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14. ABSTRACT
Diamond Blackfan Anemia (DBA) is a disorder that results in pure red cell aplasia, congenital abnormalities, and predisposition to cancer. The current treatment of steroids and chronic transfusions leads to significant morbidity. To understand the mechanism by which RPS19 insufficiency leads to defects in erythropoiesis, we identified a p53 target, microRNA34a (miR34a), as being upregulated in human CD34+ fetal liver cells transduced with RPS19shRNA lentivirus. We hypothesize that RPS19 insufficiency mediates defects in erythropoiesis through upregulation of p53 and miR34a. We have further characterized the role of miR-34a in RPS-19 deficient CD34+ human fetal liver cells. We have identified additional microRNAs that are deregulated in RPS19-deficient CD34+ human fetal liver cells. We have also characterized downstream signaling pathways that regulate erythropoiesis in RPS19-deficient hematopoietic progenitor cells, in particular those that involve inflammation and DNA damage. These studies will provide new insights into the molecular pathways downstream of ribosomal protein insufficiency in hematopoietic stem cells and potentially novel targets for therapy.

15. SUBJECT TERMS
RPS19, DBA, signaling, pathways, RNA-seq, microRNAs, CD34+ cells

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Abstract

Diamond Blackfan Anemia (DBA) is a disorder that results in pure red cell aplasia, congenital abnormalities, and predisposition to cancer. The current treatment of steroids and chronic transfusions leads to significant morbidity. Approximately 25% of patients with DBA have mutations in RPS19. We previously generated a zebrafish model with RPS19 insufficiency that the phenotype is similar to that observed in patients with DBA. We also first described that p53 is upregulated in these fish injected with RPS19 morpholinos. To understand the mechanism by which RPS19 insufficiency leads to defects in erythropoiesis, we identified a p53 target, microRNA34a (miR34a), as being upregulated in human CD34+ fetal liver cells transduced with RPS19shRNA lentivirus. This not only led to decreased erythroid colony formation, but also aberrant erythroid differentiation. We hypothesize that RPS19 insufficiency mediates defects in erythropoiesis through upregulation of p53 and miR34a. To more rigorously test this hypothesis and identify new downstream targets and microRNAs, we propose three specific aims. In Aim 1, we will characterize the role of miR34a in RPS19 insufficient primary human hematopoietic stem cells in vitro. In Aim 2, we will study the role of miR34a in RPS19 insufficient primary human hematopoietic stem cells in vivo. In Aim 3, RNA-seq will be performed to identify novel transcripts and microRNAs that are aberrantly regulated downstream of RPS19 insufficiency in primary human hematopoietic stem cells. We have further characterized the role of miR-34a in RPS-19 deficient CD34+ human fetal liver cells. We have identified additional microRNAs that are deregulated in RPS19-deficient CD34+ human fetal liver cells. We have also characterized downstream signaling pathways that regulate erythropoiesis in RPS19-deficient hematopoietic progenitor cells, in particular those that involve inflammation and DNA damage. These studies will provide new insights into the molecular pathways downstream of ribosomal protein insufficiency in hematopoietic stem cells and potentially novel targets for therapy.

Table of Contents

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The goal of this proposal is to understand the signaling pathways that lead to the pathogenesis of DBA. In the first aim, the role of miR34a and other relevant microRNAs will be investigated. In Aim 2, we proposed to perform RNA-seq and microRNA-seq to identify novel pathways. We will knock down RPS19 in human CD34+ fetal liver and cord blood cells and study genes identified by RNA-seq that are up- or down-regulated. In this manner, we hope to identify novel pathways and approaches to treat DBA.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

RPS19, DBA, signaling, pathways, RNA-seq, microRNAs, CD34+ cells

3. **ACCOMPLISHMENTS:**

Major goals of the project:

a. We will transduce human CD34+ fetal liver hematopoietic stem cells with RPS19 shRNA lentiviral constructs and examine levels of miR34a and target genes c-Myb, c-Myc, Sirt1, and Notch1 at different stages of erythroid development (Months 1-3).

We have transduced human CD34+ fetal liver HSCs with RPS19 shRNA and showed that RPS19 deficiency leads to decreased expression of the miR34a targets c-myb and c-myc through a p53-dependent pathway. We are continuing to analyze the expression and role of Sirt1 and Notch1. This milestone is 50% completed.

b. Study miR34a target gene expression (c-Myb, c-Myc, Sirt1, and Notch1) in lymphoblastoid cell lines (LCL) and CD34+ bone marrow progenitor cells from DBA patients with RPS19 mutations (Months 1-3).

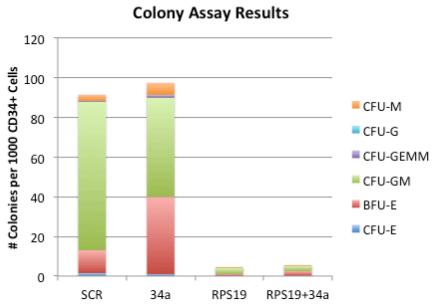
These experiments have been performed and show there is significant variability in expression of c-Myb, c-Myc, Sirt1, and Notch1. The reason for this is most likely because of the fact that the LCL cells are immortalized by EBV and have different characteristics than primary normal HSCs. We are in the process of obtaining and analyzing bone marrow samples from patients with DBA. This milestone is 50% completed.

c. We will infect CD34+ fetal liver cells with both RPS19 and miR34a shRNA lentivirus and examine effects on erythroid differentiation and proliferation by methylcellulose colony assays and FACS analysis (Months 3-6).

We demonstrated that downregulation of miR34a was not sufficient to rescue the defects observed in RPS19-deficient CD34+ cells. We showed this in methylcellulose colony assays but are currently performing experiments in a liquid

culture system, which enables us to characterize specific stages of erythroid differentiation. This milestone is 50% completed.

Downregulation of miR34a is not Sufficient to Rescue the Defects Seen in RPS19 Deficient Cells



d. We will infect CD34+ fetal liver cells with RPS19 shRNA and miR34a lentivirus and examine effects on erythroid differentiation and proliferation by methylcellulose colony assays and FACs analysis (Months 3-6).

See above.

e. Examine miR34a target gene expression in cells infected with RPS19 and miR34a shRNA or RPS19 and miR34a lentivirus during erythroid differentiation (Months 6-9).

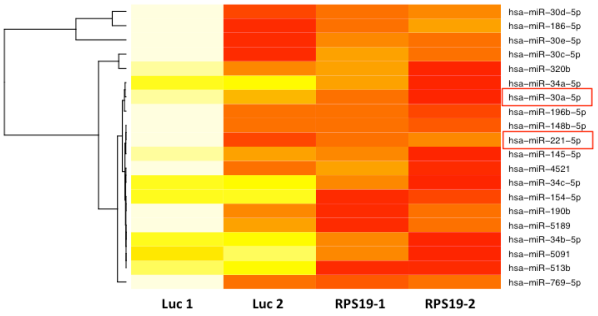
We will examine miR34a target gene expression once we have optimized liquid culture system of erythroid differentiation. These experiments are ongoing. This milestone is 50% completed.

f. Study molecules involved in apoptotic pathways (Caspases-3, -7, and -9, PARP cleavage) in RPS19 + miR34a shRNA or RPS19 + miR34a lentivirus transduced CD34+ fetal liver cells during erythropoiesis (Months 9-12).

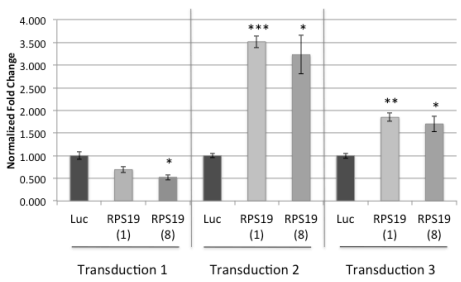
We will perform these experiments once we have optimized the liquid culture system of erythroid differentiation. This milestone is 50% completed.

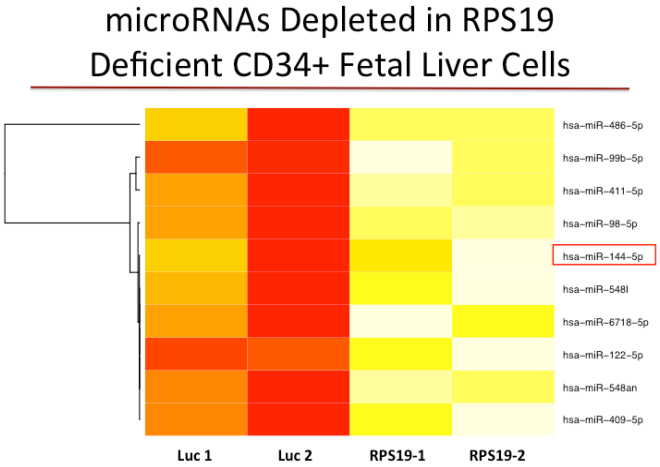
We have also explored additional microRNAs identified in RPS19-deficient human fetal liver cells from microRNA-seq experiment, including miR30 and miR221.

microRNAs Enriched in RPS19 Deficient CD34+ Fetal Liver Cells



miR30a Expression in RPS19 Deficient Fetal Liver CD34+ Cells





Summary of what was accomplished under these goals:

- RPS19 deficiency leads to upregulation of miR34a and decreased expression of c-myb and c-myc through a p53-dependent pathway. Furthermore, additional microRNAs are deregulated in RPS19-deficient CD34+ human fetal liver cells.

- We attempted experiments proposed in Aim 2 with primary human CD34+ fetal liver cells injected into NSG mice. We confronted difficulties in that these cells did not engraft. Therefore, we are continuing to troubleshoot these experiments.

- We have also performed RNA-seq experiments and submitted mRNA from RPS19 knockdown human fetal liver cells. We have identified two interesting pathways involved downstream of RPS19 deficiency in human hematopoietic progenitor cells. These pathways involve FoxM1, which appears to be deregulated specifically in RPS19 deficient cells. The other protein that is deregulated is GDF15, which is expressed in response to stress erythropoiesis. These two new mechanisms are novel and have not been previously studied.

- We also identified that that RSP19 deficient hematopoietic progenitors have decreased GATA1 expression through a TNFalpha and p53-dependent pathway and this paper is *in press* in Blood.

What opportunities for training and professional development have the project provided?

Graduate student: Former graduate student Elena Bibikova defended her Ph.D. thesis based on this work on December 19th, 2013. Dr. Bibikova now works as a research scientist at Acerta Pharma, Inc.

Postdoctoral fellows: Postdoctoral fellow Minyoung Youn, PhD. She received funding from the Child Health Research Institute Fellowship (Stanford University) to study signaling pathways in RPS19-deficient hematopoietic stem and progenitor cells for two years 9/1/2013 to 8/30/2015.

Postdoctoral fellow Joseph Park, M.D. applied for and received a CHRI fellowship for two years to study the role of RPS14 deficiency in del(5q) myelodysplastic syndrome. Techniques learned from the CDMRP project will be used for Dr. Park's project.

NIH training grant: As a result of the work funded by the CDMRP, I was able to obtain an NIH T32 training grant to support postdoctoral fellows studying Pediatric Nonmalignant Hematology and Stem Cell Biology.

T32DK098132 4/1/2014-3/31/2019

National Institutes of Health (NIDDK)
Training in Pediatric Nonmalignant Hematology and Stem Cell Biology
Sakamoto, PI
- The goal is to train postdoctoral fellows in nonmalignant hematology and stem cell biology

How were the results disseminated to communities of interest?

The data were presented at UCLA during graduate student Elena Bibikova's thesis defense and at the American Society of Hematology meeting.

What do I plan to do during the next reporting period to accomplish the goals?

I plan to continue to focus on microRNAs that are deregulated in RPS19-deficient CD34+ fetal liver cells as described in the SOW and milestones above.

4. IMPACT:

What was the impact on the development of the principal disciplines of the project?

Our results could lead to new insights into the pathogenesis and treatment of DBA. The implications are far reaching. Some of the deregulated genes that we identified in DBA, have also been found to be deregulated in RPS14 del(5q) myelodysplastic syndromes (MDS). We plan to further investigate downstream signaling pathways, in particular, those that might lead to new therapies for DBA and other bone marrow failure syndromes.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:
Changes in approach and reasons for change

None.

Actual or anticipated problems or delays and actions or plans to resolve them

One problem is the inability for RPS19-shRNA transduced human CD34+ cells to engraft in NSG mice. We are currently troubleshooting this. Another problem is inconsistent results with LCLs from DBA patients. We are now focusing on studying primary bone marrow cells from DBA patients.

Changes that had a significant impact on expenditures

None.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None.

6. PRODUCTS:

Journal publications

1. Bibikova E, Youn MY, Danilova N, Konto-Ghiorghi Y, Ono-Uruga Y, Ochoa R, Narla A, Glader B, Lin S*, and KM Sakamoto* (*co-senior authors). TNF-mediated inflammation represses GATA1 and activates p38 MAP kinase in RPS19-deficient hematopoietic progenitors. Bibikova et al. Blood*, in press* (see attached). Acknowledgement of federal support: yes.

2. Danilova N, Bibikova E, Covey TM, Nathanson D, Dimitrova E, Lindgren A, Glader B, Radu CG, Sakamoto KM, and S Lin. The Role of DNA damage response in zebrafish and cellular models of Diamond Blackfan Anemia. Disease Models and Mechanisms, *in press* (see attached). Acknowledgement of federal support: yes.

3. Danilova N, Bibikova E, Youn MY, Sakamoto KM* and S Lin* (*co-senior authors). Aberrant regulation of innate immunity in zebrafish and cellular models of Diamond Blackfan Anemia. *Revised manuscript submitted to Exp Hematol*. (see attached). Acknowledgement of federal support: yes.

Books, conference papers, and presentations

Youn MY, Bibikova E, Danilova N, Ono-Uragi Y, Konto-Ghiorghi Y, Ochoa R, Narla A, Glader B, Lin S, and KM Sakamoto. RPS19 Deficiency Leads to GATA1 Downregulation through TNF-mediated p38 MAPK Activation. Accepted for a poster presentation. American Society of Hematology, San Francisco, CA 2014 (see attached poster).

Website or other internet sites

None.

Technologies or techniques

See publications.

Inventions, patent applications, and /or licenses

None.

Other products

None

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name: Kathleen M. Sakamoto, MD, PhD
Project Role: PI
No change in effort.

Name: Elena Bibikova, PhD
Project Role: Graduate Student
Nearest person month worked: 3 (over the past year)
Contribution to Project: Dr. Bibikova performed all the work proposed in the project.
Funding support: CDMRP grant

Has there been a change in the active other support of the PI or key personnel since the last reporting period.

New grants for the PI since the last reporting period are:

Cure Search Grand Challenge Grant 1/1/2014-12/31/2016
Development of CD47 monoclonal antibody therapy for pediatric tumors
Sakamoto, PI and Weissman, co-PI
- The goal is to perform pre-clinical studies and Phase I clinical trials with anti-CD47 antibody for relapsed pediatric tumors.

T32DK098132 4/1/2014-3/31/2019
National Institutes of Health (NIDDK)
Training in Pediatric Nonmalignant Hematology and Stem Cell Biology
Sakamoto, PI
- The goal is to train postdoctoral fellows in nonmalignant hematology and stem cell biology

R13 CA 186539
National Institutes of Health (NHLBI/NCI) 5/1/2014-5/30/2015
Career Development and Increasing Diversity in Pediatric Hematology/Oncology
Sakamoto, PI
-The goal of this application is to support a workshop at the annual American Society of Pediatric Hematology/Oncology meeting to discuss late career transitions for faculty in our field.

Acerta, Inc. 8/1/2014-7/31/2015
Analysis of BTK and PI3Kd inhibitors in Normal and Neoplastic Myeloid cells
Sakamoto, PI
- The goal is to study the effects of BTK and PI3Kdelta inhibitors in normal hematopoietic stem and progenitor cells and acute myeloid leukemia cells.

Hyundai Hope on Wheels 1/1/2015-12/31/2016
The Role of CREB in the Pathogenesis of ALL and as a Target for Therapy.
Sakamoto, PI
- The goal is understand how CREB regulates growth of ALL cells and how it can be targeted for ALL patients.

Since Dr. Bibikova has graduated, postdoctoral fellow, Dr. Minyoung Youn has taken over this project.

Name: Minyoung Youn, PhD
Project Role: Postdoctoral Fellow

Nearest person month worked: 6
Contribution to Project: Will perform experiments proposed in the project.
Funding support: Stanford Child Health Research Institute postdoctoral fellowship

What other organizations were involved as partners?

We collaborate with researchers who are experts in zebrafish, Dr. Shuo Lin, at UCLA. We also collaborate with Dr. Stan Nelson for the microRNA-seq data (as proposed in the original proposal).

Organization Name: UCLA
Location of Organization: Los Angeles, CA.
Partner's contribution to the project: collaboration

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES:

See attached.

Regular Article

RED CELLS, IRON, AND ERYTHROPOIESIS

Q:A1

TNF-mediated inflammation represses GATA1 and activates p38 MAP kinase in RPS19-deficient hematopoietic progenitors

Elena Bibikova,[1] Min-Young Youn,[1] Nadia Danilova,[2] Yukako Ono-Uruga,[1] Yoan Konto-Ghiorghi,[1] Rachel Ochoa,[1]
Q:1 Anupama Narla,[1] Bertil Glader,[1] Shuo Lin,[2] and Kathleen M. Sakamoto[1]

[1]Department of Pediatrics, Stanford University School of Medicine, Stanford, CA; and [2]Department of Molecular, Cell and Developmental Biology, University of California Los Angeles, Los Angeles, CA

Key Points

Q:2
- GATA1 is downregulated in RPS19-deficient cells and zebrafish through upregulation of p53, TNF-α, and p38 MAPK.
- Treatment of ~~RPS19~~-deficient zebrafish with the TNF-α inhibitor etanercept rescues their erythroid and developmental defects.

Q:3

Diamond-Blackfan anemia (DBA) is an inherited disorder characterized by defects in erythropoiesis, congenital abnormalities, and predisposition to cancer. Approximately 25% of DBA patients have a mutation in *RPS19*, which encodes a component of the 40S ribosomal subunit. Upregulation of p53 contributes to the pathogenesis of DBA, but the link between ribosomal protein mutations and erythropoietic defects is not well understood. We found that RPS19 deficiency in hematopoietic progenitor cells leads to decreased GATA1 expression in the erythroid progenitor population and p53-dependent upregulation of tumor necrosis factor-α (TNF-α) in nonerythroid cells. The decrease in GATA1 expression was mediated, at least in part, by activation of p38 MAPK in erythroid cells and rescued by inhibition of TNF-α or p53. The anemia phenotype in ~~RPS19~~-deficient zebrafish was reversed by treatment with the TNF-α inhibitor etanercept. Our data reveal that RPS19 deficiency leads to inflammation, p53-dependent increase in TNF-α, activation of p38 MAPK, and decreased GATA1 expression, suggesting a novel mechanism for the erythroid defects observed in DBA. (*Blood*. 2014;00(00):1-8)

Introduction

Diamond-Blackfan anemia (DBA) is characterized by macrocytic anemia, congenital abnormalities, and predisposition to cancer.[1,2] Approximately 70% of DBA patients have mutations in ribosomal proteins, most frequently in RPS19.[3] Previous studies in human CD34$^+$ cells, zebrafish, and mice have shown that haploinsufficiency of RPS19 is associated with upregulation of the tumor suppressor p53.[4-6] However, the link between ribosomal protein insufficiency and specific defects in erythropoiesis is not well understood.

Several transcription factors play a critical role in erythroid development, including GATA1,[7] an essential erythroid protein comprising two zinc finger domains and a transactivation domain, which can activate or repress transcription of downstream targets by binding its consensus motif WGATAR in their promoters.[8,9] Targets of GATA1 include *EPOR*, which encodes the EPO receptor, adult globin genes, heme biosynthesis enzymes, and erythroid membrane proteins.[10]

Recently, *GATA1* splicing mutations have been found in three rare X-linked cases of DBA,[11] suggesting an association between this transcription factor and ribosomal protein deficiency.[12] We therefore investigated whether RPS19 deficiency affects GATA1 expression in human hematopoietic progenitor cells and in zebrafish.
Q:4 Our results demonstrated that GATA1 messenger RNA (mRNA) and protein levels are downregulated in human hematopoietic progenitor CD34$^+$ cells transduced with RPS19 short hairpin RNA (shRNA). Furthermore, we found that downregulation of GATA1 is p53 dependent and mediated, at least in part, through activation of the inflammatory cytokine tumor necrosis factor α (TNF-α) and its downstream signaling target p38 MAPK. Treatment of RPS19-deficient primary hematopoietic cells and zebrafish with the TNF-α inhibitor etanercept improved erythroid colony formation in vitro and rescued the anemia phenotype in vivo. Our studies suggest that inflammatory pathways play an important role in the erythroid defects and GATA1 regulation in DBA.

Methods

Cell culture

Primary human CD34$^+$ hematopoietic stem and progenitor cells were purified from cord blood (New York Blood Center) or from human fetal liver tissue (Advanced Bioscience Resources and University of California at Los Angeles Center for AIDS Research) by using magnetic-activated cell sorting (Miltenyi Biotec) and were cryopreserved. Upon thawing, cells were cultured in x-Vivo15 media (Lonza) containing 10% fetal bovine serum, ~~FMS-like tyrosine kinase-3~~ (50 ng/mL), thyroid peroxidase (50 ng/mL), interleukin-3 (IL-3; 20 ng/mL), ~~IL-6 (20 ng/mL),~~ and stem cell factor (50 ng/mL).

Submitted June 26, 2014; accepted September 12, 2014. Prepublished online as *Blood* First Edition paper, September 30, 2014; DOI 10.1182/blood-2014-06-584656.

E.B. and M.-Y.Y. contributed equally to this work.

The online version of this article contains a data supplement.

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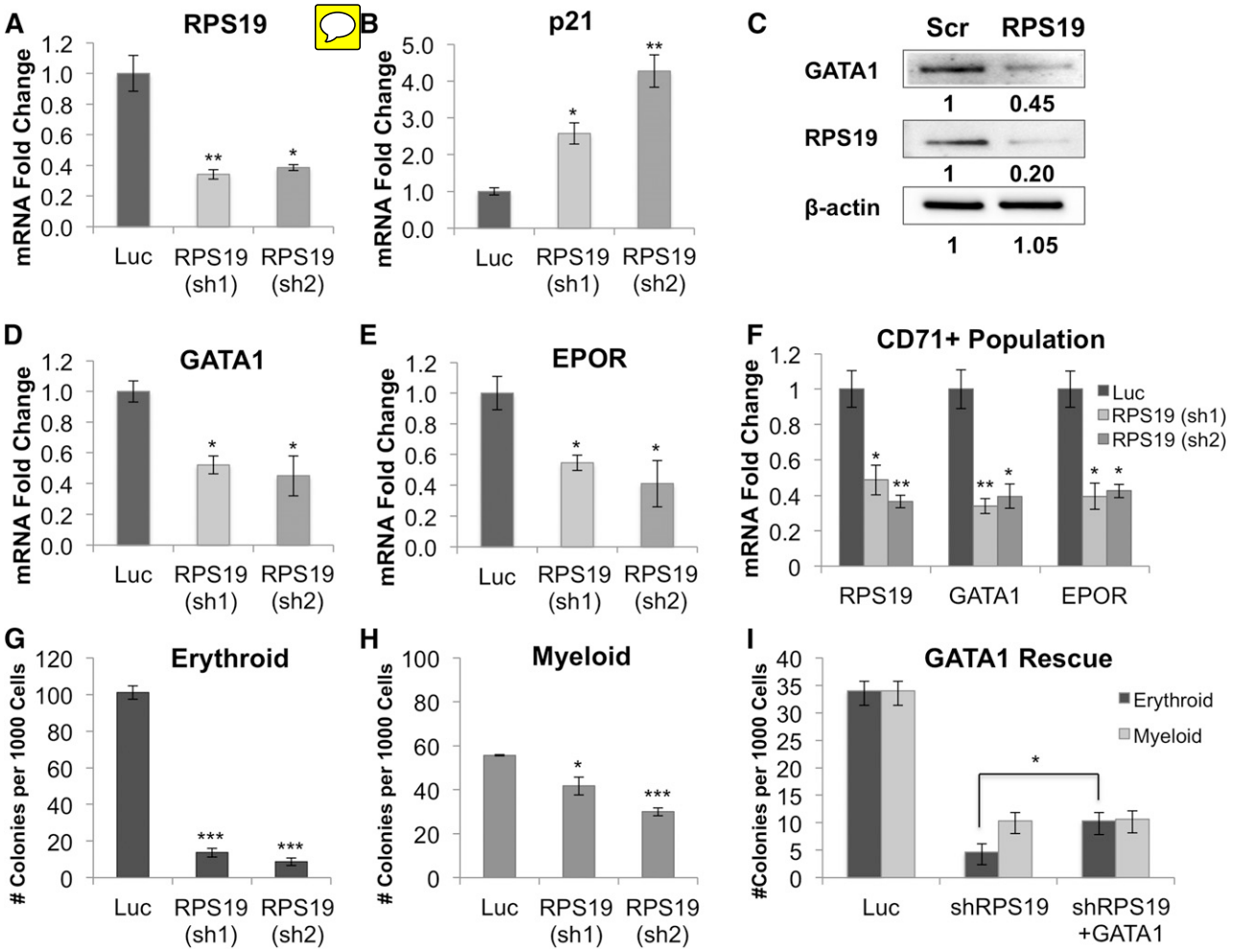


Figure 1. GATA1 expression and erythroid colony formation is decreased in RPS19-deficient hematopoietic progenitor cells. Human CD34$^+$ hematopoietic progenitor cells were infected with lentivirus carrying shRNA against RPS19 or ~~luciferase (Luc)~~ control, sorted for GFP$^+$ cells after 3 days, and analyzed 3 or 5 days after transduction, as stated for each panel. (A) shRNA knockdown of RPS19 reduces RPS19 mRNA levels by approximately 60% compared with control 5 days after transduction. (B) p21 mRNA expression is upregulated in RPS19-deficient cells 5 days after transduction. (C) GATA1 protein levels decreased in RPS19-deficient fetal liver cells [Scr, scrambled control shRNA; RPS19, RPS19 (sh2)] 3 days after transduction. (D) GATA1 and (E) EPOR mRNA levels decrease in RPS19-deficient cells 5 days after transduction. (F) GATA1 and EPOR expression is decreased in CD71$^+$ RPS19-deficient erythroid progenitor cells 5 days after transduction. (G) Erythroid colony formation in methylcellulose is reduced in cells with RPS19 knockdown. (H) Myeloid colony formation is decreased in RPS19-deficient cells. (I) Exogenous expression of GATA1 in RPS19-deficient cord blood cells partially rescues their ability to form erythroid colonies in methylcellulose. Data are representative of 2 independent transduction experiments. *$P < .05$; **$P < .01$; ***$P < .001$.

