion (Figure 7b).

**p53 activation is responsible for apoptosis and the G₀/G₁ cell cycle arrest.** To further define the role of p53 activation, we performed a dual shRNA infection, first with either green fluorescent protein (GFP)-shRPS19 or GFP-shRPL11 on day 1, then by a Cherry-shp53 infection on day 2. The efficiency of the shp53 was confirmed in UT7 and CD34⁺ cells by documenting decreases in p53 mRNA and in protein expression levels (Figure 8a). Following decreased expression of p53, proliferation of both
**RPS19**- and **RPL11**-depleted erythroid precursors increased after day 9 (Figure 8b). Strikingly, we were also able to inhibit the activation of apoptosis induced by **RPL11** depletion (Figure 8c). p53 mRNA level returned to normal after p53 depletion in **RPS19**- and **RPL11**-depleted cells (Figure 8d). Increased expression levels of p21, Bax and Noxa mRNA noted following infection of CD34⁺ cells with either shRPL11 or shRPS19 was significantly reduced following p53 knockdown (Figure 8d). These results confirm that the erythroid phenotypes due to **RPS19** and **RPL11** depletion depend on p53 activation. Of particular interest, the finding that there is no increase in **RPL11** expression levels following depletion of either **RPS19** or **RPL11**, implies that increased levels of **RPL11** is not necessary for p53 activation pathway (Figure 8e).

**Discussion**

In spite of the significant progress made in identifying mutations in ribosomal genes **RPS19** and **RPL11** in approximately 30% of DBA patients, the mechanistic understanding of how these mutations account for DBA pathogenesis including erythroblastopenia remains to be fully defined. Erythroblastopenia in DBA is the result of an intrinsic defect with blockade in erythroid differentiation that occurs just following the BFU-e/CFU-e (burst-forming unit erythroid/colony-forming unit erythroid) transition or between erythropoietin (EPO)-independent and EPO-dependent stages. To identify the link between depletion of a RP and the erythroblastopenia in DBA-affected individuals, we investigated proliferation and differentiation of primary hematopoietic cells obtained from DBA patients in the French DBA registry. We obtained two distinct phenotypes, decreased proliferation with either no apoptosis or increased apoptosis, in vitro depending on the RP mutated. Primary cells from patients harboring mutations in **RPS19** gene or normal cells following depletion of **RPS19** by specific shRNA exhibited decreased proliferation of erythroid cells. However, there was neither alteration of the terminal erythroid differentiation as already reported in **RPS19**-deficient TF1 cell lines nor significantly increased apoptosis. In marked contrast, in patients with **RPL11** mutation or following **RPL11** depletion by specific shRNA, we identified a more pronounced phenotype: a greater extent of decrease in cell proliferation, delayed erythroid differentiation and marked increase in apoptosis. The fact that we recapitulated exactly the same phenotypes in primary cells from patients harboring the RP mutations and in normal cells following RP depletion reflects true differential effects of different RPs on erythroid differentiation.

**Figure 6**

p53 activation in DBA patients carrying mutations in **RPS19** or in **RPL11** gene or after CD34⁺ cell infection with shRPS19 or shRPL11. (a) p53 mRNA and p21 mRNA expression level by quantitative RT-PCR (ratio to GAPDH mRNA level) in DBA patients carrying mutation in **RPS19** (dark gray plot) or **RPL11** (light gray plot) genes compared with the controls (black plot) at day 7 and day 10 during erythroid differentiation. (b) Western blot analyses of p53 in two patients who carried, respectively, a mutation in **RPS19** and in **RPL11** gene at D7 and D10 compared with controls. (c) mRNA expression level of p53, p21, Bax and Noxa compared with GAPDH mRNA by quantitative RT-PCR at day 7 of the erythroid differentiation after cord blood CD34⁺ infection with the shRPS19 or shRPL11 compared with shScramble infection (three independent experiments). (d) Protein expression levels of p53, phosphorylated p53, and p21 and Bax determined by western blot analysis following infection with shRSP19 or shRPL11 compared with the Scramble at day 7 of the erythroid differentiation (representative of three independent experiments).
Earlier studies using model organisms have implicated a role for p53 pathway in DBA pathogenesis. In a Dark skin (Dsk3+/−/C0) mouse model with a RPS19 missense mutation and in a morpholino-induced RPS19-deficiency in zebrafish, elevated expression levels of p53 were found to account for the observed phenotype, which included growth retardation, a delay in erythroid differentiation and congenital abnormalities that mimic some DBA phenotypes in humans. The zebrafish morpholino RPL11 also induced p53 activation and developmental abnormalities. The present study was performed in primary cells from a large cohort of 15 DBA patients in which we document p53 activation and cell accumulation in G0/G1 in all the DBA patients analyzed and in normal cells depleted from RPS19 or RPL11. The extent of p53 activation was consistently higher in RPL11-depleted cells compared with RPS19-depleted cells. We validated that the observed decrease in cell proliferation and increase in apoptosis are directly related to p53 activation by showing that both these defects could be reversed by RNA interference-mediated p53 knockdown.