Lentiviral transduction

Q:5 Primary CD34$^+$ cells were transduced with lentivirus expressing shRNA against RPS19 (RPS19-1, RPS19-2, RPS19-3) or luciferase (Luc) shRNA at a multiplicities of infection score of 10 after 24 hours in culture. Cells were sorted for green fluorescent protein (GFP) after 3 to 5 days and harvested for downstream assays as indicated in the "Results" section. For p53 knockdown experiments, cells initially transduced with RPS19 shRNA were infected with lentivirus expressing p53[13] or ~~luciferase~~ shRNA with mCherry and sorted for GFP$^+$mCherry$^+$ cells 5 days after the initial transduction. For GATA1 rescue experiments, full-length ~~GATA1~~ complementary DNA (cDNA) was obtained from K562 cells by reverse transcriptase polymerase chain reaction (RT-PCR) and cloned into a lentiviral vector containing mCherry. Cells were cotransduced with lentivirus expressing GATA1 cDNA and RPS19 shRNA and sorted for GFP$^+$mCherry$^+$ cells 5 days after transduction. A list of shRNA target sequences is provided in supplemental Table 1, available at the *Blood* Web site.

Compounds

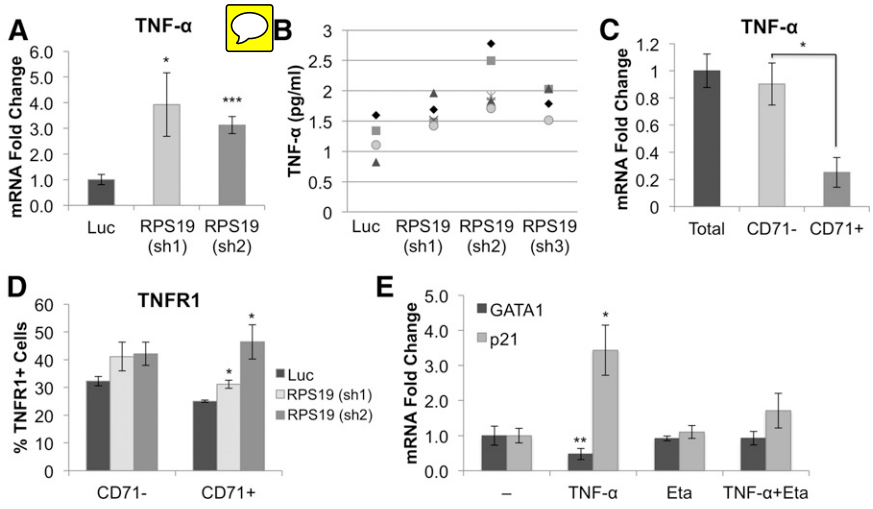
Nutlin-3 (N6287; Sigma-Aldrich) was diluted in dimethylsulfoxide to a 10 mM stock and added to cells at final concentrations of 10 μM and 25 μM for 24 hours. Etanercept (Amgen) was diluted according to manufacturer's instructions, added to cells at a concentration of 10 μg/mL, and injected into zebrafish embryos at 2 ng per embryo. SB203580 (Selleck Chemicals) was added to cells at 1 μM, 5 μM, or 10 μM concentrations for 18 to 22 hours. Cycloheximide (Sigma-Aldrich) was used at a concentration of 1 μg/mL for 2 hours. MG132 (Calbiochem) was added to cells at a 10μM concentration for 6 hours.

Colony assays

GFP$^+$- or GFP$^+$mCherry$^+$-sorted hematopoietic cells were seeded in methylcellulose medium containing IL-3, stem cell factor, granulocyte macrophage–colony-stimulating factor, and erythropoietin (H4434; STEMCELL Technologies) in triplicate, with 1000 cells per plate. Erythroid (burst-forming unit erythroid) and myeloid (colony-forming unit, granulocyte-macrophage) colonies were counted 14 days later.

qRT-PCR

RNA was extracted by using TRIzol (Life Technologies). RNA was transcribed into cDNA by using the iScript cDNA Synthesis Kit (Bio-Rad).



Q:11 **Figure 2. RPS19-deficient cells show increased expression of TNF-α.** CD34$^+$ hematopoietic progenitor cells were transduced with lentivirus carrying shRNA against RPS19 or ~~luciferase~~ control, sorted for GFP$^+$ cells at 72 hours, and analyzed 5 days after infection. (A) TNF-α mRNA and (B) protein levels are increased in the media from RPS19 deficient cells, compared with control, as measured by enzyme-linked immunosorbent assay (ELISA). Each ~~point~~ represents a separate measurement [n = 4 for Luc and RPS19 (sh3); n = 5 for RPS19 (sh1); n = 6 for RPS19 (sh2)]. (C) TNF-α is predominantly expressed in the CD71$^-$ (nonerythroid) fraction of cord blood cells in culture. (D) Cells were analyzed for CD71 and TNFR1 expression by flow cytometry. TNFR1 expression increased on the surface of RPS19-deficient CD71$^+$ cells compared with ~~luciferase~~ control but did not change in the CD71$^-$ population. (E) Addition of 100 ng TNF-α to CD34$^+$ cord blood cells results in decreased GATA1 and increased p21 expression after 24 hours. This effect is rescued by addition of 10 μg etanercept (Eta). Data are representative of 2 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$.

The quantitative RT-PCR (qRT-PCR) reaction was run with iQ SYBR Green MasterMix (Bio-Rad) using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). 7SL ~~sc~~RNA[14] was used as an internal control. Fold change **Q:6** of mRNA was calculated by using the ~~ΔΔ~~C_t method. A list of all primers used is provided in supplemental Table 2.

Western blot

Antibodies against RPS19 (#AB40833; Abcam; 1:200 dilution) and GATA1 (#sc-266; Santa Cruz Biotechnology; 1:200 dilution and #3535; Cell Signaling Technology; 1:1000 dilution) were used according to manufacturer's instructions. β-actin mouse monoclonal immunoglobulin G2a (A5316; Sigma-Aldrich) was used as a control at a 1:5000 dilution. The target proteins were analyzed by using WesternBright Sirius Chemiluminescent Substrate for horseradish peroxidase (Advansta). Densitometry was performed by using Image J software (http://rsb.info.nih.gov/ij/) to quantify the data.

Zebrafish

Zebrafish were reared at 28.5°C at a cycle of 14 hours of light/10 hours of dark. Embryos were obtained by natural spawning. Thirty embryos were injected with ~~rps19~~-specific morpholino (MO) at the 1-cell stage as previously described[5] and with 2 ng of etanercept at 4 to 5 hours post fertilization (hpf). For phenotypic analysis, embryos were scored blindly, without knowledge of whether they belonged to the control or the treatment group, and scoring was repeated 3 times. RNA was prepared from embryos at 18 and 40 hpf for qRT-PCR. At day 3, embryos were stained with **Q:7** ~~o~~-dianizidine to detect hemoglobin. For western blot, embryos were collected at 18 hpf, dechorionated with pronase, deyolked by pipetting in cold Ca-free Ringer's solution, washed 3 times with the same solution, and placed in cold radioimmunoprecipitation assay buffer with proteinase inhibitors. Protein content was detected by using bovine serum albumin assay. Ten micrograms of protein was loaded per lane and stained with antibodies against ~~gata1~~ (#55507; AnaSpec) and mouse anti-α-tubulin antibody (Sigma) followed by horseradish peroxidase anti-mouse antibody (Santa Cruz Biotechnology).

TNF-α detection

Cells were sorted for GFP 72 hours after transduction, and culture media was harvested 5 days after transduction. TNF-α was detected with a human TNF-α high sensitivity enzyme-linked immunosorbent assay kit (BMS223HS; eBioscience) according to the manufacturer's instructions.

Flow cytometry

For cell surface flow cytometry, cells were incubated with human Fc receptor binding inhibitor (#14-9161-73; eBioscience, Inc.) followed by primary antibodies CD71-APC (#551373; BD Biosciences) and biotinylated CD120a (TNFR1) (#552536; BD Biosciences). After washing, Streptavidin-PE-Cy7 (#557598; BD Biosciences) was added; all incubation times were 20 minutes on ice. For intracellular phospho-flow cytometry, cells were fixed in 3.2% paraformaldehyde and permeabilized with 100% methanol. The cells were then stained for CD71 (#551143 or #551374; BD Biosciences), phosphorylated p38 (p-p38) (#612565; BD Biosciences), phosphorylated nuclear factor κB (pNF-κB) (#4887; Cell Signaling Technology), phosphorylated ERK1/2 (pERK1/2) (#612566; BD Biosciences), phosphorylated STAT5 (pSTAT5) (#612599; BD Biosciences), and pSTAT1 (#560113; BD Biosciences). Data were collected on a DxP10 flow cytometer (Cytek) and analyzed by using FlowJo Software, v.9.7.2.

Statistics

P values for statistical significance were obtained by using an unpaired Student *t* test. The data are representative of at least 2 independent experiments.

Results

RPS19-deficient primary hematopoietic cells show reduced GATA1 expression

To investigate the link between RPS19 deficiency and erythropoietic defects, we examined whether RPS19 knockdown affects GATA1 expression. Primary human CD34$^+$ cord blood and fetal liver hematopoietic stem and progenitor cells were transduced with lentivirus expressing RPS19 or ~~luciferase~~ control shRNA. On day 5 after transduction, RPS19 expression decreased by approximately 60% (Figure 1A), with increased levels of the p53 targets p21 (Figure 1B), GADD45A, WIG-1, and BAX (supplemental Figure 1). RPS19-deficient cells also showed a decrease in the antiapoptotic gene BCL-2 (supplemental Figure 1), as well as increased apoptosis, **Q:8** seen through morphology (supplemental Figure 2) and increased cleaved poly(ADP-ribose) polymerase (supplemental Figure 3). Expression of GATA1 protein (Figure 1C), mRNA (Figure 1D), and transcriptional targets EPOR (Figure 1E), ERAF, HBB, and HBG2 (supplemental Figure 4) was decreased in RPS19-deficient cells. A time course of RPS19, GATA1, and EPOR expression (supplemental Figure 5) shows this decrease was steady at several time points and was not a result of delayed differentiation in RPS19-deficient or control cells. Additionally, the decrease in GATA1

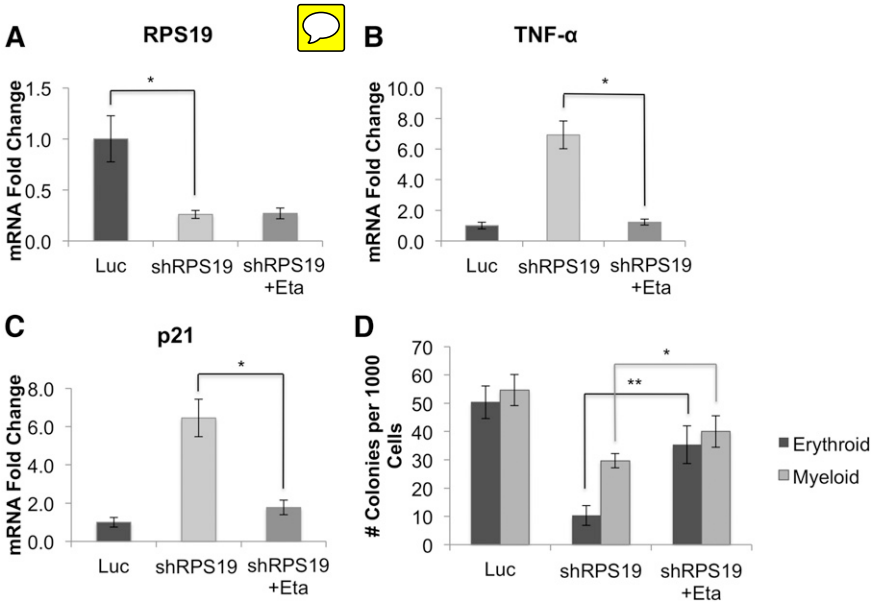


Figure 3. Inhibition of TNF-α rescues erythroid defects in RPS19-deficient cord blood cells. Human CD34$^+$ hematopoietic progenitor cells were infected with lentivirus carrying shRNA against RPS19 or ~~luciferase~~ control, sorted for GFP$^+$ cells after 72 hours, and treated with etanercept for 48 hours. (A) shRNA knockdown of RPS19 reduced RPS19 mRNA levels by approximately 70% compared with control. (B) Treatment of RPS19-deficient cord blood cells with etanercept reduced TNF-α and (C) p21 expression in these cells. (D) Etanercept treatment rescued both erythroid and myeloid colony formation of RPS19-deficient cells. Data are representative of 2 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$.

was EPO independent, because EPO was not added to the liquid culture until after day 5, and the addition of EPO showed little effect on erythroid differentiation during the early phase of liquid culture (supplemental Figure 6). The decrease in GATA1 expression was also observed in cells deficient for other ribosomal proteins, including RPS14 and RPL11 (supplemental Figure 7).

To confirm that GATA1 downregulation in RPS19-deficient cells was due to decreased ~~GATA1~~ transcription and not a consequence of fewer erythroid progenitors in the population, we sorted the RPS19-deficient hematopoietic cells for CD71, a marker of erythroid progenitors. We observed a decrease in GATA1 and EPOR expression in this population of cells (Figure 1F), consistent with our hypothesis that GATA1 transcription is decreased in erythroid progenitors. We further confirmed that the CD71$^+$ population gives rise to primarily erythroid colonies in methylcellulose (supplemental Figure 8), and we analyzed the maturation stage of the CD71$^+$ population by using late-stage erythroid cell surface markers α4-integrin and band 3,[15] which showed no difference between control and RPS19-deficient cells that could otherwise affect GATA1 expression (supplemental Figure 9).

RPS19 downregulation results in decreased erythroid (Figure 1G) and myeloid (Figure 1H) colony formation. To examine whether exogenous expression of GATA1 could rescue the erythroid defects in RPS19-deficient cells, we cotransduced CD34$^+$ cord blood cells with shRNA against RPS19 and a construct carrying full-length GATA1 cDNA (supplemental Figure 10) and observed a 2.2-fold increase in erythroid colony formation compared with cells transduced with RPS19 shRNA and empty vector control (Figure 1I). Our studies demonstrate that GATA1 levels are reduced in RPS19-deficient cells, that this reduction is specific to erythroid progenitors, and that expression of exogenous GATA1 improves erythropoiesis in our cell model of DBA.

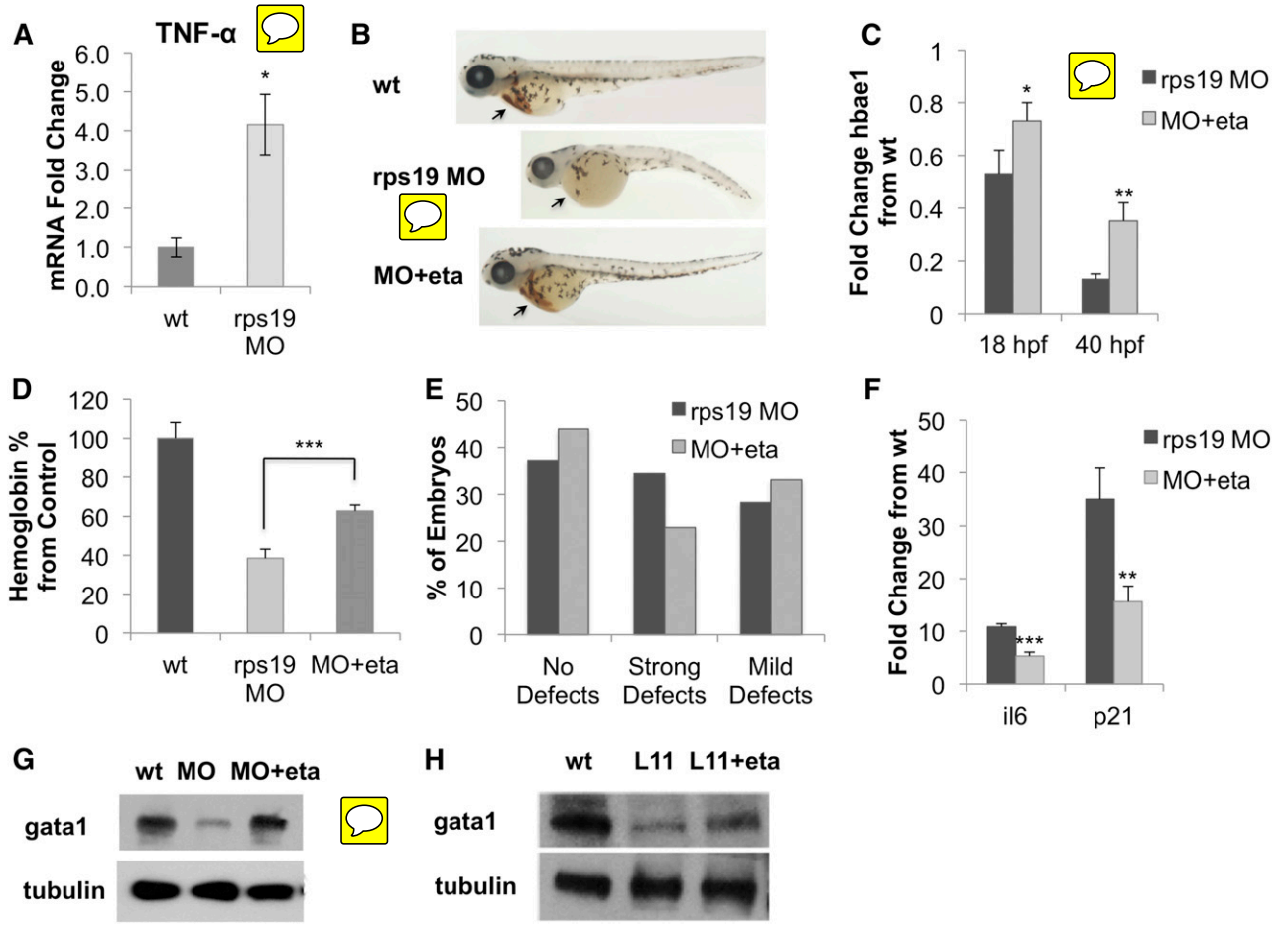
Inflammatory cytokines TNF-α and IL-6 are upregulated in RPS19-deficient primary hematopoietic cells

Bone marrow failure has been reported to be associated with an increased inflammatory response,[16] and inflammatory cytokines such as TNF-α are known to be associated with inhibition of erythropoiesis.[17] Therefore, it is possible that GATA1 expression may be reduced in RPS19-deficient cells through inflammation and activation of TNF-α, which has been shown to inhibit GATA1 in TF-1 cells.[18] In agreement with this hypothesis, we found that both TNF-α mRNA and protein are upregulated in RPS19-deficient cells (Figure 2A-B). We also saw increased levels of IL-6 in response to RPS19 knockdown but did not observe an increase in other cytokines, including IL-1 and ~~TNF-related apoptosis-inducing ligand~~ (supplemental Figure 11), or any significant changes in the baseline expression of TNF-α in our culture system (supplemental Figure 12).

To determine which cells produced TNF-α in our system, we separated the hematopoietic progenitor cells into erythroid (CD71$^+$) and nonerythroid (CD71$^-$) cell fractions 5 days after culture and analyzed levels of TNF-α and its receptor TNFR1 in the 2 cell populations. TNF-α was primarily expressed in the CD71$^-$ fraction (Figure 2C), containing macrophages, monocytes, stem cells, myelomonocytic progenitors, and promyelocytes (supplemental Figure 13). TNFR1 was upregulated on the cell surface of CD71$^+$ cells, as measured by flow cytometry (Figure 2D). Treatment of hematopoietic progenitors with TNF-α decreased GATA1 levels 2.1-fold and led to upregulation of p21, which could be attenuated by the addition of the TNF-α inhibitor etanercept (Figure 2E). Inhibition of TNF-α in RPS19-deficient cells by shRNA knockdown resulted in a restoration of GATA1 expression and increased formation of erythroid colonies in methylcellulose (supplemental Figure 14).

Etanercept improves erythropoiesis in RPS19-deficient primary hematopoietic cells

Because TNF-α is known to inhibit erythropoiesis, we examined whether suppressing TNF-α with etanercept in RPS19-deficient cells would improve their erythropoietic defects. We treated RPS19-deficient cord blood cells (Figure 3A) with etanercept and observed a strong reduction in TNF-α (Figure 3B) and p21 (Figure 3C) expression. Additionally, etanercept treatment rescued erythropoiesis, and to a lesser extent, myelopoiesis in RPS19-deficient cells, with a 3.5-fold increase in erythroid colony formation compared with untreated cells (Figure 3D).



Q:12 **Figure 4. Inhibition of TNF-α rescues erythroid defects in ~~rps19~~-deficient zebrafish.** (A) TNF-α mRNA is upregulated in ~~rps19~~ MO zebrafish at 18 hpf. (B) Treatment of ~~rps19~~ MO zebrafish with the TNF-α inhibitor etanercept rescued the erythropoietic (see arrows) and developmental defects. The average phenotype of each group is shown. (C) Expression of hbae1 is increased in ~~rps19~~ MO zebrafish after etanercept treatment. (D) Etanercept treatment restores hemoglobin levels in zebrafish, as measured by Drabkin's reagent at 54 hpf. (E) Morphologic defects in ~~rps19~~-morphant zebrafish are alleviated with etanercept treatment. "Strong defects" are defined as kinks, curved body, absent/rudimentary eyes, and other severe growth abnormalities. "Mild defects" are defined as smaller or shorter embryos with both eyes present. "No defects" indicates absence of visible defects in zebrafish morphology. Embryos were scored 3 times, with the average shown in the figure. (F) ~~rps19~~-morphant zebrafish treated with etanercept showed reduced expression of p53 ~~target~~ p21 and il-6. (G) Treatment with etanercept restored ~~gata1~~ expression in ~~rps19~~ MO zebrafish. (H) Levels of ~~gata1~~ also increased in ~~rpl11~~-mutant zebrafish following etanercept treatment. Data are representative of 2 independent experiments. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

Etanercept rescues erythroid defects and restores ~~GATA1~~ expression in ~~RPS19~~-deficient zebrafish

To investigate whether etanercept would improve the DBA phenotype in vivo, we used a zebrafish model of DBA, in which ~~rps19~~ knockdown is achieved through injection of MO.[5] We first confirmed upregulation of TNF-α in ~~rps19~~ MO zebrafish by qRT-PCR and found that TNF-α mRNA expression was increased 4.2-fold (Figure 4A), consistent with our primary hematopoietic cell model. We next treated ~~rps19~~ MO zebrafish with etanercept and observed a rescue of the DBA phenotype (Figure 4B), with improvements in erythropoiesis, as measured through hbae1 expression (Figure 4C) and hemoglobin content (Figure 4D), as well as a slight improvement in the morphology of the fish (Figure 4E).

Additionally, the fish showed decreased levels of il-6 and p21 expression (Figure 4F) and increased expression of ~~gata1~~, which was observed in both ~~rps19~~ morphants (Figure 4G) and ~~rpl11~~-mutant fish (Figure 4H). However, because purifying erythroid cells from zebrafish embryos was not technically feasible, we cannot conclude that the increase in ~~gata1~~ is specific to the erythroid population rather than a consequence of increased erythropoiesis in the fish. Overall, these data demonstrate that etanercept is effective in improving erythropoietic defects associated with DBA both in vitro and in vivo.

GATA1 downregulation and TNF-α upregulation are p53 dependent

To determine whether GATA1 downregulation and TNF-α upregulation were p53 dependent, we treated $CD34^+$ cord blood cells for 24 hours with Nutlin-3, a drug that leads to p53 stabilization (supplemental Figure 15) by blocking MDM2.[19] Nutlin-3 treatment increased expression of the p53 targets p21 (Figure 5A) and TNF-α (Figure 5B) and inhibited GATA1 expression (Figure 5C). Next, we cotransduced RPS19-deficient $CD34^+$ cord blood cells with lentivirus expressing p53 or ~~luciferase~~ control shRNA and an mCherry selection marker, and analyzed $GFP^+mCherry^+$ cells 5 days after infection. We observed a decrease in p21 (Figure 5D) and TNF-α (Figure 5E) mRNA levels in cells transduced with p53 shRNA as well as an increase in GATA1 levels (Figure 5F). Additionally, p53 knockdown increased erythroid colony formation twofold in RPS19-deficient cord blood cells (Figure 5G).

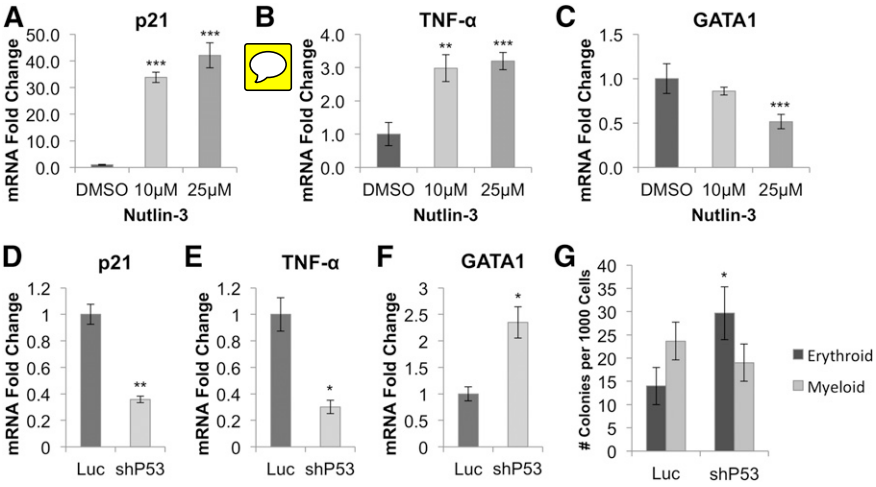


Figure 5. GATA1 downregulation is mediated through activation of p53 and TNF-α. (A) Stabilization of p53 by Nutlin-3 in CD34$^+$ hematopoietic progenitor cells leads to dose-dependent upregulation of p21 and (B) TNF-α, as well as (C) downregulation of GATA1. (D) shRNA-mediated knockdown of p53 in RPS19-deficient cord blood cells reduces p21 and (E) TNF-α expression while (F) increasing expression of GATA1 5 days after transduction. (G) Erythroid colony formation is increased in RPS19-deficient CD34$^+$ cord blood cells cotransduced with p53 shRNA compared with luciferase control shRNA. Data are representative of 2 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$.

Effect of TNF-α in RPS19-deficient primary hematopoietic cells is mediated, in part, through p38 MAPK

We next investigated the mechanism by which TNF-α affects erythropoiesis in RPS19-deficient cells by examining phosphorylation of downstream target pathways using phospho-flow cytometry before and after stimulation with TNF-α. We found a significant increase in phosphorylation of p38 MAPK in CD71$^+$ RPS19-deficient cells compared with control cells, both at baseline and after a 10-minute stimulation with 100 ng/mL TNF-α (Figure 6A). Levels of p38 were also mildly elevated at baseline in the CD71$^-$ population but did not show greater increase upon TNF-α stimulation in RPS19-deficient cells compared with control (supplemental Figure 16). Other downstream signaling pathways, including NF-kB, ERK1/2, STAT5, and STAT1, showed no difference in activation between control and RPS19-deficient CD71$^+$ cells (supplemental Figure 17), suggesting that the effect of TNF-α in RPS19-deficient cells is, at least in part, mediated through p38 MAPK.

To test this hypothesis further, we treated CD34$^+$ cord blood cells with the p38 MAPK inhibitor SB203580 and observed increased GATA1 protein in cells treated with increasing concentrations of SB203580 (Figure 6B-C). In line with this hypothesis, treatment of RPS19-deficient cells with SB203580 partially rescued both erythroid and myeloid colony formation (Figure 6D).

To test whether p38 pathway inhibition improves the stability of GATA1 protein, we treated CD34$^+$ cord blood cells with SB203580 with or without the translation inhibitor cyclohexamide and observed GATA1 stabilization in the presence of SB203580 (Figure 6E). These data demonstrate that GATA1 has a short half-life by rapid turnover but can be stabilized through inhibition of p38 MAPK. To show that GATA1 levels are proteasome dependent in the cells, we treated CD34$^+$ cord blood cells with MG132, a 26S proteasome inhibitor, in the presence or absence of SB203580 (Figure 6F). Our data show that MG132 treatment leads to increased stability of the GATA1 protein, suggesting that GATA1 is regulated through a proteasome-dependent mechanism and that p38 MAPK contributes to its degradation.

Overall, our data support a model in which deficiency of RPS19 causes p53-dependent inflammation and increased production of

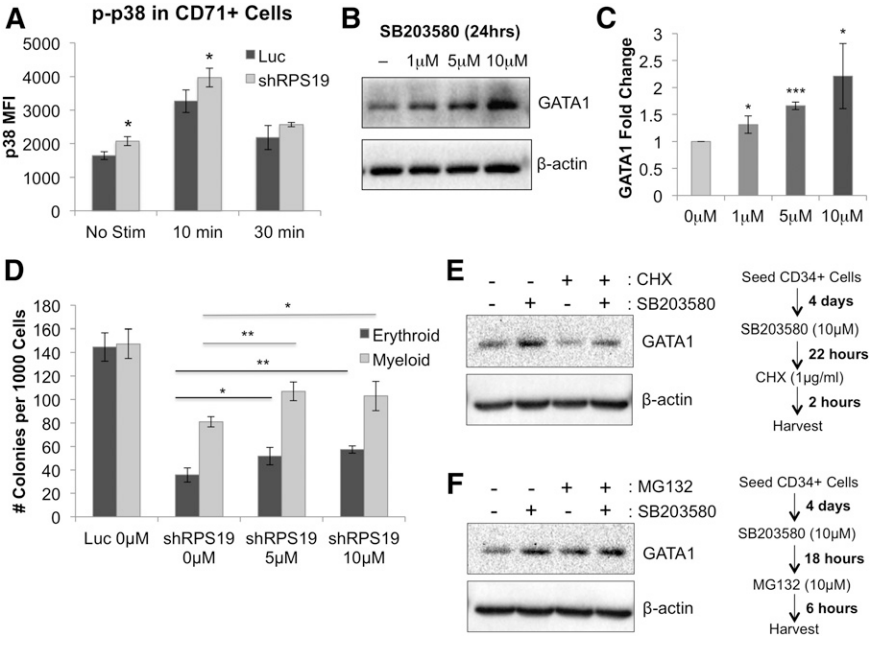


Figure 6. Effect of TNF-α on GATA1 expression is partially mediated through p38 MAPK activation. (A) p38 MAPK phosphorylation is increased in RPS19-deficient CD71$^+$ fetal liver cells before and after a 10-minute stimulation with 100 ng/mL TNF-α, as measured by phospho-flow cytometry 5 days after transduction. (B) Treatment of CD34$^+$ cord blood cells with SB203580 for 24 hours increased GATA1 protein expression in the cells. (C) Quantification of GATA1 protein increase from western blot in (B). (D) Treatment of RPS19-deficient cord blood cells with SB203580 partially rescues their erythropoiesis and myelopoiesis in methylcellulose. (E) Treatment of CD34$^+$ cord blood cells with SB203580, a p38 MAPK inhibitor, increases the level of GATA1 protein in the cells, whereas treatment with CHX, a translation inhibitor, shows decreased GATA1 protein levels in the absence of SB203580. (F) Treatment of CD34$^+$ cord blood cells with SB203580 and/or MG132, a proteasome inhibitor, increases GATA1 stability in the cells. Data are representative of 2 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$.

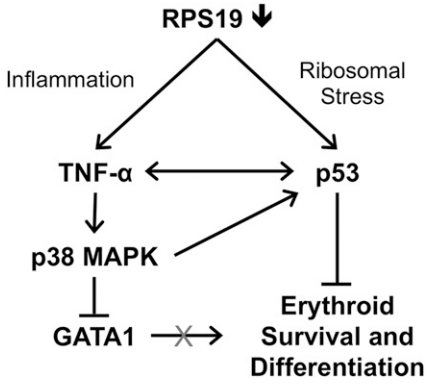


Figure 7. Model of GATA1 downregulation through the combined effects of p53 and TNF-α in RPS19-deficient cells. Deficiency of RPS19 in hematopoietic progenitors leads to ribosomal stress, which increases p53 levels as well as inflammation, which increases production of TNF-α by nonerythroid cells. TNF-α represses GATA1 through activation of p38 MAPK and may also contribute to increased p53 levels, which results in reduced survival and differentiation of erythroid cells.

TNF-α in the bone marrow, which activates p38 MAPK and contributes to GATA1 degradation in erythroid cells (Figure 7).

Discussion

On the basis of these data, we concluded that GATA1 expression is decreased in RPS19-deficient erythroid progenitors, which may contribute to the erythroid-specific lineage defects characteristic of DBA, and that restoration of GATA1 expression in RPS19-deficient cells can improve erythroid colony formation in vitro. Additionally, we found upregulation of inflammatory cytokines TNF-α and IL-6 in both primary cell and zebrafish models of DBA, signifying a novel role for inflammation in DBA pathogenesis. Finally, we show that downregulation of GATA1 can be alleviated by the TNF-α inhibitor etanercept as well as through knockdown of p53 and p38 MAPK, suggesting potential novel therapies for the treatment of DBA.

A recently published study by Ludwig et al[20] supports our findings that GATA1 is downregulated in hematopoietic cells with ribosomal protein haploinsufficiency and that expression of exogenous GATA1 in RPS19-deficient cells can partially rescue their erythroid defects. However, there are several differences in our findings, likely arising as a result of the different methods and model systems used in our studies. First, Ludwig et al did not observe changes in GATA1 mRNA or in expression levels of p53 target genes despite prior data from several groups showing p53 pathway upregulation as a feature of DBA.[4-6] Second, the study focused on cell-intrinsic factors such as altered translation mechanisms rather than extrinsic factors such as cell signaling and inflammation to explain GATA1 downregulation in RPS19-deficient cells. The difference in GATA1 expression is likely due to timing of cell analysis. Ludwig et al analyzed GATA1 mRNA levels in $CD34^+$-derived erythroid progenitors at 4 days after infection, whereas we did not observe significant changes in GATA1 expression until after day 5 (supplemental Figure 5). It is likely that the decrease in GATA1 protein precedes a decrease in GATA1 mRNA, which occurs as a result of GATA1 autoregulation. Differences in p53 pathway activation may be a result of distinctive model systems used (patient samples vs transduced hematopoietic progenitors), or a result of different methods used for gene expression analysis (qRT-PCR vs global gene profiling).

Taken together, our data support a model of GATA1 downregulation being a consequence of inflammation and activation of proinflammatory cytokines such as TNF-α, which has been shown to inhibit erythroid differentiation in multiple cell lines[18,21] and is associated with reduced GATA1 expression in TF-1 cells through p38 MAPK.[18] The erythroid niche in vivo consists of erythroid progenitors surrounding a central macrophage,[17] which may secrete inflammatory cytokines in response to ribosomal stress, inhibiting erythroid progenitor growth around it. Consistent with this mechanism, we observed increased TNF-α production in $CD71^-$ cells in our culture system (which supports both myeloid and erythroid development), with increased expression of TNFR1 on the surface of $CD71^+$ erythroid cells.

TNF-α signaling has been shown to activate p38 MAPK,[22] which phosphorylates acetylated GATA1 at Ser26 and Ser178, facilitating its degradation.[23] Our data support this mechanism of GATA1 degradation in response to increased TNF-α, because we saw increased phosphorylation of p38 MAPK in RPS19-deficient cells by phospho-flow. Treatment of primary hematopoietic cells with the p38 MAPK inhibitor SB203580 improved GATA1 protein stability in the cells. This model, in which TNF-α upregulation in RPS19-deficient cells contributes to GATA1 degradation through p38 MAPK, may also explain why dexamethasone, which reduces TNF-α levels, would have a beneficial effect in DBA patients.[24]

Increased expression of p53 in RPS19-deficient cells could also antagonize the transcriptional activity of GATA1. Exogenously expressed p53 has been shown to bind to GATA1 directly in K562 cells and inhibit its function.[25] Therefore, it is plausible that p53 activated in RPS19-deficient cells binds to GATA1, inhibiting its ability to transactivate erythroid-specific genes. This would also reduce GATA1 transcription, since *GATA1* has binding sites in its own promoter,[26] forming a negative feedback loop between GATA1 and p53 in RPS19-deficient cells. Our studies confirm that GATA1 downregulation is p53 dependent and can be rescued by shRNA-mediated knockdown of p53 in RPS19-deficient cells.

Previous work supports the relationship between p53 and TNF-α, and each may contribute to DBA pathogenesis, because p53 can activate TNF-α expression by binding to its promoter,[27] and TNF-α can activate the p53 pathway through p38 MAPK.[28] Thus, p53 and TNF-α could form a positive feedback loop, acting together to repress erythropoiesis in response to ribosomal stress. This mechanism is consistent with our data showing that repression of p53, p38 MAPK, or TNF-α in RPS19-deficient cells can alleviate their erythroid defects.

In summary, our results demonstrate a novel link between RPS19 deficiency, inflammation, and GATA1 downregulation, which could explain the erythroid defects observed in DBA patients. We show that GATA1 downregulation is p53 dependent and is, at least in part, mediated by increased TNF-α production and p38 MAPK activation in RPS19-deficient cells. Additionally, our results suggest that etanercept and other TNF-α antagonists may provide a potential approach to treating erythroid defects in patients with DBA.

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Authorship

Contribution: E.B., M.-Y.Y., Y.O.-U., Y.K.-G., and R.O. performed the experiments with human hematopoietic cells; N.D. performed experiments with zebrafish; the research was designed by E.B., M.Y.-Y., N.D., S.L., A.N., and K.M.S.; and the manuscript was written by E.B., B.G., S.L., and K.M.S.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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AUTHOR QUERIES

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RESEARCH ARTICLE

The role of the DNA damage response in zebrafish and cellular models of Diamond Blackfan anemia

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ABSTRACT

Ribosomal biogenesis involves the processing of pre-ribosomal RNA. A deficiency of some ribosomal proteins (RPs) impairs processing and causes Diamond Blackfan anemia (DBA), which is associated with anemia, congenital malformations and cancer. p53 mediates many features of DBA, but the mechanism of p53 activation remains unclear. Another hallmark of DBA is the upregulation of adenosine deaminase (ADA), indicating changes in nucleotide metabolism. In RP-deficient zebrafish, we found activation of both nucleotide catabolism and biosynthesis, which is consistent with the need to break and replace the faulty ribosomal RNA. We also found upregulation of deoxynucleotide triphosphate (dNTP) synthesis – a typical response to replication stress and DNA damage. Both RP-deficient zebrafish and human hematopoietic cells showed activation of the ATR/ATM-CHK1/CHK2/p53 pathway. Other features of RP deficiency included an imbalanced dNTP pool, ATP depletion and AMPK activation. Replication stress and DNA damage in cultured cells in non-DBA models can be decreased by exogenous nucleosides. Therefore, we treated RP-deficient zebrafish embryos with exogenous nucleosides and observed decreased activation of p53 and AMPK, reduced apoptosis, and rescue of hematopoiesis. Our data suggest that the DNA damage response contributes to p53 activation in cellular and zebrafish models of DBA. Furthermore, the rescue of RP-deficient zebrafish with exogenous nucleosides suggests that nucleoside supplements could be beneficial in the treatment of DBA.

KEY WORDS: Ribosomal protein deficiency, Rps19, Rpl11, p53, ATR, RNR, Chk1, ATP, AMPK, Exogenous nucleosides

INTRODUCTION

Ribosome biogenesis is the most energy-consuming process in the cell (Thomas, 2000). It starts with the transcription of pre-ribosomal RNA (pre-rRNA) by RNA polymerase I (Kressler et al., 2010). The nascent pre-rRNA is assembled co-transcriptionally (Osheim et al., 2004) with a subset of ribosomal proteins (RPs) and other factors that facilitate pre-rRNA modification, cleavage and processing to 40S and 60S ribosomal subunits (Tafforeau et al., 2013). A growing set of genetic diseases is linked to mutations in genes that are involved in ribosome biogenesis (Narla and Ebert, 2010).

Mutations in some RPs impair the processing of pre-rRNA, leading to the accumulation of defective rRNAs in cells (Choesmel et al., 2007; Flygare et al., 2007; Léger-Silvestre et al., 2005). It causes the Minute phenotype in Drosophila and Diamond-Blackfan anemia (DBA) syndrome in humans (Lipton and Ellis, 2009; Marygold et al., 2007). RPS19 is the most frequently mutated RP in DBA (Boria et al., 2010; Draptchinskaia et al., 1999; Farrar et al., 2011). DBA is associated with anemia, malformations and predisposition to cancer. Physical abnormalities are especially frequent in individuals that have mutations in RPL11 and RPl5 (Gazda et al., 2008). Upregulation of the transcription factor p53 is involved in DBA pathogenesis as p53 inhibition ameliorates hematopoietic and developmental defects in animal models of DBA (Danilova et al., 2011; Danilova et al., 2008; Dutt et al., 2011; McGowan et al., 2008). Several mechanisms of p53 activation that suggest a special checkpoint controlling ribosomal biogenesis have been proposed, but the exact mechanism remains unclear (Fumagalli and Thomas, 2011).

A common diagnostic feature of DBA is upregulation of adenosine deaminase (ADA) (Fargo et al., 2013), which is also observed in animal models (Danilova et al., 2011; Danilova et al., 2008). ADA catabolizes ATP and dATP by removing an amino group from the adenine moiety. ADA deficiency leads to the accumulation of toxic deoxyadenosine and severe combined immunodeficiency syndrome (Sauer and Aiuti, 2009), whereas overexpression of ADA causes ATP depletion and hemolytic anemia (Chen and Mitchell, 1994). ADA overactivation in DBA suggests changes in nucleotide metabolism that might contribute to DBA pathophysiology. Although hemolysis is not a distinctive feature of DBA, the changes in plasma proteins indicate mild hemolytic anemia (Young and Alter, 1994). Other aspects of nucleotide metabolism might be changed in DBA, such as the balance of deoxynucleotide triphosphates (dNTPs), which has been shown in other models to be important for normal cell function (Bester et al., 2011; Hastak et al., 2008; Mannava et al., 2013). Nucleotide metabolism in DBA, however, has never been investigated.

dNTPs are synthesized during the S phase of the cell cycle by ribonucleotide reductase (RNR), or through the nucleoside salvage pathways (Nordlund and Reichard, 2006) (Fig. 1A). Both dNTP deficiency and excess are dangerous for cells. Deficiency of any dNTP leads to replication stress and the activation of the ATR-p53 pathway, whereas an excess of dNTPs inhibits RNR and arrests proliferation (Kunz et al., 1994).

RNR is also induced in response to DNA damage in order to produce dNTPs for DNA repair (Chabes et al., 2003). RNR is a downstream target of ATR, the major kinase responsible for the DNA replication checkpoint (Branzei and Foiani, 2009; Elledge et al., 1993; Håkansson et al., 2006; Nordlund and Reichard, 2006).

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TRANSLATIONAL IMPACT

Clinical issue

Diamond Blackfan anemia (DBA) is a congenital disorder characterized by anemia, various malformations and an increased incidence of cancer. DBA is caused by mutations in ribosomal proteins (RPs), most frequently in RPS19, which interfere with rRNA processing and ribosome biogenesis. DBA is the most studied representative of several disorders that are associated with defects in ribosome biogenesis. Corticosteroids remain the major treatment option in DBA; hematopoietic stem cell transplantation is used in corticosteroid-resistant individuals. p53 activation appears to be a crucial mediator of many clinical features of DBA, but the molecular basis for p53 activation is unclear. A better understanding of the molecular mechanism underlying DBA is necessary for the development of new treatments for DBA and related disorders.

Results

A frequent feature in DBA is upregulation of adenosine deaminase, which suggests that DBA involves changes in nucleotide metabolism. Therefore, in this study, which uses zebrafish deficient in Rps19 and Rpl11 as models of DBA, the authors first examine nucleotide metabolism and show that enzymes involved in both nucleotide catabolism and biosynthesis are upregulated. RP deficiency also results in the arrest of cell proliferation. In spite of this, the authors find that both the expression of enzymes involved in dNTP biosynthesis and the incorporation of exogenous nucleotides into their DNA is increased in RP-deficient zebrafish. These findings point to increased DNA repair. In accordance with this interpretation, the authors find that markers for DNA damage are upregulated in RP-deficient zebrafish and in human fetal liver cells that are deficient in RPS19. Moreover, the application of inhibitors of the kinases involved in the DNA damage checkpoint decreases p53 upregulation and apoptosis and improves hematopoiesis in RP-deficient zebrafish. Finally, exogenous nucleosides also rescue RP-deficient zebrafish by resulting in the downregulation of pro-apoptotic p53 targets and a decrease in the activation of AMP-activated protein kinase.

Implications and future directions

These findings reveal the involvement of DNA damage pathways in the upregulation of p53 and in other molecular changes that are observed in a zebrafish model of DBA and in a human cellular model. They suggest that drugs that help to decrease DNA damage or that help to increase DNA repair might be effective in the treatment of DBA. They also suggest that nucleoside supplements work *in vivo* to increase DNA repair and might therefore be beneficial to individuals with DBA and related conditions.

Salvage pathways of dNTP synthesis are also induced in response to DNA damage through the ATM kinase (Smal et al., 2006).

Nucleotides are not only the building blocks for RNA and DNA but are also important signaling molecules; moreover, ATP is the major source of energy in cells. ATP shortage leads to activation of the energy sensor AMP-activated protein kinase (AMPK) (Hardie et al., 2012). Activated AMPK then switches on catabolic pathways to generate ATP and switches off biosynthetic pathways and cell-cycle progression.

In this study, we identify a link between RP deficiency, nucleotide metabolism and p53 activation. Our data suggest that zebrafish and human RP-deficient cells activate the ATR, ATM, CHK1 and CHK2, p53 pathway. We also uncover complex but coherent changes in nucleotide metabolism in RP-deficient cells that stems from their need to catabolize defective rRNAs, produce NTPs for more rRNAs and make extra dNTPs for DNA repair in response to ATR and ATM activation. RP-deficient cells exhibited an imbalance of dNTPs, ATP depletion, and upregulation of AMPK and p53. Although cells in RP-deficient zebrafish had a lower proliferation rate than controls, they incorporated more deoxycytidine into their DNA, suggesting the activation of nucleoside salvage pathways and increased DNA repair. A mixture of exogenous nucleosides rescued hematopoietic and morphological defects in RP-deficient zebrafish.

Our data suggest that activation of ATR, ATM, CHK1 and CHK2 contributes to p53 upregulation in DBA. The finding that exogenous nucleosides rescue RP-deficient zebrafish suggests that nucleoside supplements could be beneficial in individuals with DBA.

RESULTS

Embryos switch from salvage to *de novo* dNTPs synthesis during development

To explore the role of the DNA damage response in DBA, we first investigated nucleotide metabolism in wild-type and RP-deficient zebrafish embryos (Fig. 1A). Zebrafish eggs have a supply of maternal dNTPs, the amount of which decreases during embryo development, reaching a steady-state level at about 24 hours post-fertilization (hpf) (Fig. 1B). At the same time, *de novo* synthesis of dNTPs gradually increases, as illustrated by the expression of the gene *rrm1*, which encodes an RNR subunit (Fig. 1C). By contrast, expression of the gene *dck*, which encodes a salvage enzyme, decreases with age (Fig. 1D). Therefore, early embryos mostly recycle their nucleotides through the salvage pathways, whereas the *de novo* dNTP production gradually increases over time.