Figure 7 Depletion of RPS19 and RPL11 induces cell cycle arrest in G0/G1 phase. (a) Cell cycle analysis of erythroid cells from a patient carrying mutation in RPL11 during erythroid differentiation. Cell cycle arrest in G0/G1 phase with an increased percentage of cells in subG1 phase, corresponding to apoptotic cells can be seen. (b) Representative histograms from cell cycle analysis following depletion of RPS19 and RPL11 by specific shRNA. Percentages of cells in various phases of cell cycle: G0/G1, S, G2M. Following infection with the shRPS19, there is an increased number of cells blocked in G0/G1 phase compared with the controls. Depletion in RPL11 induced an even larger increase in cells blocked in G0/G1 phase with an accumulation of cells in subG1 phase, which reflects increased numbers of apoptotic cells (three independent experiments).
Our findings with primary human DBA cells validate the findings from murine and zebrafish models that p53 has a key role in the decreased erythroid proliferation in DBA pathophysiology. Kuramitsu et al.28 have previously shown that decreased expression of RPS19 in CD34+ cells induces cell cycle arrest with an accumulation of cell in G0 and no apoptosis, findings consistent with data from the present study. More recently, Badhai et al.29 showed that RPS19 insufficiency in primary fibroblasts of DBA patients causes cell cycle arrest in G1 phase, which correlated with marked decreases in CDK2, cyclin D and phosphorylated pRB, indicating an impaired progression into S phase. Interestingly, this phenotype was associated with a normal level of p53 in primary fibroblasts of DBA patients, probably because fibroblasts are less sensitive to stress induced by mutations in R P genes. Our results are also in agreement with those of Dutt et al.,30 who observed cell cycle arrest and an activation of p53 pathway following depletion of RPS19. The activation...
of p53 pathway due to mutations in RPS19 may be mediated by nucleolar stress due to a defect in rRNA maturation such as that identified in the 3′UTR of the ITS1 at the A2 cleavage site of the 18S rRNA.\textsuperscript{13,15,16} It is well established that a rRNA maturation defect generates nucleolar stress or ribosomal stress. After ribosomal stress, levels of RPL5, RPL11,\textsuperscript{31–33} RPL23,\textsuperscript{34} RPS3\textsuperscript{35} and RPS7\textsuperscript{36} are increased in the nucleolus, which in turn sequester MDM-2 (or human double minute 2 (HDM-2) in human) and prevent it from binding to p53, its natural inhibitor. MDM-2 (or HDM-2), an E3 ubiquitin ligase, has a role in ubiquitinating p53 and its resultant degradation by the proteasome. In the event of a ribosomal stress, MDM-2 (or HDM-2) fails to bind p53, and as a result, p53 is not ubiquitinated and thus not degraded by the proteasome.\textsuperscript{31–36} In addition, Fumagalli et al.\textsuperscript{34} have shown that, without nucleolar stress in hepatocytes, haploinsufficiency in RPS6, RPS23 and RPL7a leads to an upregulation of RPL11. Recently, Dutt et al.\textsuperscript{30} has shown that RPS14 or RPS19 depletion after CD34\textsuperscript{+} infection with specific shRNAs leads to p53 activation without nucleolar disruption but through HDM-2 binding to RPL11. In DBA patient CD34\textsuperscript{+} cells, it is too speculative at this time to define how a mutation in a RPN gene leads to an activation of p53 and if HDM-2 is involved but our future studies will focus on deciphering these mechanisms. However, we were able to show that p53 is activated in RPL11-mutated DBA patients and in native CD34\textsuperscript{+} cells infected by shRPL11, which in both cases led to a large decrease in RPL11 protein expression level. As such, it appears that p53 can be activated without increased RPL11 expression level. There is preliminary evidence that p53 may be activated by different mechanisms during a ribosomal stress, one through the RPL11-HDM2-p53 pathway and the other through p38 activation. It is thus possible that p53 activation mechanism(s) following RPS19 or RPL11 depletion may not be the same, which may account for the observed differences in the extents of p53 activation.

In summary, our findings imply that deficiencies in RPS19 and RPL11 activate p53 to different extents, leading to differential effects on erythropoiesis. Furthermore, our findings have enabled us to understand that increased level of RPL11 expression is not necessary to activate p53 in erythroid cells. An unanswered question is whether the entire erythroid phenotype of DBA is related to increased expression levels of p53 or if additional p53-independent mechanisms may also have a role in the pathogenesis of the disease.

Materials and Methods

Population. A total of 15 unrelated patients affected with DBA and 15 hematologically normal individuals were studied. The DBA patients are registered in the French DBA registry. DBA was diagnosed according to the established criteria.\textsuperscript{1} All cases were reviewed in detail at the time of analysis, and Table 1 shows the biological and clinical data of the 15 DBA patients, which have been obtained from the institutions and physicians responsible for the patient’s management. Informed consent was obtained in accordance with the Declaration of Helsinki. Human umbilical cord blood was collected from normal full-term deliveries after maternal informed consent was approved according to institutional guidelines (AP-HP, Paris, France).

Lentiviral vector construction, production and cell infection.

shRNA cloning. The RPS19 shRNA used in the present study was previously described by Flygare.\textsuperscript{25} Three different shRNA sequences against RPL11 were synthesized (Eurogentec, Angers, France) and cloned into a pBlue Script containing the human H1 promoter (Généthon, Evry, France). The H1-shRPS19C, H1-shRPL11A, -L11B, -L11C and H1-SCR (scramble control sequence) cassettes were cloned into a lentivector, pRSLin-PGK-eGFP-WPRE (Généthon; Supplementary File 1A). The sequence of the various cloned fragments was confirmed by cDNA sequencing. Oligonucleo-hairpins sequences of RPS19 and RPL11 short hairpins (shRNAs) used in the present study are available upon request. The R3 shRNA sequence was described Brummekamp et al.\textsuperscript{13}\textsuperscript{13} We modified the pRSLin-PGK-eGFP-WPRE including a Cherry sequence instead of eGFP to collect by cell sorting the positive cells for both GFP (shRPS19- and shRPL11-infected cells) and cherry (shp33-infected cells), pm-Cherry C1 plasmid (Conetech, Saint-Germain-en-Laye, France) was digested by SgrAl/Xmal then religated. The modified Cherry cassette was amplified by PCR introducing Sall/BamHI restriction sites and used as replacement of the Sall/BamHI GFP cassette of pRRL-PGK GFP. Blunt vector from a XhoI digest of pRRL-PGK-Cherry and blunt inserts of pH1-shRPS15 from a XclII/EcoRI digest of SuperRepto-ph1-shp33-PGK-Puro were ligated using standard ligaction protocol.

Lentiviral production. Lentiviral stocks were prepared as previously described\textsuperscript{25} and stored at ~ 80 °C. Titers of viral particles were determined by quantifying the number of GFP or mCherry-positive cells following infection of HEK 293 T cell lines.