RP deficiency changes the expression of genes involved in nucleotide metabolism

Rps19-deficient zebrafish were generated using a morpholino, as previously described (Danilova et al., 2008; Torihara et al., 2011; Uechi et al., 2008). We also used mutant *rpl11*^{hi3820bTg} (Amsterdam et al., 2004), which has been characterized previously as a model of DBA in our lab (Danilova et al., 2011). Embryos have a supply of maternal ribosomes, and RP-deficient zebrafish can develop normally throughout early developmental stages. The morpholino generates Rps19 deficiency, which is most pronounced from 18 to 48 hpf (Danilova et al., 2008). Rpl11 mutants were predominantly used at 48 hpf – when at least 50% of Rpl11 protein remains (Danilova et al., 2011), mimicking haploinsufficiency of RPL11 in DBA. Most experiments were performed using both Rps19- and Rpl11-deficient models to reveal features common for deficiency of RPs from both small and large ribosomal subunits. Rps19 deficiency was also generated in a p53-null background, by using (*tp53*$^{zdf1/zdf1}$) zebrafish (Berghmans et al., 2005).

RP deficiency resulted in the upregulation of enzymes that are involved in both nucleotide catabolism and biosynthesis. In Rpl11 mutants, the expression of the genes encoding the catabolic enzymes Ada and Xdh was increased (Fig. 2A). *ada* and *xdh* were also upregulated in Rps19 morphants (Fig. 2B). These changes are consistent with the need to catabolize the aberrant rRNAs in RP-deficient cells and are in accord with the increased activity of ADA in DBA individuals (Fargo et al., 2013; Léger-Silvestre et al., 2005). Increased expression of *ada* in Rps19-deficient embryos was not mediated by p53, because *ada* was also increased in *tp53*−/− zebrafish (supplementary material Fig. S1). In wild-type embryos, *ada* expression increased during development, starting at 19 hpf, and the increase was higher in embryos that had been injected with a morpholino against Rps19 (Fig. 2C).

Both in Rpl11 mutants and Rps19 morphants, the expression of the *ppat* and *cad* genes, the products of which are involved in purine and pyrimidine biosynthesis, was increased (Fig. 2A,B), which is consistent with an increased demand for NTPs in RP-deficient cells that need to replace defective rRNAs. This notion is also supported by the increased expression of enzymes involved in nucleotide biosynthesis in DBA individuals (Halperin and Freedman, 1989) and by the increased expression of genes encoding factors involved in ribosome biogenesis in zebrafish Rpl11 mutants, these include PolI

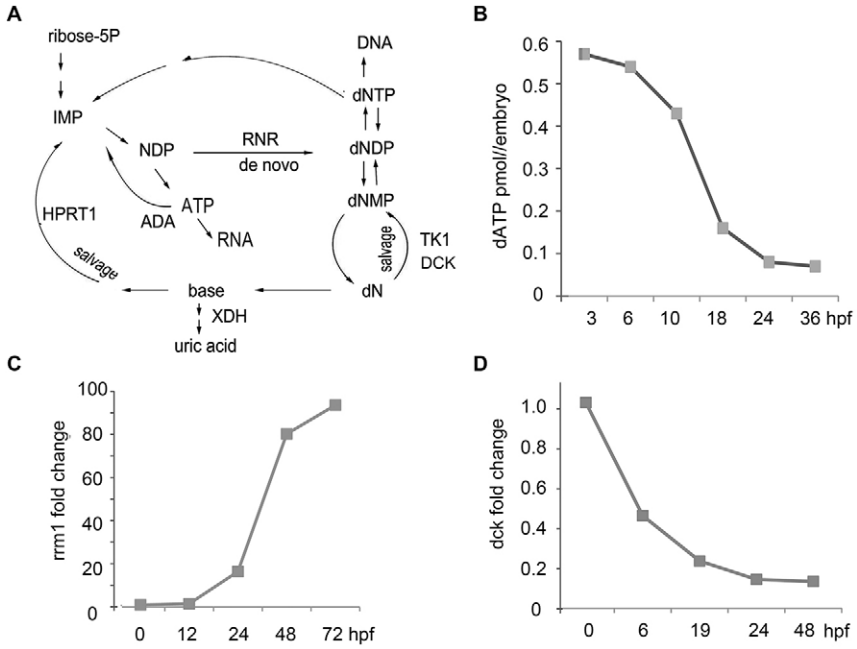

Fig. 1. Zebrafish embryos switch from a salvage to a *de novo* pathway of dNTP synthesis during development. (A) dNTPs are synthesized *de novo* by the RNR enzyme or via the nucleoside salvage pathways. ADA, adenosine deaminase; NDP, nucleoside diphosphate; dNMP, dNDP and dNTP are deoxynucleoside mono-, di- and triphosphate, respectively; XDH, xanthine dehydrogenase; HPRT1, hypoxanthine phosphoribosyltransferase; TK1, thymidine kinase; DCK, deoxycytidine kinase. (B) Embryos have maternal supplies of dNTPs to support rapid cell division. The amount of free dATP in embryos decreased with age. hpf, hours post-fertilization. Means of three replicates were used to generate the graph. (C) *De novo* synthesis was low in early embryos; *rrm1* expression increased through development, as measured using RT-qPCR. The fold change of expression was calculated relative to the expression at 0 hpf. qPCR in panels C and D was performed in triplicate, and the means were used to generate the graphs. (D) The expression of *dck*, encoding the salvage enzyme, was high in early-stage embryos and decreased with age. The results of RT-qPCR analyses are shown. The fold change of expression was calculated relative to the expression at 0 hpf.

and PolII, Ddx family, and nucleolar factors among others (Danilova et al., 2011). Among salvage enzymes, the expression of *hprt1* was increased, whereas expression of *tk1* and *dck*, which are expressed only in growing cells, was decreased, consistent with the decreased proliferation of RP-deficient cells (Fig. 2A,B). Altogether, these results show that nucleotide metabolism is activated in RP-deficient zebrafish.

Expression of RNR, which is involved in *de novo* dNTP synthesis, is upregulated in RP-deficient zebrafish

RP-deficient cells are characterized by proliferation arrest (Fumagalli et al., 2012). Therefore, it was an unexpected finding that the expression of the gene *rrm1*, encoding an RNR subunit, was increased 2- to 7-fold in RP-deficient zebrafish (Fig. 2D). RNR is a downstream target of ATR kinase, which is responsible for the replication stress checkpoint, thus indicating ATR activation. To study the possibility that p53 has an effect on *rrm1* upregulation, we compared timecourses of *tp53* and *rrm1* expression in Rps19-deficient zebrafish embryos. In wild-type embryos, expression of a transactivating isoform of *tp53* peaked at gastrulation (Fig. 2E). In Rps19-deficient embryos, this peak was much larger and remained high at later timepoints, but no difference in *tp53* expression was noted between wild-type and Rps19-morphant zebrafish until after 6 hpf. Conversely, the expression of *rrm1* had already increased in Rps19 morphants by 3.5 hpf and continued to increase at later timepoints (Fig. 2F). The injection of a 5-base-mismatch morpholino did not lead to *rrm1* upregulation (Fig. 2F). *rrm1* expression was also increased in *tp53*$^{-/-}$ zebrafish that had been injected with the morpholino against Rps19 (Fig. 2G). Therefore, *rrm1* upregulation in Rps19-deficient zebrafish takes place soon after zebrafish embryos start zygotic transcription at ~3 hpf and is p53 independent.

RP-deficient zebrafish upregulate the ATR/ATM-Chk1 pathway

To determine whether *rrm1* upregulation in RP-deficient zebrafish embryos is caused by activation of the DNA damage checkpoints, we evaluated the phosphorylation of histone H2A.X at residue Ser139, which is a marker of DNA damage. Phosphorylation of H2A.X was not detectable in wild-type embryos but was induced in embryos that had been injected with Rps19-specific morpholino (Fig. 3A). Chk1 kinase acts downstream from ATR and ATM kinases to regulate the cell cycle in response to blocked DNA replication and genotoxic stress, and p53 is a Chk1 target. In RP-deficient zebrafish, we found increased phosphorylation of Chk1 at residue Ser345, a marker of Chk1 activation (Fig. 3B). To confirm Chk1 involvement in p53 activation, we treated Rps19-deficient embryos with a Chk1 inhibitor (PF477736) and found downregulation of p53 (Fig. 3C; supplementary material Fig. S2). The ATR and ATM inhibitor CGK733, and ATM inhibitor KU6009, also downregulated p53 in Rps19-deficient embryos. These inhibitors were also effective in Rpl11-mutant embryos, as shown by downregulation of the p53 targets *p21* and *puma* in Rpl11 mutants (Fig. 3D). Treatment with these inhibitors decreased the severity of morphological defects, improved survival (supplementary material Fig. S3) and increased the amount of red blood cells in RP-deficient embryos (Fig. 3E). These data pointed to activation of the ATR/ATM-Chk1/Chk2/p53 pathway in RP-deficient zebrafish.

DNA damage checkpoints are activated in RPS19-deficient human fetal liver cells

Next, we examined whether RPS19-deficient human fetal liver cells also show signs of DNA damage response. Previously, a cellular model of DBA has been developed that utilized transduction of human CD34^{+} cells from umbilical cord blood and bone marrow with lentiviral vectors expressing short hairpin RNA (shRNA) against *RPS19* (Flygare et al., 2005). We used the same vectors to transduce human CD34^{+} fetal liver cells. Transduction with the shRNA vectors strongly reduced the *RPS19* mRNA levels, reduced levels of the protein and induced expression of p53 targets, such as *p21* (supplementary material Fig. S4). The level of the RPS19 protein decreased more slowly than that of the mRNA, so that 5 days after transduction the cells still had ~50% of RPS19 protein relative to controls, which is similar to previous reports (Flygare et al., 2005). At that stage, we determined the expression of markers

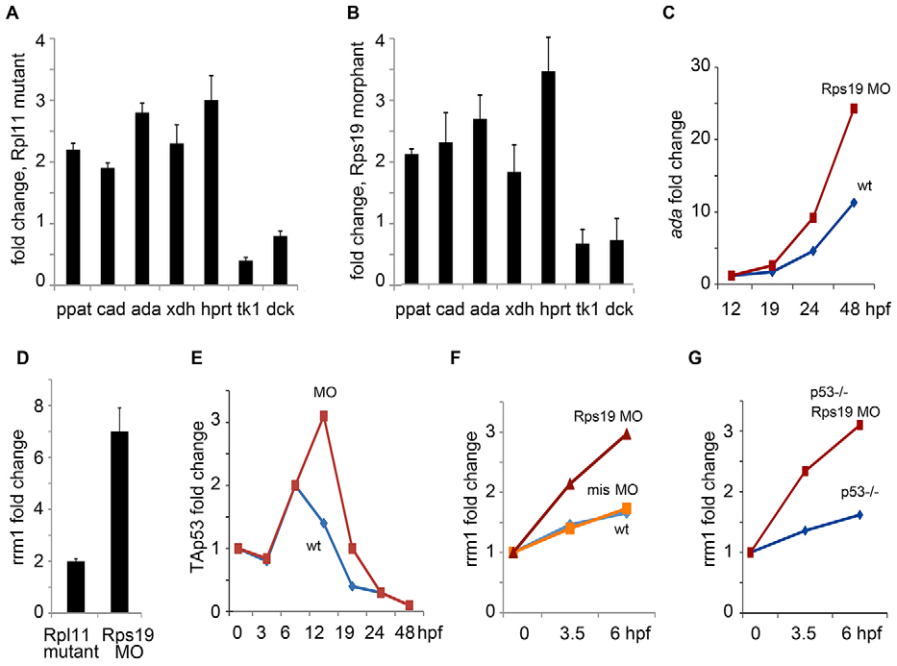

Fig. 2. The expression of genes involved in nucleotide metabolism changes in RP-deficient zebrafish. (A) *ada* and *xdh*, which are involved in nucleotide catabolism, were upregulated in Rpl11 mutants. Genes encoding enzymes involved in nucleotide biosynthesis, such as *ppat* (phosphoribosyl pyrophosphate amidotransferase) and *cad* (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase) were also upregulated. With regards to salvage enzymes, expression of *hprt* was increased. The levels of *tk1* and *dck*, which are expressed only in proliferating cells, were decreased. Gene expression was measured by using RT-qPCR analyses of embryos at 48 hpf. The fold change of expression in Rpl11 mutants was calculated relative to the expression in wild-type siblings. (B) Embryos that had been injected with a morpholino targeting Rps19 had similar changes in expression to those in Rpl11 mutants. RT-qPCR analyses were performed at 24 hpf. The fold change of gene expression in Rps19 morphants was calculated relative to expression of the gene wild-type embryos. (C) An increase in *ada* expression in Rps19-deficient embryos was detected, starting at 19 hpf. Red, embryos injected with a morpholino against Rps19 (Rps19 MO); blue, uninjected control (wt). Analyses were performed by using RT-qPCR. The fold change of expression was calculated relative to gene expression at 0 hpf. In panels C,E,F,G, qPCR was performed in triplicate and the means were used to generate the graphs. (D) *rrm1* was upregulated in Rpl11 mutants at 48 hpf and in Rps19 morphants at 24 hpf. Analyses were performed by using RT-qPCR. The fold change of gene expression for Rpl11 mutants was calculated relative to expression in wild-type siblings. The fold change in *ada* expression in Rps19 morphants was calculated relative to expression in wild-type embryos. In A,B,D, the means±s.d. are shown. (E) Timecourse of expression of a transactivating isoform of *tp53* (TAp53) in wild-type embryos (wt, blue) and in embryos that had been injected with Rps19 morpholino (MO, red). The results of RT-qPCR analyses are shown. The fold change of gene expression was calculated relative to expression at 0 hpf. (F) *rrm1* expression was increased at 3.5 and 6 hpf in embryos that had been injected with an Rps19-specific morpholino (red) but not in embryos that had been injected with a 5-base-mismatch morpholino (mis MO, orange). Expression of the gene in wild-type embryos is shown in blue. The results of RT-qPCR analyses are shown. The fold change of expression was calculated relative to expression of the gene at 0 hpf. (G) Upregulation of *rrm1* was also observed in *tp53*$^{-/-}$ embryos that had been injected with an Rps19-specific morpholino (Rps19 MO, red), expression in control *tp53*$^{-/-}$ embryos is in blue. The results of RT-qPCR analyses are shown. The fold change of expression was calculated relative to the expression at 0 hpf.

of DNA damage checkpoint pathways using corresponding antibodies and the phosphoflow technique. As a positive control for DNA damage, we treated our cells with etoposide (supplementary material Fig. S5). We found a consistent response that involved an increase in p53 phosphorylation at residues Ser15 and Ser37, phosphorylation of ATM at residue Ser1981, phosphorylation of 53BP1 at residue Ser1778, phosphorylation of Chk1 at residue Ser345 and phosphorylation of Chk2 at residue Thr68 (Fig. 4). Upregulation of these markers was proportional to RPS19 downregulation (supplementary material Fig. S6). These data indicate that RP-deficient cells function under conditions of chronic DNA damage.

Rps19- and Rpl11-deficient zebrafish have imbalanced dNTP pools

In order to analyze the consequences of changes in the expression of enzymes involved in nucleotide metabolism and activation of the DNA damage response in RP-deficient zebrafish, we measured concentrations of dNTPs in Rpl11 mutants and Rps19 morphants. We found that the levels of dTTP were increased in RP-deficient zebrafish embryos, dATP and dGTP were not significantly altered, and dCTP levels were slightly decreased compared with those of controls (Fig. 5A,B). Therefore, Rps19- and Rpl11-deficient zebrafish have imbalanced dNTP pools, suggesting that RP-deficient cells are incapable of maintaining a proper balance of nucleotides.

ATP levels are lower in RP-deficient zebrafish

It is known that upregulation of ADA results in ATP depletion (Chen and Mitchell, 1994) and ADA is upregulated in our DBA models. ATP is produced by glycolysis, which is suppressed in our DBA models due to p53 upregulation (Danilova et al., 2011). Therefore, both of these factors might affect ATP concentrations in RP-deficient zebrafish. We measured ATP levels in Rpl11 mutants and in zebrafish embryos that had been injected with a morpholino against Rps19, and we found decreased amounts of ATP in both models (Fig. 5C).

AMPK is activated in RP-deficient zebrafish

AMPK senses cellular energy levels (Hardie et al., 2012). Low ATP levels signal metabolic stress to AMPK, leading to its

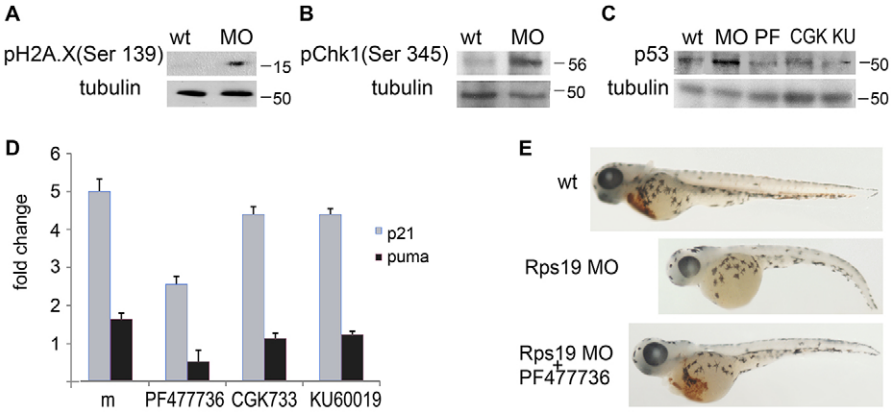

Fig. 3. RP-deficient zebrafish show activation of the DNA damage checkpoint pathway. (A) Phosphorylation of residue Ser139 of histone H2A.X was induced in embryos that had been injected with an Rps19-specific morpholino (MO). Western blotting was performed at 24 hpf. Staining of tubulin was used for a loading control. Molecular masses are shown on the right. Wt, wild type. (B) Phosphorylation of residue Ser345 in Chk1 kinase was increased in Rps19-deficient embryos (MO). Western blotting was performed at 24 hpf. (C) Embryos that had been injected with an Rps19 morpholino had increased levels of p53; the treatment of morphants with 3 nM of Chk1 inhibitor PF477736 (PF), 10 nM of the ATR and ATM inhibitor CGK733 (GCK), or 3 nM of the ATM inhibitor KU60019 (KU) reduced p53 levels. Western blotting was performed at 24 hpf. (D) Treatment of Rpl11 mutants with inhibitors of the ATR-ATM-Chk1 pathway resulted in downregulation of the p53 targets *p21* and *puma*. Gene expression was analyzed by using RT-qPCR. The fold change of gene expression was calculated relative to expression in wild-type siblings. Means±s.d. are shown. (E) Embryos that had been injected with a morpholino against Rps19 (Rps19 MO) had few red blood cells at 3.5 days post-fertilization. The treatment of Rps19 morphants with PF477736 partially rescued this hematopoietic defect.

phosphorylation at residue Thr172 and activation. We found that, in Rps19-deficient embryos, AMPK was phosphorylated at residue Thr172, indicating that it was activated (Fig. 5D). p53 is an AMPK target (Jones et al., 2005), suggesting that in RP-deficient cells, AMPK contributes to p53 activation. The activation of AMPK explains the decreased expression of genes that are involved in the biosynthesis of lipids and proteins in Rpl11-mutant zebrafish (Danilova et al., 2011).

Nucleoside salvage pathways are activated in RP-deficient zebrafish

DNA damage stimulates the production of dNTPs not only through the *de novo* pathway by upregulation of RNR, but also through activation of the salvage pathways. Specifically, ATM kinase activates DCK by phosphorylation on residue Ser74 in response to DNA damage (Smal et al., 2006; Yang et al., 2012), DCK then catalyzes phosphorylation of deoxynucleosides. In the absence of stress, only a small percentage of nucleotides in DNA originate from salvage pathways. In RP-deficient zebrafish, we observed increased incorporation of [^{3}H]deoxycytidine into DNA. Rpl11-mutant fish incorporated 2.5-fold more deoxycytidine into DNA than their wild-type siblings (Fig. 6A), whereas embryos that had been injected with a morpholino against Rps19 incorporated sevenfold more deoxycytidine in comparison with controls (Fig. 6B). These findings point to activation of the Dck enzyme and, furthermore, suggest activation of its upstream kinase ATM in RP-deficient zebrafish. Because RP-deficient zebrafish activated nucleotide salvage pathways, this suggests that nucleosides could be used as a treatment to decrease DNA damage in these zebrafish.

Exogenous nucleosides rescue RP-deficient zebrafish

Previously, exogenous supply of nucleosides has been shown to rescue cells experiencing oncogene-induced replication stress (Bester et al., 2011) and senescence (Mannava et al., 2013), and to downregulate p53 in the Caco-2 cell line (Ortega et al., 2011). Nucleosides, however, have never been used *in vivo* for this purpose. Therefore, we treated Rpl11-mutant and Rps19-morphant zebrafish with a mixture of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine at concentrations of 10, 25, 50 and 100 μM. The 50 μM concentration was chosen as being optimal for subsequent treatments. The efficiency of *rps19* downregulation by the morpholino was not affected by treatment with nucleosides (supplementary material Fig. S7). We also found that treatment with nucleosides downregulated the expression of p53 and p53 targets (Fig. 6C,D). For comparison, we used treatment with leucine, which has been shown to rescue animal and cellular DBA models (Jaako et al., 2012; Payne et al., 2012). *tp53* mRNA was only slightly decreased after nucleoside treatment, but at the protein level, the effect was more pronounced (Fig. 6E). Treatment with nucleosides also decreased the level of AMPK phosphorylation (Fig. 5D), indicating downregulation of its activity.

The expression of the genes encoding the enzymes involved in nucleotide metabolism, such as *rrm1* and *ada*, returned closer to normal levels after treatment with exogenous nucleosides (Fig. 6F).

Zebrafish that had been injected with a morpholino against Rps19 had high levels of apoptosis at 24-30 hpf, especially in the head and blood (Fig. 6G). Apoptosis is part of normal development, but it peaks at earlier stages, and only a few apoptotic cells are seen after 19 hpf in wild-type embryos. Treatment with nucleosides resulted in a reduction of the number of apoptotic cells (Fig. 6G; supplementary material Fig. S8). The treatment increased the amount of red blood cells in Rps19-deficient zebrafish (Fig. 6H); of 40 Rpl11-mutant embryos, 36 (90%) did not have blood cells at 80 hpf, and after the supply of exogenous nucleosides, blood was present in 29 (72%) embryos.

Treatment with nucleosides also improved survival and decreased morphological defects in RP-deficient zebrafish (supplementary material Fig. S9). These effects suggest that exogenous nucleosides provided sufficient dNTPs for DNA repair and reduced stress in RP-deficient zebrafish. A treatment comprising a mixture of adenosine, guanosine, uridine, cytidine and thymidine was slightly less efficient than treatment with deoxyribonucleosides (supplementary material Fig. S10), but also rescued RP-deficient zebrafish embryos, pointing to the activation of multiple nucleoside salvage pathways in these embryos.

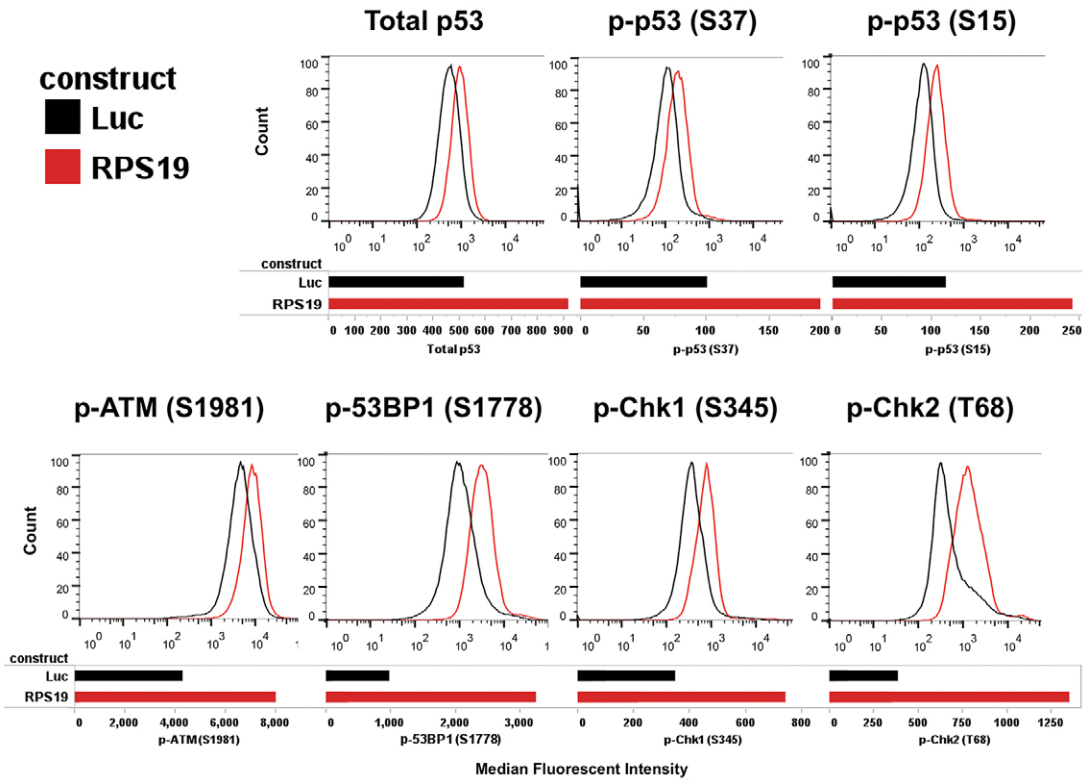

Fig. 4. Human RPS19 deficient cells have an activated DNA damage checkpoint. Intracellular phosphoflow cytometry of RPS19-deficient human CD34^{+} cells from fetal liver 5 days after transduction with lentiviral vectors expressing shRNA against *RPS19*. A vector targeting luciferase was used as a control. All vectors also expressed GFP. Histograms show GFP-positive gated cells from luciferase control (Luc, black) or RPS19-knockdown cells (RPS19, red). Bar graphs show the median fluorescent intensity obtained from the histograms for each antibody-fluorochrome conjugate. We used primary antibodies against phosphorylated p53 at residue S37 [p-p53 (S37)], total p53, phosphorylated p53 at residue S15 [p-p53 (S15)] that were conjugated to Alexa Fluor 647. We also used the following unconjugated antibodies: mouse against phosphorylated ATM at residue S1981 [p-ATM (S1981)], rabbit against phosphorylated 53BP1 at residue S1778 [p-53BP1 (S1778)], rabbit against phosphorylated Chk1 at S345 [p-Chk1 (S345)], and rabbit against phosphorylated Chk2 at T68 [pChk2 (T68)] with a secondary labeling step using either anti-mouse IgG antibody conjugated to PE or anti-rabbit IgG antibody conjugated to PE.