Cell infection and culturing of UT-7 cells. UT-7 cells were infected at day 1 at multiplicity of infection (MOI) of 10 in Iscove’s 4 modified Dulbecco’s media (IMDM; Invitrogen; Invitrogen, Cergy-Pontoise, France), 10% of fetal bovine serum (FBS) and 5 ng/ml granulocyte macrophage colony-stimulating factor. GFP- or mCherry-positive cells were sorted 2 days later using a FACS/Vantage Cell Sorter (Becton Dickinson Biosciences, Le Pont de Claix, France). The effectiveness of shRNA in downregulating specific mRNA and the protein was assessed 2 days following isolation of sorted cells (Supplementary File 1B and C).

Erythroid cell proliferation and differentiation of CD34\textsuperscript{+} cells, apoptosis and cell cycle analysis. CD34\textsuperscript{+} cells from peripheral blood of DBA patients and control subjects or cord blood were isolated by the immunomagnetic technique (Milenyi Biotec, Paris, France). Purified CD34\textsuperscript{+} cells from DBA patients and controls were cultured in IMDM medium (Invitrogen) containing 1% L-glutamine (Invitrogen), 1% penicillin–streptomycin (Invitrogen), 5 × 10\textsuperscript{−}6 M xanthohighetrol (Sigma-Aldrich, Lyon, France), 1.5% bovine serum albumin (Stemcell Technologies, Sheffield, UK), iron-saturated transferrin (Sigma-Aldrich) and 2% liposomes, 50 ng/ml stem cell factor (SCF), 1 U/ml interleukin-3 (IL3) (I-3), 1 U/ml EPO and 10% FBS. At day 7, cultures were switched to the same medium but with 30% FBS (from D7 to D12). For some DBA patients, we performed in vitro erythroid cultures in two phases: a first phase of 7 days in methylcellulose with SCF (50 ng/ml) and IL3 (50 U/ml) and a second phase in liquid culture.

For lentiviral infection, CD34\textsuperscript{+} cells were cultured for 2 days in the presence of 10% FBS, 100 U/ml IL3-13, 10 ng/ml IL6, 25 mg/ml SCF, 10 ng/ml thrombopoetin and 10 ng/ml Flt3-L (Fms-related tyrosine kinase 3 ligand). A first infection was then performed at a MOI of 50. In the case of shRNA-RPS19, a second infection was performed 6 h after the first infection (Supplementary File 2). Cells were cultured for 2 more days and GFP-positive cells were sorted using the FACS DIVA Cell Sorter. Sorted cells were cultured to the same IMDM medium with SCF (50 ng/ml), IL3 (1 U/ml) and EPO at 1 U/ml till D7 when the FBS concentration was increased to 30% (from D7 to D14). Viable cells were counted using the trypan blue dye exclusion test as a function of time in culture. Following May–Grünewald–Giemsa staining, extent of terminal erythroid differentiation was evaluated by morphological assessment.

Erythroid differentiation was also evaluated by flow cytometry (FACS Canto, Becton Dickinson Biosciences). Cells were immunophenotyped from D7 to D14 using several antibodies: phycoerythrin (PE)-coupled anti-GPA (Caltag, Burlingame, CA, USA), allophycocyanin (APC)-conjugated anti-CD36, PerCP-cy5.5-conjugated anti-CD34, APC-anti-CD71 (Becton Dickinson Biosciences) and PC7-conjugated anti-CD34 (Beckman Coulter, Villepinte, France). Isotype controls were obtained from Becton Dickinson Biosciences.

For determining apoptosis, cells were stained with PE-conjugated Annexin-V (Becton Dickinson Biosciences) and with DAPI (Sigma-Aldrich, Saint-Quentin-Fallavier, France) according to the manufacturer’s protocol. In addition, in DBA patients, the TUNEL method was performed according to the manufacturer’s
protocol (kit ApopDETEK, DAKOCytomation, Enzo Diagnostics Inc., Farmingdale, NY, USA) on the cells isolated from erythroid colonies and the percentage of apoptotic cells was determined based on microscopic counting of 200 cells. Cell cycle was studied at day 7, 10 and D12 following propidium iodide staining (Sigma-Aldrich) by flow cytometry.

**Real-time quantitative RT-PCR.**

RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with RNase-free DNase (Qiagen, Courtaboeuf, France). The quality of the RNA was verified on an agarose gel and quantified with a Nanodrop spectrophotometer (ND-1000 UV-Vis; Thermo Scientific, Wilmington, DE, USA).

Reverse transcription-PCR. cDNA was synthesized from extracted RNA with SuperScript II RNase H-Reverese transcriptase kit (Invitrogen).

**Real-time quantitative RT-PCR.** Primers and internal probes for quantitative reverse-transcription (QRT)-PCR were designed using Primer Express Software (Perkin-Elmer Applied Biosystems, Foster city, CA, USA) and are available upon request. PCR was carried out in the ABI Prism GeneAmp 5700 Sequence Detection System (Perkin-Elmer) using the TaqMan Universal PCR Master Mix (ABI) containing the specific primers (1.5 μM) and specific probe (0.1 μM). The expression level of each gene was normalized using three housekeeping genes—hypoxiane-guanine-phosphoribosyl-transferase (HPRT) gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and 1/2 microglobulin (1/2 microglobulin) gene.

**Western blot analysis.** Each pellet of 50,000 cells from the DBA patients and 125 000 cells from shRNA-infected CD34+ cells was lysed in Laemmli buffer (100 mM Tris pH 6.8, 10% glycerol, 4% SDS, 0.2% blue Bromophenol, 20 mM dithiothreitol), sonicated (five times, 15 s) in ice water and boiled for 5 min. Samples were run on a Tris-glycine 10% SDS-PAGE resolving gel. Following electrophoresis, proteins were transferred onto a PVDF Hybond-P membrane (GE Healthcare, EC Europe, Orsay, France) and stained with Porcine S solution (Sigma-Aldrich, Lyon, France) to assess quantity of transferred proteins. Following washing of the membranes with Tris-buffered saline with 1% Tween-20 (TBST 1 ×), membranes were soaked in blocking buffer (4% milk, 1% bovine serum albumin, 0.2% sodium azide in TBST 1 ×) for 1 h and then immunolabeled overnight at 4 °C with the different antibodies. Antibodies used for immunoblotting were as follows: p53, phospho p53 (ser15), caspase 3, cleaved caspase 3, Bcl-X, PARP antibodies (all rabbit polyclonal antibodies from Cell Signaling, Ozyme, Saint Quentin Yvelines, France); p21 (C2) (rabbit monoclonal, Cell Signaling, C with the different antibodies. Antibodies used for immunoblots (GE Healthcare). Following several washes with TBST 1 ×, membranes were incubated with appropriate secondary antibodies for 1 h. After several washes with TBST 1 ×, the membranes were soaked in ECL solution (GE Healthcare) and the expression level of various proteins determined on the Amersham hyperfilm ECL (Amersham).

**Statistical analysis.** Data are presented as the mean ± standard error of the mean (± S.E.M.) or standard deviation (S.D.). Student’s t-test was used to compare the data from different populations.

**Conflict of Interest**

The authors declare no conflict of interest.

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**Author Contributions**

Hélène Moniz performed the vast majority of experiments and analyzed the data. Marc Gastou performed most of the experiments with the shp53. Thierry Leblanc provided us the DBA patient data and samples. Corinne Hurtaud helped in the purification of CD34+ cells and performing erythroid cell cultures. Aurélie Créteil performed methyleneblue cultures of CD34+ cells and analyzed erythroid differentiation from the DBA patients. Marlene Flaubladier produced and provided us the antibody against RPL11. Laure Croisille performed the clonogenicity assays. Yann Lécluse performed the sorting of the GFP-positive cells by flow cytometry. Jérome Larghero provided cord blood samples. Hansen Raslova helped us with the design of the lentiviruses. Olivier Bluteau and Larissa Lorrider generated the mCherry-shp53. Gil Tchernia and William Vanichenker provided clinical and scientific insights. Narla Mohandas participated in study design and wrote the article with Lydie Da Costa and Hélène Moniz. Lydie Da Costa designed the studies, analyzed the data and directed the research project.

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