DISCUSSION

Our data suggest that failure of ribosome biogenesis in RP-deficient cells results in upregulation of the ATR-ATM-Chk1-Chk2-p53 pathway (Fig. 7). Upregulation of *rrm1* in the early stages of the development of RP-deficient zebrafish suggests that activation of ATR takes place soon after the start of zygotic transcription, before p53 upregulation. The exact mechanism of ATR and ATM upregulation in RP-deficient cells requires further studies, especially the role of aberrant processing of pre-rRNA. Several reports have shown that pre-rRNA processing starts before its transcription is finished (Huertas and Aguilera, 2003; Osheim et al., 2004; Schneider et al., 2007). The architectural organization of ribosomal (r)DNA is very complex, and obstructions of pre-rRNA processing have been suggested to interfere with its transcription and ultimately with replication (Bermejo et al., 2012). In addition, the increased demand for rRNA in RP-deficient cells might hyper-activate rDNA units, increasing the probability of topological stress occurring that would result in fork reversal and R-loop accumulation (Bermejo et al., 2012). Transcriptional defects might lead to transcription elongation impairment, increased formation of DNA-RNA hybrids, breaks and hyper-recombination, which will impair replication (Bermejo et al., 2012; Huertas and Aguilera, 2003). Similarly, mRNA splicing happens co-transcriptionally, and splicing defects increase the formation of DNA double-strand breaks (Li and Manley, 2005; Wahba et al., 2011).

The ATR-Chk1 axis has recently been implicated in cell-cycle arrest that is induced by a prolonged treatment with low levels of actinomycin D, a selective inhibitor of rRNA synthesis (Ma and Pederson, 2013). The exact mechanism of ATR induction by this treatment was not uncovered.

The phosphorylation of p53 at residues Ser15 and Ser37 observed in RPS19-deficient human CD34^{+} fetal liver cells is probably performed by multiple protein kinases, including ATR, ATM, Chk1 and Chk2, the activation of which was observed in these cells. Phosphorylation at these sites is known to stabilize and activate p53, but other mechanisms could also contribute to p53 activation (Ashcroft et al., 2000; Lane and Levine, 2010).

Changes in the expression of enzymes involved in nucleotide metabolism in models of DBA are consistent with the requirement of RP-deficient cells to catabolize the defective rRNA, produce more nucleotides to make new rRNAs and to make increased amounts of dNTPs sufficient for DNA repair. Activity of the salvage enzyme DCK is regulated at the post-translational level. DCK is activated by phosphorylation in response to DNA damage (Smal et al., 2006; Yang et al., 2012), and increased incorporation of radioactive deoxycytidine into the DNA of RP-deficient zebrafish suggests that Dck activity was increased in our models.

Decreased or imbalanced dNTP pools can be a source of DNA damage (Bester et al., 2011; Hastak et al., 2008; Kunz et al., 1994; Mannava et al., 2013). In RP-deficient zebrafish, we found changes in the dNTP pool composition, most notably, a significant increase in dTTP levels. Elevated dTTP can induce replication stress through the induction of a relative dCTP deficiency (Austin et al., 2012).

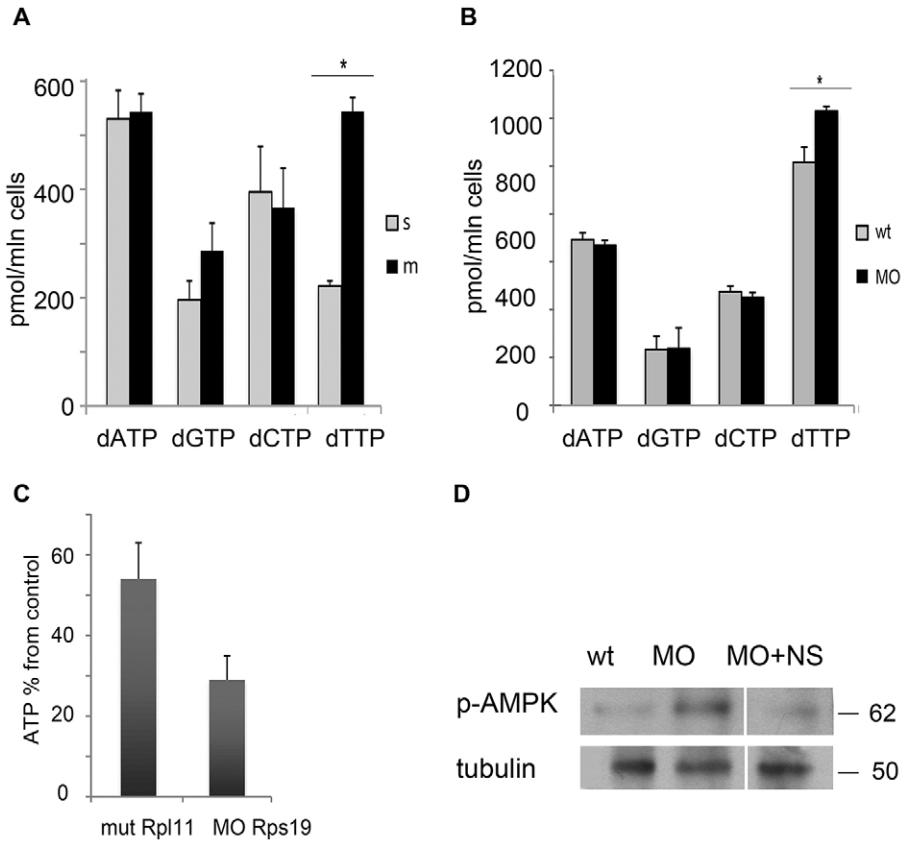

Fig. 5. RP-deficiency results in an imbalanced dNTP pool, ATP depletion and AMPK activation. (A) Levels of dNTPs in Rpl11 mutants (m, black) and wild-type siblings (s, gray) at 48 hpf. dTTP was increased in Rpl11 mutants. Student's *t*-test, **P*<0.01. (B) At 18 hpf, dTTP was increased in embryos that had been injected with an Rps19 morpholino (MO, black). Student's *t*-test, **P*<0.05. wt (gray), wild-type embryos. (C) The ATP levels in Rpl11 mutants (mut Rpl11) and Rps19 morphants (MO Rps19) were decreased. The ATP level is shown relative to that of siblings for Rpl11 mutants, and relative to that of wild-type embryos for Rps19 morphants. ATP levels were measured 48 hpf in mutants and 24 hpf in morphants. In A-C, the means±s.d. are shown. (D) Rps19 deficiency led to the activating phosphorylation of AMPK at Thr172 (p-AMPK), as analyzed by western blotting. Treatment with nucleosides reduced AMPK phosphorylation. wt, wild-type embryos; MO, embryos that had been injected with a morpholino against Rps19. Molecular masses are indicated on the right.

Similarly, disproportional increases in dCTP and decreases in dTTP have been shown to result in replication fork collapse and genomic instability (Sánchez et al., 2012). Therefore, although an imbalance of the dNTP pool can be a result of the efforts of a cell to compensate for ribosomal stress and DNA damage, such an imbalance can induce further damage.

Upregulation of the ADA enzyme is one of the most important changes in RP-deficient cells because ADA can deplete the ATP pool and lead to AMPK activation. Indeed, we found decreased ATP levels and increased AMPK phosphorylation at residue Thr172 in Rps19-deficient zebrafish. Activated AMPK can phosphorylate p53 at residue Ser15, contributing to the activation of p53 by the ATR-ATM pathway.

One of the consequences of p53 activation is the suppression of glycolysis (Matoba et al., 2006), which we also observe in Rpl11-mutant fish (Danilova et al., 2011). When p53 expression is low, such as in tumor cells, glycolysis is increased to provide biosynthetic intermediates for fast growing tumor cells, this is known as the Warburg effect. Glycolysis is also increased in normal cells during proliferation (Lunt and Vander Heiden, 2011). One of the main cellular energy expenditures is the production of nucleotides for RNA and DNA. DNA synthesis is strongly dependent on glycolysis, thus the S phase of the cell cycle is accompanied by a surge of glycolysis (Lunt and Vander Heiden, 2011), and suppression of glycolysis results in decreased production of ATP and precursors for biosynthesis (Lunt and Vander Heiden, 2011). Decreased production of ATP due to suppressed glycolysis would synergize with overactivated ADA in decreasing the ATP pool in RP-deficient cells and activating AMPK.

Activated AMPK induces energy saving measures in the cell, which includes inhibition of translation. Pre-rRNA processing requires hundreds of various proteins, so when translation is inhibited, fewer such proteins are produced and supplied to ribosomes (Talkish et al., 2012). This might be one of the reasons why treatment with leucine improves anemia in animal models of DBA (Jaako et al., 2012; Payne et al., 2012). However, overactivation of mTOR, especially for prolonged periods of time, might lead to cellular transformation.

Here, exogenous nucleosides rescued RP-deficient zebrafish. In healthy cells, the dNTP pool is limited and, in response to DNA damage, cells activate RNR to produce more dNTPs. However, when cells fail to produce a balanced pool through *de novo* pathways, salvage pathways might become essential. We found that RP-deficient zebrafish incorporated 2.5- to 7-fold more exogenous deoxycytidine into their DNA than controls. Because proliferation is arrested in RP-deficient cells, the increase must be due to DNA repair. In support of the ability of salvage pathways to compensate for the deficiency of *de novo* nucleotide biosynthesis, exogenous deoxynucleosides have been shown to facilitate DNA repair during ribonucleotide reductase blockade in cancer cells (Kunos et al., 2011) and to reduce replication stress and DNA damage that is induced by oncogenes (Bester et al., 2011; Burrell et al., 2013; Mannava et al., 2013).

Previous studies of nucleotide biosynthesis suggest that only a small fraction of dietary nucleosides and nucleotides become incorporated into DNA. Our data, together with recent data from other labs, suggest that exogenous dietary sources might become vital in a state of genotoxic stress. Blood cells depend more on salvage pathways for the production of dNTPs than other cells (Austin et al., 2012); thus, they are more vulnerable to the adverse effects of a stressed metabolism. RP-deficient red blood cells have increased activation of p53 in comparison to other cell types (Dutt et al., 2011); therefore, they might benefit the most from exogenous nucleosides.

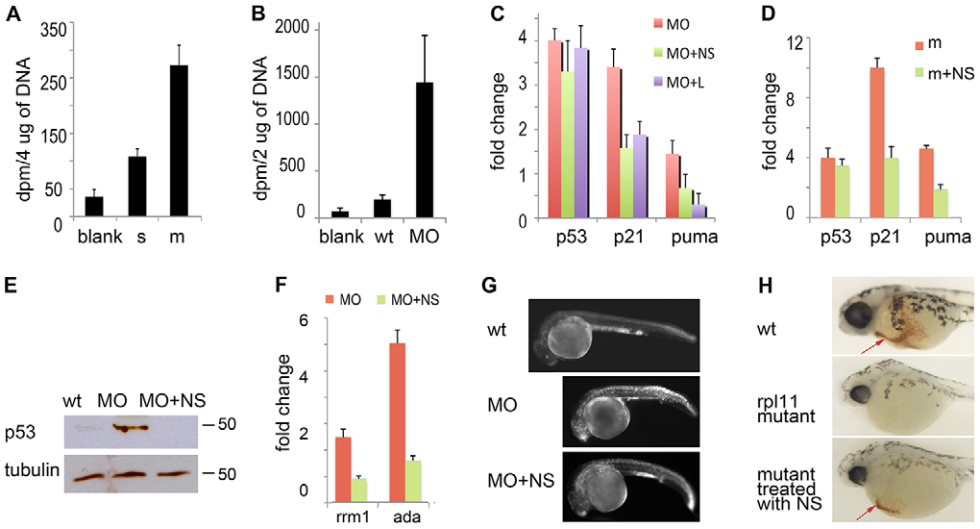

Fig. 6. Rescue of RP-deficient zebrafish with exogenous nucleosides. (A) At 48 hpf, Rpl11 mutants incorporated more [^{3}H] deoxycytidine into DNA than their wild-type siblings (P<0.002). The count adjusted to 4 µg of genomic DNA is shown. (B) At 48 hpf, embryos that had been injected with a morpholino against Rps19 incorporated more [^{3}H]deoxycytidine into DNA than wild-type embryos (P<0.005). The count adjusted to 2 µg of genomic DNA is shown. (C) Supplementation with exogenous nucleosides (NS) decreased the expression of *tp53*, *p21* and *puma* in zebrafish embryos that had been injected with a morpholino against Rps19 (MO). L, treatment with 0.5 mg/ml leucine was used for comparison. RT-qPCR analyses were performed at 22 hpf. The fold change in gene expression was calculated relative to expression in wild-type embryos. (D) The addition of exogenous nucleosides reduced the expression of *tp53*, *p21* and *puma* in Rpl11-mutant zebrafish embryos. RT-qPCR analyses were performed at 48 hpf. The fold change in expression was calculated relative to expression in wild-type siblings. (E) Nucleoside treatment decreased the level of p53 protein in Rps19-deficient zebrafish embryos. 22 hpf. Western blot. (F) The addition of exogenous nucleosides normalized the altered expression of genes that are involved in nucleotide metabolism – *rrm1* and *ada*. RT-qPCR analyses were performed at 22 hpf. In A-D and F, means±s.d. are shown. (G) Treatment with nucleosides decreased the amount of apoptosis. Embryos were injected with a morpholino against Rps19, and half of these were treated with nucleosides. Representative images are shown of fish at 28 hpf. Acridine orange staining shows fewer apoptotic cells in morphants that had been treated with nucleosides. (H) The addition of exogenous nucleosides increased the amount of red blood cells in Rpl11 mutants. Representative images are shown of O-dianizidine staining of fish at 80 hpf. Rpl11 mutants were confirmed by genotyping after staining. Arrows point to erythroid cells.

Our findings suggest that individuals with DBA can benefit from nucleoside supplements. Nucleoside mixtures are safe, and many infant formulas already include nucleosides and nucleotides (Singhal et al., 2010). Nucleoside supplementation in enteral nutrition has been shown to improve outcomes in physiologically stressed individuals (Hess and Greenberg, 2012).

Nucleoside supplementation might be beneficial not only in DBA but in many other conditions that involve activation of DNA damage checkpoints. For example, we found that treatment with nucleosides improved the survival of irradiated zebrafish (N.D. and S.L., unpublished data). Many genetic disorders are associated with the activation of DNA damage checkpoints; therefore, nucleoside supplementation might find wide therapeutic application.

MATERIALS AND METHODS

Zebrafish

Zebrafish (*Danio rerio*) lines used: AB, *rpl11*^{hi3820bTg} and *tp53*$^{zdf1/zdf1}$. Embryos were obtained by natural spawning. The University of California, Los Angeles Animal Committee approved the study.

Genotyping zebrafish embryos with a mutation in *rpl11*

Individual zebrafish embryos were placed into 20 µl of 50 mM NaOH and heated for 20 minutes at 95°C to dissolve them. The lysates were neutralized with 2 µl of 1 M Tris-HCl buffer, pH 8 and 1 µl of each lysate was used in PCR analyses. Genotyping was performed with primers that had been used in the initial screen (Amsterdam et al., 2004); forward primer 5′-CTCTTCTAGTGATCAAACATGGCG-3′ from the first exon, and reverse primer 5′-GCTAGCTTGCCAAACCTACAGGT-3′ corresponding to the viral insertion in the first intron. The forward primer described above and a reverse primer from the first intron 5′-TGCTCATCCGGAATCTGTACA-3′ (corresponding to the unmodified genomic sequence) were used in PCR analyses to discriminate between homozygous and heterozygous embryos.

Human fetal liver cells

Human fetal liver tissues were obtained from Advanced Bioscience Resources (Alameda, CA). Cells were sorted for CD34$^+$ using MACS cell separation (Miltenyi Biotec, Auburn, CA) and then grown in x-Vivo15 medium (Lonza, Basel, Switzerland) containing 10% fetal bovine serum, 50 ng/ml of FLT-3 ligand, thrombopoietin and stem cell factor, and 20 ng/ml of IL-3 and IL-6. Cells were transduced with lentivirus containing shRNA against RPS19 or luciferase, sorted for green fluorescent protein (GFP) 72 hours later and then harvested at 5 days post-transduction.

RT-qPCR

RNA for reverse-transcription quantitative PCR (RT-qPCR) was prepared using Trizol (Invitrogen, Carlsbad, CA) from 30-40 embryos; 2 µg of RNA was then used for reverse transcription with the random hexamer primers. PCR was performed in triplicates using iQ SYBR Green Super Mix. Primers are shown in supplementary material Table S1. Levels of mRNA were normalized to β-actin and calculated by using the Ct method.

Morpholinos

At the one-cell stage, 3 ng of the following morpholinos were injected – Rps19, a specific morpholino targeting the exon 3 splicing site 5′-GCTT-CCCCGACCTTTCAAAAGACAA-3′; and 5-base-mismatch: 5′-GATTCC-TCGAACTCTCAATAGACAA-3′ (Gene Tools, Philomath, OR).

Flow cytometry of human fetal liver cells

CD34$^+$ cells were stained with Aqua-Amine (Life Technologies, Carlsbad, CA), fixed in 1.6% paraformaldehyde and permeabilized with 100% methanol. Staining was performed with antibodies against phosphorylated

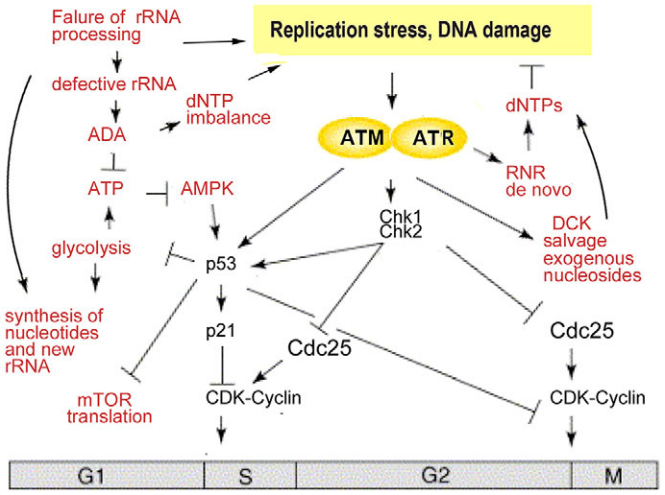

Fig. 7. Schematic of the changes that are induced in cells by a deficiency of RPs. The nascent rRNA cannot be processed correctly in the absence of some RPs, which hypothetically leads to replication stress and DNA damage. Alongside this stress, global changes in nucleotide metabolism arise from (i) the necessity to catabolise defective rRNAs; (ii) the need to produce more rRNAs; (iii) the need to produce more dNTPs for DNA repair; (iv) the decreased availability of ATP and precursors for biosynthesis, caused by suppressed glycolysis; and (v) ADA activity destroying ATP. Altogether, metabolism perturbations lead to dNTP imbalance and ATP depletion. These factors can further exacerbate replication stress and DNA damage. The pattern of p53 phosphorylation that we observed is consistent with inputs from several kinases from the ATR-ATM-Chk1-Chk2 pathway. In addition, the activation of AMPK, caused by low ATP levels, can contribute to p53 activation. During replication stress and DNA damage, ATR kinase activates RNR (*de novo* pathway) to increase production of dNTPs, which are necessary for DNA repair. At the same time, ATM kinase activates the salvage enzyme DCK to produce more dNTPs through salvage pathways. Salvage pathways are barely used in healthy cells, but for stressed cells they are much more important, as illustrated by the increased incorporation of radioactively labeled deoxycytidine into the DNA of zebrafish that are deficient in RPs. Exogenous nucleosides rescue cells that are deficient in RPs by decreasing replication stress through providing additional dNTPs for DNA repair.

p53 (at residue S37) conjugated to Alexa Fluor 647 (BD Biosciences, San Jose, CA), p53 conjugated to Alexa Fluor 647, phosphorylated p53 (at residue S15) conjugated to Alexa Fluor 647, or unconjugated antibodies to phosphorylated ATM (at residue S1981), phosphorylated 53BP1 (at residue S1778), phosphorylated Chk1 (at residue S345) and phosphorylated Chk2 (at residue T68) (Cell Signaling Technology, Beverly, MA). The secondary antibodies used were conjugated to phycoerythrin (PE) and were against mouse IgGs or rabbit IgGs (Life Technologies, Carlsbad, CA). An LSR II flow cytometer (BD Biosystems, San Jose, CA) was used.

dNTP measurement

Fifty embryos were homogenized in 1 ml of ice-cold 60% methanol, maintained overnight at −20°C, boiled for 3 minutes, centrifuged for 15 minutes at 17,000 ***g***, evaporated in a SVC100H SpeedVac Concentrator (Savant, Waltham, MA), resuspended in 100 µl of water and centrifuged for 15 minutes at 17,000 ***g***, 5 µl of the supernatant was then used with Klenow Fragment DNA polymerase and [^{3}H]dNTP, as described previously (Mathews and Wheeler, 2009).

ATP measurement

Twenty embryos were homogenized in 150 µl of 0.5% trichloroacetic acid, neutralized by 1 M Tris-acetate buffer, pH 7.75 and then diluted to 1.5 ml. Of this solution, 50 µl was mixed with 50 µl of rL/L reagent from ENLITEN ATP bioluminescence assay system (Promega, Madison, WI), and light output was measured by using a luminometer.

Western blot

At 22-24 hpf, 30-40 embryos per group were lysed, and 30 µg of protein was separated on a 12% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with rabbit antibodies against phosphorylated H2A.X (at residue Ser139), phosphorylated Chk1 (at residue Ser345), phosphorylated AMPK (at residue Thr172) (Cell Signaling, Beverly, MA), or p53 (AnaSpec, Fremont, CA) followed by a horseradish peroxidase (HRP)-conjugated antibody against rabbit IgGs (Santa Cruz Biotechnology, CA). The membrane was stripped and re-probed with a mouse antibody against α-tubulin (Sigma) followed by a HRP-conjugated antibody against mouse IgGs (Santa Cruz Biotechnology). SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) was used for detection.

Deoxycytidine incorporation into DNA

At 10 hpf, 10 µl of 2′-Deoxycytidine[5-3H(N)], containing 16.3 Ci/mmol (Movarek Biochemicals, Brea, CA), was added to 100 embryos in 25 ml of water. At 48 hpf, genomic DNA was prepared from 25 embryos using DNA purification kit (Zymo Research, Irvine, CA). DNA was eluted in a 50 µl volume, and 40 µl of this solution was added to a liquid scintillator and the radioactivity bound to DNA was measured.

Treatments

Deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine were prepared in water at a concentration of 2.5 mM and then added to fish water to final concentrations of 10-100 µM. The ATM inhibitor KU60019, the ATM and ATR inhibitor CGK733 and the Chk1 inhibitor PF477736 were dissolved in dimethylsulfoxide to 30 mM and added to fish water at final concentrations of 10 nM, 100 nM and 20 nM, respectively.

Statistics

Each experiment was repeated at least twice. In most cases, the results of a representative experiment are shown. Data are presented as the means of at least three measurements±s.d.; Student's *t*-test was used for comparisons of a variable between two groups.

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Competing interests

The authors declare no competing financial interests.

Author contributions

N.D. and S.L. conceived and designed the experiments; N.D., E.B., T.M.C., E.D., D.N. and Y.K. performed experiments; N.D., S.L., K.M.S., B.E.G. and K.R. analyzed results; N.D., S.L., K.M.S. and K.R. wrote the paper.

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Supplementary material

Supplementary material available online at
http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.015495/-/DC1

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From: "Experimental Hematology" <exphem@elsevier.com>
Subject: Experimental Hematology Editorial Decision
Date: October 15, 2014 8:07:24 AM PDT
To: shuolin@ucla.edu

Ref.: Ms. No. 14- 159
Aberrant regulation of innate immunity in zebrafish and cellular models of Diamond Blackfan Anemia
Experimental Hematology

Dear Dr. Lin,

Thank you for submitting your manuscript for consideration. Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you feel that you can address the concerns of the reviewers, we would invite you to submit a revised version of your paper for further consideration. Please note that this does not guarantee publication.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please 1) respond to each criticism of the reviewers, point by point, indicating precisely (using red type) where in the manuscript the changes have been made in response to each critique, 2)give reasons for any suggested changes that were not implemented (Note that failure to address a reviewer's concern without good reason may be cause to reject the manuscript), and 3)identify additional changes made or sections shortened to reduce the manuscript length.

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Response to Reviewers (mandatory)

This should be a separate file labeled "Response to Reviewers" that carefully addresses, point-by-point, the issues raised in the comments appended below. You should also include a suitable rebuttal to any specific request for change that you have not made. Mention the page, paragraph, and line number of any revisions that are made.

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We cannot accommodate PDF manuscript files for production purposes. We also ask that when submitting your revision you follow the journal formatting guidelines. Figures and tables may be embedded within the source file for the submission as long as they are of sufficient visual quality. For any figure that cannot be embedded within the source file (such as *.PSD Photoshop files), the original figure needs to be uploaded separately. Refer to the Guide for Authors for additional information.

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Highlights consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See the

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Yours sincerely,

David Traver, PhD
Associate Editor
Experimental Hematology

Reviewers' comments:

Reviewer #1: In this manuscript, Danilova et al use a morpholino-based strategy to model RPS19 deficient Diamond Blackfan anemia (DBA) in zebrafish embryos and demonstrate an RNA expression profile consistent with upregulated innate immunity and inflammation, including activation of the complement cascade. rps19 morphant embryos display anemia as exhibited by reduced o-dianisidine staining that can be partially restored by inhibiting complement, tlr3 or activin.
This paper exploits the ease of transient gene knockdown in the zebrafish, and the temporal separation of innate and adaptive immune system development to examine in vivo potential pathways at play in the pathogenesis of DBA caused by ribosomal protein disruption. While the data presented are of value and may even provide novel therapeutic targets in DBA, there are a number of issues with the manuscript in its current form that preclude publication.

Major comments:
1. While morpholinos provide a rapid and efficient means of gene knockdown and the particular morphant phenotype employed in this paper has been previously published, confirmation of the phenotype using CRISPR-based technology is now readily available and should be considered for inclusion as validation.
2. The majority of the data presented is a series of RT-PCR expression assays that are difficult to follow as written. The text should be supplemented with either a table or schema (or preferably both), highlighting the pathways connecting the various factors being examined and summarizing the expression levels observed.
3. Gene expression data is provided for whole embryo but no data is provided on the cell populations in which these genes are differentially expressed. For example, are increased tlr1, 3 and 9 expression levels found specifically in immune cells? Whole mount in situ hybridization for the genes of interest should be performed to complement the PCR data. Co-localization studies with known markers of specific cell populations (eg. mpeg for macrophages) would be particularly informative.
4. TGF-beta signaling data is interesting but most of the evidence is circumstantial. Can more direct evidence for TGF-beta signaling be provided?
5. Controls should be consistent in all figures. Figures 1 and 2 and Figure 4A and B show wild type embryos as controls, while Figure 4C and D show scrambled morpholino, which is probably a better control and should be included in all figures.
6. Numbers of embryos used and number of experimental replicates should be included for each figure.
7. In addition to including numbers of embryos and experiments in Figure 6, o-dianisidine staining needs to be quantified according to some numeric or descriptive scale and non-parametric methods employed for statistical analysis. The x-axis in Figure C is also misleading as all groups were treated with a morpholino +/- rescue. Data on SCR morpholino-injected embryos following treatments should also be shown and quantified.

Minor comments:

1. It is unclear why the rpl11 mutant is mentioned in the Methods section as it appears that no experiments were conducted using these fish. Thus, the paragraph,"We also used mutant rpl11hi3820bTg[36], which was characterized in our lab [11]. We combined these two models, one gene from small ribosomal subunit, another from large ribosomal subunit with their deficiency created by different mechanisms to study common features of RP deficiency" should be removed from the results section and references to these studies removed from the Results section and included in the Discussion.
2. In the Introduction, paragraphs 2-4 should be combined into a single paragraph.
3. Concentration for NaOH is missing in the Methods.
4. Acetyl acid should be acetic acid.
5. μ should be used rather than u for μM or μL.
6. Labeling of axes in the RT-PCR figures should be consistent throughout the manuscript.
7. Figure 6C legend "treatment with TLR3 inhibitor, SB431542 activin inhibitor and SB290157 complement inhibitor" should say "or" rather than "and".
8. There are a number of grammatical errors throughout the manuscript, such as:

"In Rps19-deficient zebrafish, we found increase in the expression of factor…" - which should say "an increase" and "Activation of complement system…" - which should say "of the complement system". The manuscript would benefit from proofreading to correct these errors.

Reviewer #2: Aberrant regulation of innate immunity in zebrafish and cellular models of Diamond Blackfan
Anemia

The authors describe deregulation of toll-like receptor expression at the RNA level and inflammatory mediators in Rps19 morphant zebrafish . They compare this to historical gene expression profiles form Rpl11 deficient zebrafish where similar effects are observed. They demonstrate that some of these findings are also seen in human cells with knockdown of RPS19 (or in lymphoblastoid cells from a patient with DBA). Finally they are able to rescue some of the effects using inhibitors of the activated pathways. This is an interesting study and a useful adjunct to the current literature (including just published from these groups on related subject matter) suggesting immune deregulation in DBA. The presentation of the data is a not optimal (use of different time points for no clear reason and poor labeling of figures) as well as the absence of any data on protein expression (obviously challenging in fish but not so in the human cells) to confirm the findings also hold at the
protein level. This is of particular importance because there is potentially an impact on protein translation from the knockdown of a ribosomal protein. What is the level of knockdown of Rps19 in this system since patients have 50% levels of Rps19. Why are the results for Rpl11 fish not also validated by qPCR? Other more specific comments below

Figure 1

Why is the data from the mismatch morpholino not shown. This is clearly the most relevant control particularly given increasing concerns over morpholino toxicity.

Why are there qPCRs at different time points? Is this related to the time at which these genes are expressed? Also where are they expressed since ultimately the hypothesis is the expression of these genes are responsible in part for the erythroid phenotype so are they expressed in red blood cells or other hematopoietic tissue.

In B there seems to be 2 shades of grey on the control bar. Do these represent anything different?

C is missing controls - what is the effect of p53 on the expression of tlr3 in wt (or more appropriately mismatch morpholino injected embryos)

Figure 2

Same comment as above - why are they not showing the mm morpholino controls

The labeling of the experiments is poor e.g. in B and E, one column is p53-/- the other Rps19 MO but presumably this is also p53-/- but also with knockdown of Rps19. In some graphs only the fold change is shown and in others the normalized control bar is also shown. This should be consistent

What is the time point for A, and again why the use of different time points?

Figure 3

Why are the 2 graphs shown at different time points

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Response to Reviewers

Reviewers' comments:

Reviewer #1: In this manuscript, Danilova et al use a morpholino-based strategy to model RPS19 deficient Diamond Blackfan anemia (DBA) in zebrafish embryos and demonstrate an RNA expression profile consistent with upregulated innate immunity and inflammation, including activation of the complement cascade. rps19 morphant embryos display anemia as exhibited by reduced o-dianisidine staining that can be partially restored by inhibiting complement, tlr3 or activin. This paper exploits the ease of transient gene knockdown in the zebrafish, and the temporal separation of innate and adaptive immune system development to examine in vivo potential pathways at play in the pathogenesis of DBA caused by ribosomal protein disruption. While the data presented are of value and may even provide novel therapeutic targets in DBA, there are a number of issues with the manuscript in its current form that preclude publication.

Major comments:

1. While morpholinos provide a rapid and efficient means of gene knockdown and the particular morphant phenotype employed in this paper has been previously published, confirmation of the phenotype using CRISPR-based technology is now readily available and should be considered for inclusion as validation.

Response: We use both mutants and morphants depending on the goals of a particular study. We recently published data on an Rps19 mutant (Zhang et al, 2014), which exhibits a phenotype similar to Rps19 morphants. Findings in Rps19 morphants presented in this paper are supported by similar findings in Rpl11 mutants.

2. The majority of the data presented is a series of RT-PCR expression assays that are difficult to follow as written. The text should be supplemented with either a table or schema (or preferably both), highlighting the pathways connecting the various factors being examined and summarizing the expression levels observed.

Response: Thank you for your suggestion. We have now made a schema to make it clearer.

3. Gene expression data is provided for whole embryo but no data is provided on the cell populations in which these genes are differentially expressed. For example, are increased tlr1, 3 and 9 expression levels found specifically in immune cells? Whole mount in situ hybridization for the genes of interest should be performed to complement the PCR data. Co-localization studies with known markers of specific cell populations (eg. mpeg for macrophages) would be particularly informative.

Response: TLR receptors are conserved and well characterized in several species including zebrafish. For example, TLR3 is known to be expressed predominantly in dendritic cells but also in some epithelial and other cell types, which are present in all tissues in high numbers. This explains the pattern of tlr3 expression shown on ZFIN in the whole organism. Specific details of TLRs expression are outside the scope of this paper.

4. TGF-beta signaling data is interesting but most of the evidence is circumstantial. Can more direct evidence for TGF-beta signaling be provided?

Response: More detailed study of TGFb signaling in RP-deficient zebrafish is currently underway in the Shuo Lin lab and will be the subject of a separate publication.

5. Controls should be consistent in all figures. Figures 1 and 2 and Figure 4A and B show wild type embryos as controls, while Figure 4C and D show scrambled morpholino, which is probably a better control and should be included in all figures.
Response: Fig4C and 4D show a scrambled shRNA control for shRNA inhibition of RPS19 in human cells, not scrambled morpholino. In zebrafish, scrambled morpholino for rps19 was reported previously and had no effect on embryo development up to 13 ng/embryo (Danilova et al. 2008). For this reason, in our studies, we often use wild type control for older embryos, and double check with a scrambled control mostly in early developmental stages, to exclude any effect of oligo injection (See Danilova et al. Dis Mod Mech 2014). However, we agree with the reviewer's point and have added information about the specificity of our morpholino. An additional indicator of specificity of innate immune changes that we observed in rps19 morphants is that we saw analogous changes in rpl11 mutants and in human cells with RPS19 deficiency. Moreover, microarray data obtained from cells of DBA patients further support our results.

6. Numbers of embryos used and number of experimental replicates should be included for each figure.
Response: We apologize for this oversight and have now added this information.

7. In addition to including numbers of embryos and experiments in Figure 6, o-dianisidine staining needs to be quantified according to some numeric or descriptive scale and non-parametric methods employed for statistical analysis. The x-axis in Figure C is also misleading as all groups were treated with a morpholino +/- rescue. Data on SCR morpholino-injected embryos following treatments should also be shown and quantified.
Response: Figure 6B shows a representative control and treated embryos, which is described in the legend. We provided in the figure legend numerical values for Fig 6C data, including the standard deviation. We clarified in the legend that all groups were injected with morpholinos. As mentioned above, embryos injected with the scrambled morpholino do not differ from wild type, and effects of their treatments cannot be quantified.

Minor comments:

1. It is unclear why the rpl11 mutant is mentioned in the Methods section as it appears that no experiments were conducted using these fish. Thus, the paragraph,"We also used mutant rpl11hi3820bTg[36], which was characterized in our lab [11]. We combined these two models, one gene from small ribosomal subunit, another from large ribosomal subunit with their deficiency created by different mechanisms to study common features of RP deficiency" should be removed from the results section and references to these studies removed from the Results section and included in the Discussion.
Response: We indeed mostly used the rpl11 mutant as a reference point to microarray data reported in our previous paper, but in this paper, we added data on il6 expression, which was not present in the microarray results. We do not show this as a figure but mention it the text. (Results, section Inflammatory pathways, third paragraph: RT-qPCR analysis of gene expression of Rpl11 mutant showed 14 fold (sd 2.1) upregulation of *il6* in mutants in comparison to siblings at 48 hpf.)

2. In the Introduction, paragraphs 2-4 should be combined into a single paragraph.
Response: We agree and have done this.

3. Concentration for NaOH is missing in the Methods.

Response: This has been corrected

4. Acetyl acid should be acetic acid.
Response: This has been corrected.

5. μ should be used rather than u for μM or μL.
Response: We have corrected this.

6. Labeling of axes in the RT-PCR figures should be consistent throughout the manuscript.
Response: We have revised the figures.

7. Figure 6C legend "treatment with TLR3 inhibitor, SB431542 activin inhibitor and SB290157 complement inhibitor" should say "or" rather than "and".
Response: We have revised this.

8. There are a number of grammatical errors throughout the manuscript, such as: "In Rps19-deficient zebrafish, we found increase in the expression of factor…" - which should say "an increase" and "Activation of complement system…" - which should say "of the complement system". The manuscript would benefit from proofreading to correct these errors.
Response: We apologize and have now corrected the grammatical errors in the manuscript.

Reviewer #2:
The authors describe deregulation of toll-like receptor expression at the RNA level and inflammatory mediators in Rps19 morphant zebrafish. They compare this to historical gene expression profiles form Rpl11 deficient zebrafish where similar effects are observed. They demonstrate that some of these findings are also seen in human cells with knockdown of RPS19 (or in lymphoblastoid cells from a patient with DBA). Finally they are able to rescue some of the effects using inhibitors of the activated pathways. This is an interesting study and a useful adjunct to the current literature (including just published from these groups on related subject matter) suggesting immune deregulation in DBA.

1. The presentation of the data is a not optimal (use of different time points for no clear reason and poor labeling of figures) as well as the absence of any data on protein expression (obviously challenging in fish but not so in the human cells) to confirm the findings also hold at the protein level. This is of particular importance because there is potentially an impact on protein translation from the knockdown of a ribosomal protein. What is the level of knockdown of Rps19 in this system since patients have 50% levels of Rps19.
Response: In human cells, when RPS19 mRNA expression is decreased by 80-90%, the decrease in protein level is ~50% from control (Danilova,2014). We included a reference for these data. Lower degree of protein downregulation is expected, since some proteins are already included into ribosomes. In rpl11 mutants, we also observed less effect on protein levels (Danilova 2011). In Rps19 morphants, the degree of mRNA knockout at 3 ng dose is 80-90% (Danilova 2008). There are no suitable antibodies against zebrafish Rps19.

2. Why are the results for Rpl11 fish not also validated by qPCR?
Response: We mostly use data on Rpl11 fish as a reference to microarray data that have been

already published; most of them have been validated by qPCR (Danilova 2011). We added this information to the text. In this study, we showed increased expression of il6 in Rpl11 mutants by qRCR.

Other more specific comments below

Figure 1
Why is the data from the mismatch morpholino not shown? This is clearly the most relevant control particularly given increasing concerns over morpholino toxicity.
Response: The morpholino used in this study was previously reported in two papers (2008 Blood, 2014 Dis Mod Mech) and its specificity was confirmed by RNA rescue, application of a translational morpholino, and application of mismatch morpholino. Mismatch morpholino was injected to 13 ng/embryo and no difference from non-injected wild type embryos was detected. We added information about mismatch morpholinos and appropriate references in the manuscript.

Why are there qPCRs at different time points? Is this related to the time at which these genes are expressed?
Response: During development, expression of many genes induced in response to ribosomal stress changes. For example, p53 expression in wild-type embryos peaks at gastrulation, in RP-deficient embryos, it peaks at ~ 18hpf. Isg15 peaks soon after p53 and decreases afterword. In contrast, TNFalpha is initially low and increases with age. Inclusion of time-course data would make the paper unnecessary complex. Therefore we show examples of changes in line with a goal of this paper, i.e. to demonstrate that innate immune system responds to RP deficiency. The dynamic of changes would be better to study in DBA patients.

Also where are they expressed since ultimately the hypothesis is the expression of these genes are responsible in part for the erythroid phenotype so are they expressed in red blood cells or other hematopoietic tissue.
Response: Expression of most genes studied here, is known. For example, tlr3 is expressed in immune cells, epithelial cell, and in some immature cells but interferons and other factors produced by these cells, act on all cells in the body including hematopoietic tissues. We added a scheme that illustrates this.

In B there seems to be 2 shades of grey on the control bar. Do these represent anything different?
Response: No, we corrected this.

C is missing controls - what is the effect of p53 on the expression of tlr3 in wt (or more appropriately mismatch morpholino injected embryos).
Response: Expression of tlr3 in wild type embryos (with intact p53) is shown in 1A. It is increased in Rps19-deficient embryos. Fig1C shows that tlr3 level is also increased in p53-/- embryos with Rps19 deficiency.

Figure 2
Same comment as above - why are they not showing the mm morpholino controls
The labeling of the experiments is poor e.g. in B and E, one column is p53-/- the other Rps19 MO but presumably this is also p53-/- but also with knockdown of Rps19. In some graphs only the fold change is shown and in others the normalized control bar is also shown. This should

be consistent
Response: We apologize, and added the missing information to Fig. 2B. In C and D, we omitted normalized controls to make panels more compact.

What is the time point for A, and again why the use of different time points?
Response: The time point for each graph is shown in the Figure Legend.

Figure 3

Why are the 2 graphs shown at different time points.
Response: These markers are expressed at both time points, examples are shown.

Innate immune system response to ribosomal protein deficiency in zebrafish and cellular models of Diamond Blackfan Anemia

Nadia Danilova,[1] Elena Bibikova,[2] Min-Young Youn,[2] Kathleen M. Sakamoto,[2*] and Shuo Lin[1*]

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[2]Department of Pediatrics Stanford University School of Medicine, Stanford, CA, USA

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Normal Hematopoiesis

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Abstract

Deficiency of several ribosomal proteins (RPs) caused by a mutation or gene deletion leads to Diamond Blackfan Anemia (DBA), a bone marrow failure disorder associated with anemia, congenital defects, and an increased incidence of cancer. While p53 activation is responsible for many features of DBA, the role of p53-independent signaling pathways in RP-deficient cells is less defined. Here, we found that Rps19-deficient zebrafish had p53-independent upregulation of Toll like receptors Tlr3 and Tlr9. These innate immune receptors recognize RNA and DNA from pathogens, but can also be activated by endogenous nucleic acids under pathological conditions. In contrast, Tlr5α and Tlr5β, which recognize bacterial flagellin and do not have endogenous ligands, were downregulated in response to RP-deficiency. Several innate immune mechanisms such as interferons, complement system, and inflammation were upregulated in RP-deficient zebrafish. Our data suggest the upregulation of a TGFβ family member activin in RP-deficient zebrafish and in RPS19-deficient human cells, which include a lymphoid cell line from a DBA patient with an RPS19 mutation, and fetal liver cells and K562 cells transduced with RPS19 shRNA. Inhibitors of TLR3, complement, and activin decreased morphological defects in RP-deficient zebrafish and improved their hematopoiesis and survival. Our studies suggest that innate immune system contributes to the phenotype of RPS19-deficient fish and human cells.

Keywords: Diamond Blackfan Anemia, TLR3, TLR9, interferon, complement, activin

Introduction

Diamond-Blackfan Anemia (DBA) is a bone marrow failure syndrome, which is also characterized by congenital malformations and cancer [1]. DBA is caused by mutations in ribosomal proteins (RPs), most often in RPS19, while mutations in several others RPs are found at lower frequencies [2-4]. Deficiency in RPs leads to the impairment of ribosome biogenesis [5-7] and p53 activation [8-12]. Inhibition of p53 decreases hematopoietic and developmental defects in animal models of DBA suggesting that p53 upregulation is involved in the pathogenesis of DBA. Activation of p53 independent signaling pathways in DBA has also been reported [11, 13, 14]; however their role and interaction with the p53 network is not well defined. Previous reports on DBA suggested contribution of immune mechanisms to DBA pathophysiology [15, 16]. However, these studies focused on lymphoid cells with varying conclusions. Therefore, understanding the precise role of not only adaptive immunity but also innate immunity in regulation of hematopoiesis in DBA is important.

The advantage of using zebrafish to study the role of innate immunity in DBA is the absence of a functional adaptive immune system during the first several days of zebrafish development. The first T cells start to develop after day three and B cells after one week of development. Therefore, contribution of innate immune mechanisms to the RP-deficient phenotype can be studied without interference from T and B cells.

Components of injured, apoptotic, and necrotic cells can activate the innate immune system through binding to immune receptors such as Toll-like receptors (TLRs) [17, 18]. Nucleic acids escaping from damaged tissue or contained within endosomes could serve as endogenous ligands for TLR3, which recognizes viral double strand RNA, but can also be activated by mRNA [19, 20]. TLR9 recognizes bacterial DNA but can also be activated by endogenous DNA [18]. Activation of TLRs can lead to upregulation of several innate immune mechanisms including interferons, inflammation, and activin [21-23]. Upregulation of IFNs inhibits hematopoiesis: IFN-β inhibits the growth of erythroid cells [24] while IFN-γ impairs maintenance of HSCs [25]. Inflammation contributes to the pathophysiology of many diseases [26] and inflammatory cytokines have been shown to suppress hematopoiesis. For example, activation of TNFα or activin, a pro-inflammatory cytokine from the TGFβ family can inhibit hematopoiesis [23, 27, 28].

Besides TLRs, other innate immune receptors can be activated by endogenous ligands. The complement system is an intrinsically unstable cascade of enzymes that in response to infection leads to cell lysis and inflammation [29]. Self-cells are protected from complement damage by membrane-bound and soluble inhibitors. Abnormalities in membranes or plasma proteins, as well as insufficient production of inhibitors can cause complement to attack self-cells. Complement is involved in the pathogenesis of many disorders including hematological diseases such as paroxysmal nocturnal hemoglobinuria [30].

DBA is associated with accumulation of defective pre-mRNA molecules and increased apoptosis. This may provide a source of defective RNA and DNA molecules that may activate the innate immune system. Previously, in Rpl11zebrafish mutant, microarray analysis of gene expression revealed upregulation of genes involved in inflammation, interferon and complement pathways [11]. Also, in hematopoietic progenitors of DBA patients, microarray analysis revealed upregulation of interferon and tumor necrosis factor (TNF) pathways [31]. Similar findings were reported in fibroblasts of DBA patients [32]. On the other hand, Rpl11 zebrafish mutant microarray data showed decreased expression of defensins and lysozyme [11], which suggests weakened immunity. In accord with this finding, some patients with ribosomopathies demonstrate features of common variable immunodeficiency syndrome [33].

We report in this paper that in an Rps19-deficient zebrafish model of DBA, we found increased expression of Tlr3 and Tlr9 receptors activated by nucleic acids. In contrast, Tlr5α and Tlr5β, which recognize bacteria, were downregulated. Genes involved in innate immune mechanisms such as interferon signaling and inflammation were upregulated in Rps19-deficient zebrafish. Changes in expression of activin/inhibin subunits in Rps19-deficient zebrafish and RPS19-deficient human primary cells and cell lines pointed to activin upregulation. Inhibitors of TLR3, activin, and complement

rescued hematopoiesis and developmental defects in Rps19-deficient zebrafish. Our data suggest that the innate immune system could contribute to the to the pathophysiology of DBA.

Materials and Methods

Zebrafish

Zebrafish *(Danio rerio*) lines used AB, *rpl11*^{hi3820bTg} and *tp53*$^{zdf1/zdf1}$. Embryos were obtained by natural spawning. UCLA Animal Committee approved the study.

Human primary cells and cell lines

EBV-immortalized lymphoid cell line from a DBA patient with an RPS19 mutation and a cell line from a normal control were a gift from Hanna Gazda (Harvard University, Boston, MA). Both cell lines were grown in RPMI-1640, supplemented with 10% FBS. Human fetal liver tissues were obtained from Advanced Bioscience Resources Inc (Alameda, CA). Cells were sorted for $CD34^+$ using MACS cell separation (Miltenyi Biotec, Auburn, CA). Sorted cells were grown in x-Vivo15 media (Lonza, Basel, Switzerland) containing 10% FBS, 50 ng/mL of Flt-3, Tpo, and SCF and 20 ng/mL of Il-3 and Il-6. K562 cells were grown in RPMI-1640, supplemented with 10% FBS. Primary CD34+ fetal liver cells and K562 cells were transduced with lentivirus containing shRNA against RPS19 or scrambled (SCR) shRNA, sorted for GFP at 72 hours, and harvested post-transduction, as indicated in results.

Microarray

K562 cells were transduced with lentivirus carrying shRNA against RPS19 or control scrambled shRNA and sorted for the GFP marker. RNA was purified with Trizol and analyzed by microarray on an Affymetryx platform. The microarray and data analysis were performed at the UCLA DNA Microarray Core.

RT-qPCR

RNA was prepared using Trizol (Invitrogen, Carlsbad, CA) from 30-40 embryos or from 1-5 million cells; 2 μg was used for RT with the random hexamer primers. PCR was performed in triplicates using iQ SYBR Green Super Mix. Primers are shown in a supplemental Table 1. Levels of mRNA were normalized to beta-actin and calculated by $C\tau$ method.

Morpholinos

3 ng of the following morpholinos were injected at the one cell stage: Rps19 –specific morpholino targeting exon 3 splicing site 5'- gcttccccgacctttcaaaagacaa and 5 bases mismatch: 5'- gattcctcgaactctcaatagacaa (Gene Tools, Philomath, OR).

Staining of erythroid cells

6 mg of o-dianizidine was dissolved in 50 μl of acetic acid, diluted to 6 ml by water, and neutralized by 10 μl of 10M NaOH following by 4 ml of ethanol and 130 μl of 50% H_2O_2. Embryos were placed in this solution and color development was monitored under a microscope. Embryos were washed with water before imaging.

Drug treatments

100 mM stocks of TLR3 receptor inhibitor (Calbiochem), compound SB431542 (Tocris Bioscience, Minneapolis, MN), and compound SB290157 (Santa Cruz Biotechnology, CA) were prepared in DMSO and added to fish water to 3 μM concentrations.

Statistics

Each experiment was repeated at least twice, and results of a representative experiment are shown. Data are presented as the means of at least tree measurements plus/minus SD. Student's *t* test was used for comparisons of a variable between two groups.

Results

Zebrafish models of DBA

Several zebrafish models of DBA have been reported previously. We and other labs created an Rps19-deficient fish using morpholinos [8, 34, 35]. The morpholino used in this study is highly specific as was confirmed by using an alternative translational morpholino, rescue of morphant phenotype by *rps19* mRNA, and use of scrambled morpholino, which had no effect on embryos at any dose studied up to 13 ng per embryo [8]. We also used mutant *rpl11*^{hi3820bTg}[36], which was characterized in our lab [11]. We combined these two models (one gene from a small ribosomal subunit, another from a large ribosomal subunit with their deficiency created by different mechanisms) to study common features of RP deficiency.

Expression of innate immune receptors is altered in zebrafish models of DBA

RP-deficient cells accumulate non-processed pre-rRNA [5-7], have increased DNA damage [37], have altered composition of their membranes [38], and are prone to apoptosis [39, 40]. We hypothesized that non-processed pre-RNA and DNA and RNA from apoptotic cells could lead to activation of toll-like receptors. We therefore examined expression of TLRs in zebrafish with deficiency of Rps19. We found upregulation of genes encoding toll-like receptors Tlr3, which recognizes RNA and Tlr9, which recognizes DNA (Figure 1A). The gene for Tlr1, which recognizes membrane lipoproteins was also upregulated (Fig. 1A). On the contrary, expression of genes encoding Tlr5α and Tlr5β, which recognize bacterial flagellin and do not have endogenous ligands was decreased (Fig. 1B). This is consistent with microarray data of Rpl11 mutants showing decreased expression of antimicrobial peptides [11]. This pattern may suggest some degree of immunodeficiency in Rps19-deficient zebrafish, along with activation of autoimmunity.

Previously, it was shown that TLR3 could be activated by p53 [41]. However, if Tlr3 becomes upregulated by non-processed pre-RNA, then its upregulation would not be p53-dependent. Thus, we injected *tp53-/-* mutants with Rps19-specific morpholino and measured *tlr3* expression. *tlr3* was upregulated in a p53-negative background (Fig. 1C). This finding indicates that *tlr3* upregulation in RP-deficient zebrafish is p53-independent.

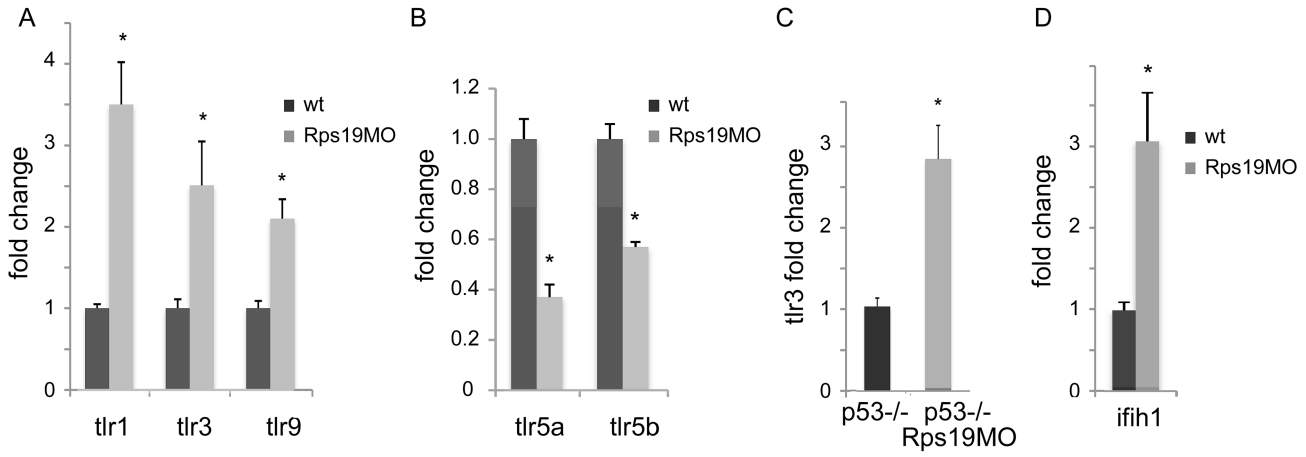

Figure 1. Expression of innate immune receptors was altered in RP-deficient zebrafish. (A) *tlr1, tlr3*, and *tlr9* were upregulated in embryos injected with Rps19-specific morpholino in comparison to wild-type controls or embryos injected with a mismatch morpholino (data not shown). 18 hpf (hours post fertilization), RT-qPCR, RNA in this and other experiments was derived from a pool of at least 30 embryos. (B) Expression of *tlr5a/b* that recognize bacterial flagellin, was decreased in Rps19 morphants. 28 hpf, RT-qPCR. (C) *tlr3* was upregulated in *tp53-/-* mutants injected with Rps19 specific morpholino. 19 hpf, RT-qPCR. (D) *ifih1* gene encoding innate immune receptor Mda5 recognizing nucleic acids was upregulated in Rps19-deficient zebrafish. The bars

represent the mean of three replicates ± sd. Asterisk indicates significant difference in comparison to controls, p<0.05.

Another gene important for the innate immunity that was induced in RP-deficient zebrafish was RNA helicase *ifih1* (Fig. 1D). *ifih1* encodes innate immune receptor Mda5 that acts as a cytosolic sensor of double-stranded RNA [42]. Mda5 belongs to a family of RNA Helicase-DEAD Box Proteins. Previously, we found that several other genes encoding proteins of this family were upregulated in Rpl11 mutant more than two fold, including *ddx18, ddx27, ddx49, ddx55, ddx3y*, and *ddx47* [11]. Proteins of this family are involved in ribosome biogenesis and in catabolism of faulty RNAs. A recent study showed that MDA5 overactivation leads to upregulated type I interferon responses and phenotypes consistent with autoimmune diseases such as systemic lupus erythematosus [42].

Therefore several classes of innate immune receptors recognizing abnormal nucleic acids were upregulated in RP-deficient zebrafish along with upregulation of Tlr1 recognizing lipoproteins. At the same time, Tlr5α and Tlrβ recognizing bacterial flagellin, were downregulated. Activation of TLRs and MDA5 leads to induction of interferons, inflammation, and activin. We therefore examined these pathways in our DBA models.

Interferon signaling is activated in RP-deficient zebrafish

All TLRs, except for TLR3, signal through MyD88 to induce NFkB and IRF7, while TLR3 also induces IRF3-dependent transcription [43]. MDA5 also signals through IRF3 to induce interferons. IRF3 and IRF7 control the transcription of IFN-alpha and beta, as well as transcription of IFN-stimulated genes (ISG) [44, 45]. We therefore examined expression of *irf3* and *irf7* in Rps19-deficient zebrafish embryos and found them to be upregulated (Fig. 2A). i*rf7* was upregulated in a p53-negative background as well (Fig. 2B). Moreover, we have previously found *irf7* upregulation in our Rpl11 mutant [11].

Next, we examined expression of interferon mediators and targets. INFs signal through STAT proteins to activate the transcription of interferon stimulated genes (ISGs). In Rps19-deficient zebrafish, *stat1b* and *stat3* were upregulated (Fig. 2C). *Socs3* (suppressor of cytokine signaling 3), a member of the STAT-induced STAT inhibitor family, was also upregulated. In a microarray of Rpl11 mutant, *Stat3* was also upregulated [11].

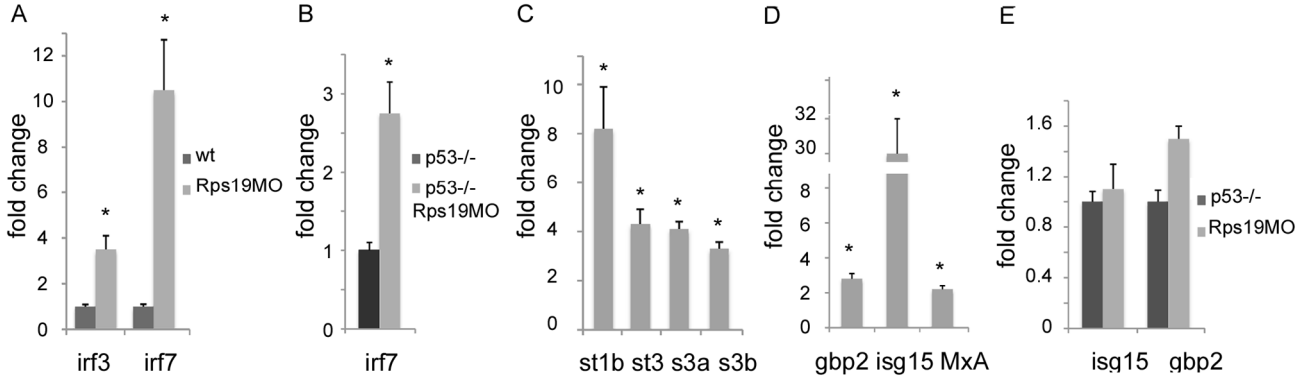

Figure 2. Interferon signaling was upregulated in Rps19-deficient zebrafish. (A) Interferon regulators *irf3* and *irf7* were upregulated in Rps19-deficient embryos, 28 hpf, RT-qPCR. Fold change in expression was calculated relative to wild-type embryos. (B) *Irf7* was upregulated in p53-/- mutant injected with Rps19 morpholino, 19 hpf, RT-qPCR. (C) Interferon mediators and their inhibitors were upregulated in Rps19-deficient zebrafish, 24 hpf, RT-qPCR. St1b, *stat1b*; st3, *stat3*; s3a, *socs3a*; s3b, *socs*3b. (D) Interferon targets *gbp2*, *isg15* and *MxA* were upregulated in Rps19-deficient zebrafish, 24 hpf, RT-qPCR. (E) There was no upregulation of *isg15* on *tp53* -/- background and only slight increase in *gbp2* expression, 19 hpf, RT-qPCR. Bars represent the mean of three replicates ±sd. Asterisk indicates significant difference in comparison to controls, p<0.05.

Upregulation of ISG genes is another indicator of interferon system activation. The interferon-induced gene, *isg15* (ubiquiting-like modifier 15), was the second-most upregulated gene in Rpl11 mutants according to microarray data verified by qPCR [11]. It was also upregulated in Rps19-

deficient zebrafish (Fig. 2D). The protein encoded by *isg15* is an ubiquitin-like protein that is conjugated to intracellular target proteins upon activation by interferons. Targets include STAT1, JAK1, EIF2AK2/PKR, SERPINA3G/SPI2A, MAPK3/ERK1, PLCG1, MX1/MxA, and RIG-1. The gene encoding Gbp2 (guanylate binding protein 2, interferon-inducible) was also upregulated in Rpl11 mutant [11] and in Rps19 morphants (Fig. 2D). Gbp2 hydrolyzes GTP to GMP, promotes oxidative killing, and delivers antimicrobial peptides to autophagolysosomes. A gene encoding another GTP-metabolizing protein, MxA, was also upregulated in Rps19 morphants (Fig. 2D).

Upregulation of some interferon responsive genes require cooperation of interferon and p53 pathways [46]. We therefore examined if interferon responsive genes in RP-deficient embryos were upregulated in *tp53-/-* mutant. *isg15* was not upregulated in a p53-negative background, while expression of *gbp2* was only slightly increased (Fig. 2E). These data suggest that these factors require cooperation between interferons and p53 for their transcriptional upregulation.

Inflammatory pathways are upregulated in RP-deficient zebrafish

Upregulation of TLRs receptors may lead to NFκB activation and induction of inflammation. We found upregulation of several pro-inflammatory genes in Rps19-deficient zebrafish embryos including cytokines *tnf* and interleukin 6 (*il6*) (Fig. 3A). An important gene involved in inflammatory innate immune response is *ptgs2* (prostaglandin-endoperoxide synthase-2, encoding for the Cox-2 enzyme). Cox-2 catalyzes the rate-limiting step of prostaglandin biosynthesis and is the target of non-steroidal anti-inflammatory drugs like aspirin. Immediate early genes *junb* and *fos,* fibrinogen, *and matrix metallopeptidase 9* involved in leukocyte migration were also upregulated in Rps19-deficient zebrafish (Fig. 3B).

Prolactin, which was recently recognized as a marker of autoimmune disease [47], was also upregulated in Rps19-deficient zebrafish (Fig. 3B).

Similarly to Rps19 deficient zebrafish, the Rpl11 mutant showed upregulation of matrix metalloproteinases *mmp9*, and *mmp13*, prolactin, *fos*, and *junb* by microarray verified by qPCR [11]. In addition, RT-qPCR analysis of gene expression in the Rpl11 mutants showed 14 fold (sd 2.1) upregulation of *il6* compared with sibling conrols at 48 hpf.

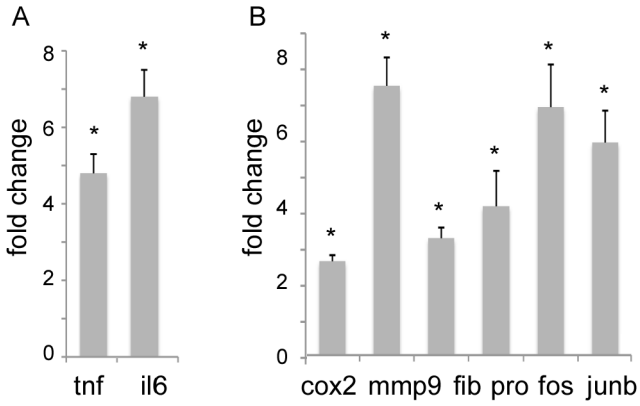

Figure 3. Pro-inflammatory genes were upregulated in Rps19-deficient zebrafish (A) Expression of genes for cytokines Tnfα and Il6 was increased, 24hpf, RT-qPCR. Fold change in expression was calculated relative to wild-type embryos. (B) Expression of genes considered to be markers of inflammation was increased, 48 hpf, fib, fibrinogen, pro, prolactin, RT-qPCR. Fold change in expression was calculated relative to wild-type embryos. Bars represent the mean of three replicates ±sd. Asterisk indicates significant difference in comparison to controls, $p<0.05$.

TGFβ signaling is altered in Rps19-deficient zebrafish and in RPS19-deficient human cells

Toll-like receptor agonists can stimulate release from macrophages of activin A, a pro-inflammatory cytokine from the TGFβ family [23]. We therefore examined expression of activin subunits in RP-deficient zebrafish. Activin is counterbalanced by inhibin, a factor with an opposite activity. Both activin and inhibin are dimers. Activin is composed of two beta subunits that can be identical or different. Inhibin shares beta subunits with the activin, but the other subunit, alpha, is unique to inhibin. Therefore, changes in expression of the alpha subunit would affect the amount of beta subunits available for activin and, consequently, affect the activin/inhibin ratio. In Rps19 deficient zebrafish, expression of a subunit InhAa, unique to inhibin, was decreased, while expression of subunits inhBa

and inhBb common for both inhibin and activin, was unchanged (Fig. 4A). It means the activin/inhibin equilibrium was shifted to activin in RP-deficient zebrafish. Another indicator of activin overproduction was upregulation of follistatin, a protein that binds and inhibits activin (Fig. 4B). We also found upregulation of *smad7*, an inhibitory SMAD that suppresses TGFβ signaling.

Inhibin alpha (*INHA*) was also among downregulated genes in cellular models of DBA. We transduced human human CD34+ fetal liver cells and K562 cells (*tp53*-/-) with lentiviral vectors expressing short hairpin RNA (shRNA) against *RPS19* [37, 48]. In this system, we achieved ~50% reduction in RPS19 protein level [37]. In K562 cells with RPS19 knockdown, microarray analysis revealed *INHA* among the most downregulated genes (Fig. 4C). Since no changes in expression of beta subunits *INHBA* and *INHBB* have been detected by microarray, these data point to activin overproduction in these cells. *INHA* expression was also decreased in RPS19-deficient human fetal liver (Fig. 4D), and in lymphoid cell lines from a DBA patient with an RPS19 mutation (Fig. 4E).

These data suggest TGFβ family member activin with proinflammatory properties was over-produced in zebrafish DBA models and in human cells deficient in RPS19. Activin overproduction was detected both in a wild type and a p53-negative background. This suggests that the increase in activin production in DBA models does not depend on p53.

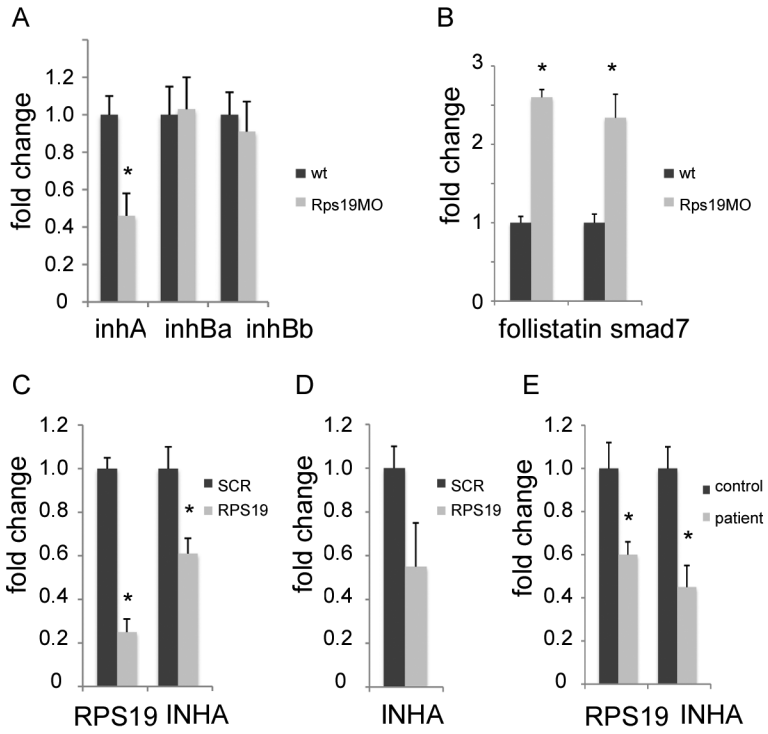

Figure 4. Expression of activin/inhibin was altered in RP-deficient zebrafish and human cells. (A) *inhA*, encoding a subunit unique to inhibin, was downregulated in Rps19-deficient zebrafish embryos while expression of genes for subunits common to both inhibin and activin was not changed, 40 hpf, RT-qPCR. (B) Follistatin, which is an activin inhibitor, was upregulated in Rps19-deficient zebrafish along with upregulation of inhibitory Smad7, 40 hpf, RT-qPCR. (C) K562 cells transduced with anti-RPS19 shRNA showed decreased expression of RPS19 and INHA subunit, SCR, scrambled shRNA control, RT-qPCR. (D) Inhibin alpha was also downregulated in human fetal liver cells deficient in RPS19, SCR, scrambled shRNA control, RT-qPCR. (E) Inhibin alpha was downregulated in a lymphoid cell line derived from a DBA patient with a mutation in the RPS19 gene, RT-qPCR. Bars represent the mean of three replicates ±sd. Asterisk indicates significant difference in comparison to controls, $p<0.05$.

Complement system is upregulated in RP-deficient zebrafish

Complement system can be activated by different mechanisms even in absence of infection. Spontaneous C3 hydrolysis and binding of C3 convertase enzyme to cells initiate the alternative pathway of complement. Normally, it is limited by activity of factor H and complement regulatory proteins acting on cell surface to clear complement complexes. In pathological conditions the balance between complement activation and inhibition is broken and C3 convertase can recruit more complement proteins onto the cell surface leading to the formation of the membrane attack complex, cell damage and activation of other immune mechanisms.

In Rps19-deficient zebrafish, we found an increase in the expression of factor B (*cfb*) from the alternative complement pathway (Fig. 5A). Complement component C6 is a part of the membrane attack complex and is also upregulated in Rps19-deficient zebrafish (Fig. 5A). On *tp53*-/- background upregulation was smaller (Fig. 5B) suggesting p53 activation contributes to complement activation.

While complement components were upregulated, expression of complement inhibitor factor H was decreased (Fig. 5B). Similarly to findings in Rps19-deficient zebrafish, we found upregulation of complement components *c6* and *cfb* in zebrafish Rpl11 mutant [11]. Moreover, RNA-seq of Rps19-deficient zebrafish was done recently, and, similar to our data, this study demonstrated upregulation of c6 complement component and downregulation of inhibitory factor H [14].

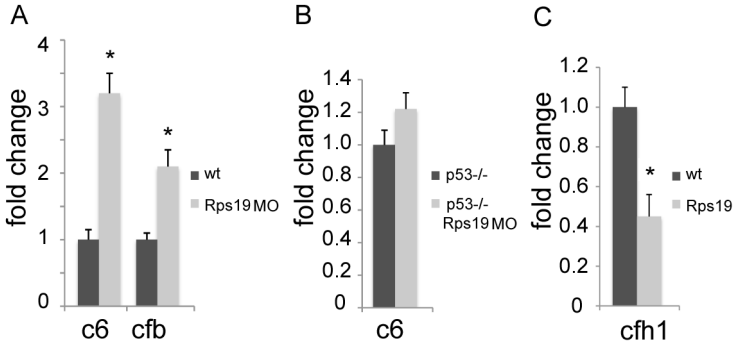

Figure 5. Complement system was upregulated in RP-deficient zebrafish. (A) Complement components *cfb* and *c6* were upregulated in Rps19-deficient zebrafish embryos, 24 hpf, RT-qPCR. (B) On *tp53*-/- background, *c6* upregulation was much smaller, 24 hpf, RT-qPCR. (C) Inhibitor of complement factor H was downregulated in Rps19-deficient zebrafish embryos, 24 hpf, RT-qPCR. Bars represent the mean of three replicates ±sd. Asterisk indicates significant difference in comparison to controls, $p<0.05$.

Application of inhibitors of TLR3 receptor, activin receptor, and complement improved the condition of RP-deficient zebrafish

Our data suggested that upregulation of some TLRs, inflammation, activin, and complement system might contribute to the RP-deficient phenotype. We therefore examined effects of inhibitors of these pathways on RP-deficient zebrafish. We injected zebrafish embryos with Rps19-specific morpholino and treated them with TLR3 receptor inhibitor, compound SB431542 that inhibits activin receptor, or compound SB290157 that acts as a competitive antagonist of anaphyloxin C3a receptor. Application of TLR3 inhibitor resulted in downregulation of interferon mediators *irf7* and *stat1b,* suggesting decrease of interferon system activation (Fig. 6A). Expression of pro-inflammatory markers *il6* and *sox2* was also decreased after treatment as well as expression of acute-phase response gene *fos*. We also observed downregulation of *p21* responsible for cell cycle arrest and a pro-apoptotic gene *bax*. As a result, we observed partial rescue of hematopoiesis (Fig. 6B) and morphology (Fig. 6C) in Rps19-deficient embryos. Inhibitors of activin and complement also improved hematopoiesis of Rps19-deficient embryos.

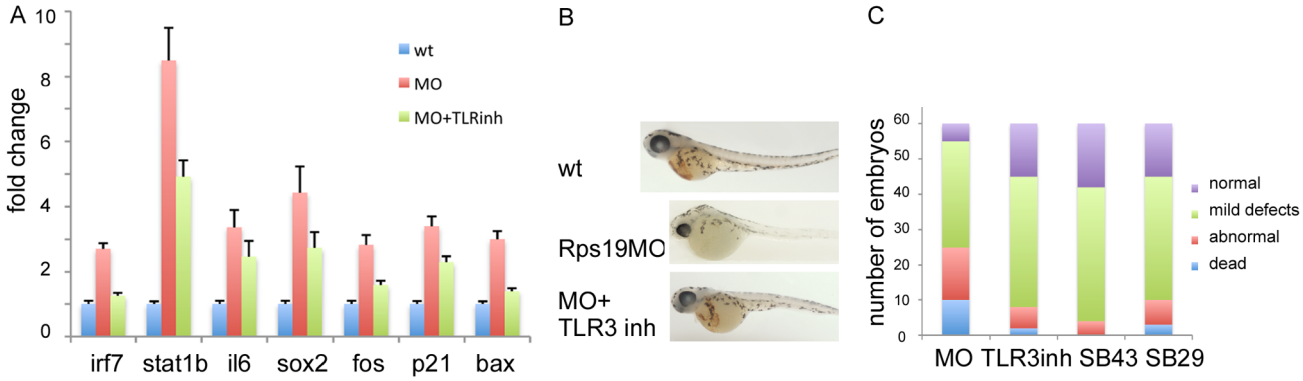

Figure 6. Inhibitors of TLR3, activin, and complement partially rescued Rps19-deficient zebrafish embryos. (A) TLR3 inhibitor decreased expression of interferon mediators *irf7* and *stat1b*, decreased expression of inflammatory markers *il6* and *sox2* and acute phase response gene *fos*. It also downregulated *p21* responsible for cell cycle arrest and pro-inflammatory gene *bax*, 40 hpf, RT-qPCR. Bars represent the mean of three replicates ±sd. Asterisk indicates significant difference in comparison to controls, $p<0.05$. (B) TLR3 inhibitor improved hematopoiesis in Rps19 morphants, day 3, o-dianizidine staining. 50 embryos per group,

representative staining is shown. (C) Embryos were injected with rps19 morpholino, MO group was left untreated, other groups were treated with TLR3 inhibitor (TLRinh), SB431542 activin inhibitor (SB43) or SB290157 complement inhibitor (SB29). Treatments decreased morphological defects and improved survival in Rps19-deficient zebrafish embryos. Embryos classified as abnormal had strong defects such as tail kinks, curved body, strongly shortened body, arrest at early developmental stage, underdeveloped eyes etc. Embryos with mild defects had smaller size, smaller eyes and heads. 24 hpf. The experiment was performed in triplicates, 60 embryos per group; bars represent the mean of three replicates. Number of dead/abnormal,mild/normal embryos for MO 10±0.6/15±0.6/30±1.2/5±1; for TLR3 inhibitor 2±1/6±1/37±1/15±0.6; for SB431542 inhibitor 0±0/4±1/38±2.5/18±1.5; for SB2990157 3±1/7±0.5/35±1.2/15±0.7.

Discussion

In RP-deficient zebrafish, we found alterations in expression of key innate immune receptors. While expression of genes encoding Tlr1, Tlr3, and Tlr9 that can be activated by endogenous ligands was increased, expression of genes encoding Tlr5α and Tlr5β that recognize bacterial flagellin was decreased. In addition, we found upregulation of *ifih1* gene encoding Mda5 receptor, which is involved in catabolism of faulty RNAs among other functions. Activation of MDA5 may also contribute to upregulation of interferon responses and inflammation [42]. This pattern suggests that inflammation and autoimmunity may be activated in our DBA models while immunity against infection may be decreased. Indeed, we found upregulation of interferon signaling, increased expression of components of complement system along with downregulation of its inhibitors, and upregulation of pro-inflammatory signaling (Fig. 7). At the same time, expression of antimicrobial peptides lyzozyme and defensin was decreased in RP-deficient zebrafish [11]. Our data are consistent with upregulation of interferons and TNF pathway in cells from DBA patients [31, 32].

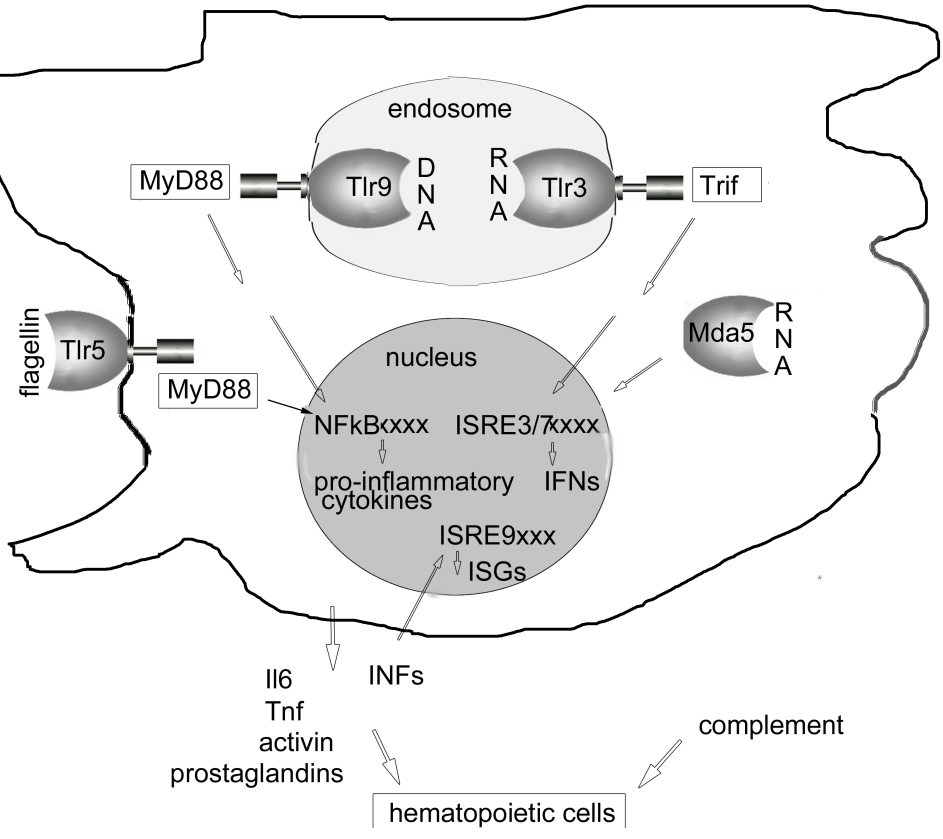

Figure 7. Response of innate immune system to RP-deficiency. Tlr3 and Tlr9 receptors are expressed in endosomes in several cell types including dendritic cells and can be activated by endogenous ligands at pathological conditions. In RP-deficient cells, such ligands could be DNA and RNA from apoptotic cells and incorrectly processed pre-rRNA. Once activated, TLR receptors activate

expression of interferons and pro-inflammatory cytokines, which are released and act on hematopoietic cells. In addition, cytoplasmic sensor of pathological nucleic acids Mda5 (encoded by *ifih1)* was upregulated in RP-deficient zebrafish, which could contribute to upregulation of IFNs and pro-inflammatory factors. In contrast, Tlr5 receptor, which recognizes bacterial flagellin, was downregulated in RP-deficient cells. Several complement components were upregulated in RP-deficient zebrafish while complement inhibitors were downregulated, which suggests complement activation. These factors may synergize in decreasing proliferation of hematopoietic cells and shortening their life span.

Many changes in RPs-deficient cells may contribute to the activation of innate immune mechanisms. They include accumulation of defective pre-rRNAs, DNA damage, increased ROS, increased apoptosis, alterations in composition of membranes and extracellular matrix, metabolic defects, and insufficient production of proteins inhibiting complement system among other causes.

Upregulation of TLRs and MDA5 pathway is likely the major source of activation of interferons. However other factors may contribute to this activation as well. For example, irradiation of macrophages was associated with enhanced expression of several interferon-stimulated genes. ISGs induction in irradiated macrophages was dependent on DNA damage and Ataxia-Telangiectasia mutated kinase (ATM) [22]. Recently, we found that replication stress and DNA damage checkpoint was activated in RP-deficient zebrafish and human cells, leading to upregulation of the ATR/ATM/Chk1/Chk2 pathway [37]. Therefore, this pathway may also be involved in upregulation of interferons.

Previously, it was noted that p53 and interferon systems could affect each other. Interferons can stimulate p53 response and some genes such as *gbp2* can be jointly regulated by interferons and p53 [46]. We also found that in RP-deficient zebrafish, interferon induced genes *isg15* and *gbp2* needed the presence of intact p53 in order to be fully upregulated.

Activation of the complement system in RP-deficient zebrafish may be caused by alteration in composition of membranes and plasma proteins that was reported in DBA [38]. Decreased expression of factors inhibiting complement, such as factor H, is another source of complement activation. Our data add DBA to the growing list of diseases with overactivated complement.

Both interferons and complement system induce inflammation. Indeed, in RP-deficient zebrafish we found upregulation of many inflammatory markers. We also found decreased expression of inhibin-specific subunits in RP-deficient zebrafish and human cells, which suggest a shift to activin in the inhibin-activin balance. This may play an important role in suppression of hematopoiesis in DBA models.

Inhibitor of TLR3 receptor, inhibitor of activin receptor SB431542, and complement inhibitor improved the condition of RP-deficient embryos.

In adult fish and in human patients, which have both innate and adaptive immune systems, activation of innate immune receptors may affect T and B cells. For example, upregulation of interferons may activate macrophages and direct T helper cell development to the pro-inflammatory Th1 pathway [49]. Increased proportion of cytotoxic T cells (decreased T4/T8 ratio) was reported in DBA and may also be connected to interferon activation [16]. Interferons may contribute to the apoptosis of hematopoietic progenitors induced by cytotoxic T cells in acquired aplastic anemia [50].

Blockade of TLR3 was recently shown to protect mice from lethal radiation induced syndrome [51]. Interestingly, agonists of TLR5 also have radioprotective activity in mouse and primate models [52]. These data suggest that the right balance between p53 and NFkB is necessary to prevent cell death and promote survival. Our data suggest that alterations of some innate immune pathways may contribute to the pathogenesis of DBA. Glucocorticoids used in DBA treatment may work in part by suppressing inappropriate immune activation. Cytokine inhibition has been successfully used to treat some rheumatic and autoimmune diseases [53] and antibodies targeting interferons and interleukins are being investigated for SLE. Sotatercept that suppresses activin signaling is investigated as a potential therapeutic for anemia [54]. Based on our findings, therapies targeting innate immune response or modulating activin signaling should further be explored as potential treatments for DBA.

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Competing interests
The authors declare no competing financial interests

Author contribution
Conceived and designed the experiments ND, SL; performed experiments ND; EB, M.Y; analyzed results ND, SL, KMS; wrote the paper ND, SL, and KMS.

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Supplemental

Table 1. **Primers specific to zebrafish cDNAs used in qPCR.**

Gene	Name	Sequence
Actin beta	ac acr	5'-TCTCTTCCAGCCTTCCTTCCT 5'-CTCATCGTACTCCTGCTTGCT
p53	53f 53r	5'-CTGAAGTGGTCCGCAGATG 5'-CGTTTGGTCCCAGTGGTGG
puma	zpm zpmr	5'-CCTCACATGATGCCTTCAGC 5'-CATTGATGGTGTCCGAGACC
p21	z21 z21r	5′-TGAGAACTTACTGGCAGCTTCA 5′-AGCTGCATTCGTCTCGTAGC
interleukin 6	il6 il6r	5'-TCCTGGTGAACGACATCAAA 5'-TCATCACGCTGGAGAAGTTG
fibrinogen	fib fibr	5'- CAGCAGTCCCCCTCTTACTG 5'-CATGACACCTGGATGCAAAC
complement factor B	cfb cfbr	5'- AAAGGAATCGAAGTGGCAGA 5'- TGCATCAAGTTTCGCTTTTG
c-fos	fos fosr1	5'-TACCAGCCTTAACGCCGACT 5'-GCTTCTCTTGTTGGAGGTCTT
inhibin A	inha inhar	5'-CCTTGAAGGTTTGGGGTTGG 5'-CCAGGTCCAGCATCAGAAGA
cox2	cox2 cox2r	5'-CATTCGCAACATGGTGGACT 5'-TGACCGTACAGCTCCTTCAG
factor H	cfh1 cfh1r	5'-TGACGCTCCACCAAAAGTTG 5'-CCTGGGACACTTTGCTTCAC
ifih1	ifih1 ifih1r	5'-GAGCCGCCGTCTAAAATCAG 5'-AATGACTCCGTTGGTCTCGT
tlr1	tlr1 tlr1r	5'-TCCTGCAGACATCCACACTT 5'-CAGAGAGGCAAAATCACGCA
tlr9	tlr9 tlr9r	5'-TCAGAGTTGGATTGCAAACGT 5'-GAGAAGTGAACCTGGGGACT
mxA	MxA MxAr	5'-GTCAGGGACCAGATCAAGCT 5'-GGCTGTAAACGATGAGCTCC
Complement factor 6	cf6	5'-AACCGAGCATTCCAAAGGAC

	cf6r	5’-AACGGTCAGCATGAAAAGCA
stat1b	stat1b stat1br	5’-ACACACTTGCTGCTCCAATG 5’-AAACCTTGCAACGGGTCTTG
Irf3	irf3 irf3r	5’-GCCGAGGTCGATCTCAATAA 5’-ACTGCAGTGAAGACGCTCCT
smad 7	sm7 sm7r	5’-CCCCTATGGGGTTTTCAGAT 5’-GTGCCCTGAGGTAGGTCGTA
tnf	TNFa TNFar	5’-GGTGTTTGGGATCATTTTGG 5’-CAAGCCACCTGAAGAAAAGG
Inhibin Ba	inhBa inhBar	5’-ACGCCATCCGTAAGGTACAC 5’-GCAGTCGAAGGAAGATCCAG
Inhibin Bb	inhBb inhBbr	5’-TTCACGCGGGTAAAGTTAGG 5’-TTTGCCTGCAACACGTAGAG

RPS19 Deficiency Leads to GATA1 Downregulation through TNF-Mediated p38 MAPK Activation

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*These authors contributed equally to this work.

Abstract

Diamond-Blackfan Anemia (DBA) is a rare inherited bone marrow failure disorder, characterized by defects in erythropoiesis, congenital abnormalities, and predisposition to cancer. Approximately 25% of DBA patients have a mutation in RPS19, which encodes a component of the 40S ribosomal subunit. While several studies have found that the tumor suppressor protein p53 contributes to DBA pathogenesis, and that certain GATA1 mutations can give rise to DBA, the link between ribosomal protein mutations and erythroid defects is not well understood.

To investigate the molecular pathways downstream of RPS19 deficiency, we infected human cord blood CD34+ cells with RPS19 shRNA lentivirus and observed that RPS19 knockdown showed decreased GATA1 mRNA and protein expression, resulting in defective erythropoiesis. We also detected increased TNF-α levels in RPS19 knockdown cells, and in zebrafish that have been treated with RPS19 morpholino. TNF-α was primarily upregulated in the non-erythroid population of RPS19 deficient cells, while its receptor, TNFR1, showed increased expression on the surface of erythroid cells.

To understand the relationship between GATA1 and TNF-α, we treated primary hematopoietic CD34+ cells with TNF-α or its inhibitor etanercept. Our results demonstrated that TNF-α treatment reduced GATA1 expression, and that this effect could be rescued by the addition of etanercept. Treatment of RPS19 deficient cells with etanercept improved their erythroid differentiation, and increased GATA1 expression. Additionally, etanercept treatment successfully reversed the anemia phenotype observed in RPS19 deficient zebrafish.

To study pathways downstream of TNF-α, we examined phosphorylation of signaling pathways such as p38 MAPK, NFκB, ERK, STAT1, and STAT5 in RPS19 deficient erythroid cells using phospho-flow cytometry. Among these pathways, we found a significant increase in phosphorylation of p38 MAPK, but not ERK, NFκB, STAT1, or STAT5, suggesting that p38 MAPK activation by TNF-α contributes to decreased GATA1 expression in RPS19 deficient cells.

We suggest a novel mechanism for the erythroid defects observed in DBA, in which RPS19 deficiency leads to increased TNF-α production in non-erythroid cells, and activation of p38 MAPK, followed by decreased GATA1 expression, in erythroid cells. Our data also suggest that TNF-α inhibitors, such as etanercept, may be beneficial in treating patients with DBA.

Results

1. GATA1 expression is decreased in RPS19 deficient hematopoietic progenitor cells.

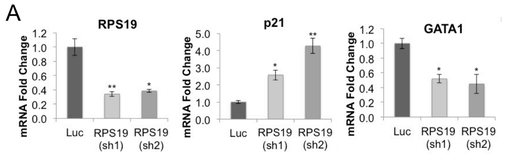


Figure 1. Human cord blood CD34+ hematopoietic progenitor cells were infected with lentivirus carrying shRNA against RPS19 or luciferase (Luc) control, sorted for GFP+ cells, and analyzed after 5 days. (A) shRNA knockdown of RPS19 reduces RPS19 mRNA levels to by approximately 60%, compared with control. p21 mRNA expression is upregulated and GATA1 mRNA level decreased in RPS19 deficient cells. *p<0.05, **p<0.01.

Results

2. RPS19 deficient hematopoietic progenitor cells show increased expression of TNF-α.

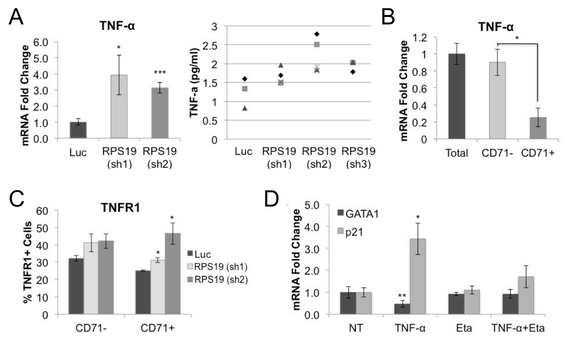


Figure 2. Human CD34+ cord blood cells were infected with lentivirus carrying shRNA against RPS19 or Luc control, sorted for GFP+ cells after 3days, and analyzed 5 days after infection. (A) TNF-α mRNA are increased in RPS19 deficient cells. Its protein levels are also increased in the media from RPS19 deficient cells, compared with control, as measured by ELISA. Each point represents a separate measurement. (B) TNF-α is predominantly expressed in the CD71- (non-erythroid) population of cord blood cells in culture. (C) CD34+ fetal liver cells were analyzed for CD71 and TNFR1 expression by flow cytometry. TNFR1 expression increased on the surface of RPS19 deficient CD71+ cells compared with luciferase control, but did not change in the CD71- population. (D) Addition of 100ng/ml TNF-α to CD34+ cord blood cells results in decreased GATA1 and increased p21 expression after 24 hours. This effect is rescued by addition of 10μg/ml etanercept (Eta). Data are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001.

3. Inhibition of TNF-α rescues erythroid defects in RPS19 deficient hematopoietic progenitor cells.

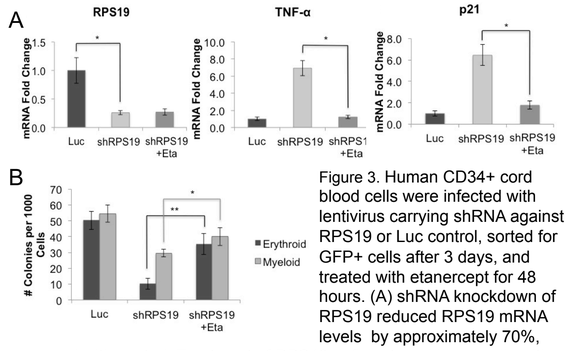


Figure 3. Human CD34+ cord blood cells were infected with lentivirus carrying shRNA against RPS19 or Luc control, sorted for GFP+ cells after 3 days, and treated with etanercept for 48 hours. (A) shRNA knockdown of RPS19 reduced RPS19 mRNA levels by approximately 70%, compared with control. Treatment of RPS19 deficient cord blood cells with etanercept reduced TNF-α and p21 expression in these cells. (B) Etanercept treatment rescued both erythroid and myeloid colony formation of RPS19 deficient cells. Data are representative of two independent experiments. *p<0.05, **p<0.01.

Results

4. Inhibition of TNF-α rescues erythroid defects in RPS19 deficient zebrafish.

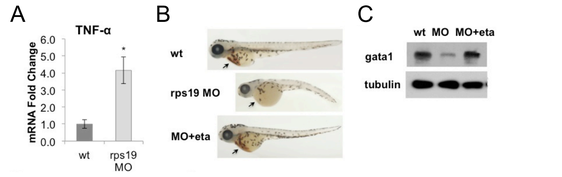


Figure 4. 30 embryos were injected with rps19-specific morpholino at the one-cell stage and with 2ng of etanercept at 4-5 hours post fertilization (hpf). (A) TNF-α mRNA is upregulated in rps19 MO zebrafish at 18 hpf. (B) Treatment of rps19 MO zebrafish with the TNF-α inhibitor etanercept rescued the erythropoietic (see arrows) and developmental defects. (C) Treatment with etanercept restored gata1 expression in rps19 MO zebrafish. Data are representative of two independent experiments. *p<0.05.

5. GATA1 downregulation is mediated through activation of p53 and TNF-α.

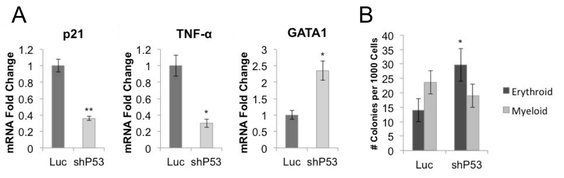
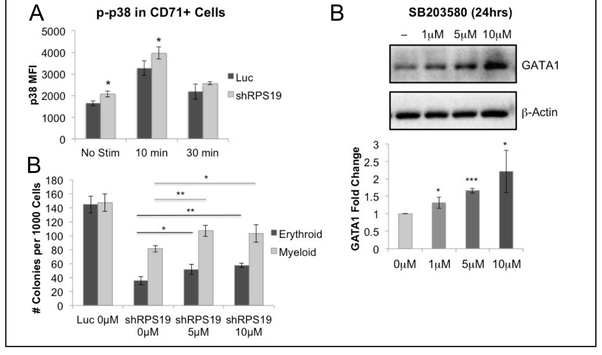


Figure 5. Human CD34+ cord blood cells initially transduced with RPS19 shRNA were infected with lentivirus expressing p53 or Luc shRNA with mCherry and sorted for GFP+mCherry+ cells 5 days after the initial transduction. (A) shRNA-mediated knockdown of p53 in RPS19 deficient cells reduces p21 and TNF-α expression, while increasing expression of GATA1. (B) Erythroid colony formation is increased in RPS19 deficient cells co-transduced with p53 shRNA, compared to luciferase control shRNA. Data are representative of two independent experiments. *p<0.05, **p<0.01.

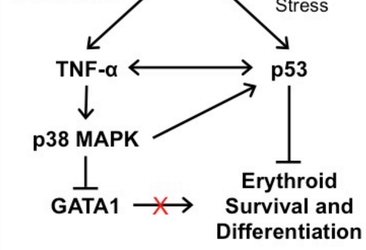
6. Effect of TNF-α on GATA1 expression is partially mediated through p38 MAPK activation.



Results

Figure 6. (A) Human CD34+ fetal lever cells were infected with lentivirus carrying shRNA against RPS19 or Luc control, and stimulated with 100ng/ml TNF-α at 5 days after transduction. p38 MAPK phosphorylation is increased in RPS19 deficient CD71+ cells before and after a 10 minute stimulation, as measured by phospho-flow cytometry. (B) Human CD34+ cord blood cells are cultured for 4 days, and then treated with SB203580, a p38 MAPK inhibitor, for 24 hours. Treatment of SB203580 increased GATA1 protein level. (C) Human CD34+ cord blood cells were infected with lentivirus carrying shRNA against RPS19 or Luc control, sorted for GFP+ cells 5 days after infection, and seeded in methylcellulose medium with SB203580. Treatment of RPS19 deficient cells with SB203580 partially rescues their erythropoiesis and myelopoiesis in methylcellulose. Data are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Model



Deficiency of RPS19 in hematopoietic progenitors leads to ribosomal stress, which increases p53 levels, as well as inflammation, which increases production of TNF-α by non-erythroid cells. TNF-α represses GATA1 through activation of p38 MAPK, and may also contribute to increased p53 levels, which results in reduced survival and differentiation of erythroid cells.

Summary

1. GATA1 is downregulated in RPS19 deficient cells and zebrafish through upregulation of p53, TNF-α, and p38 MAPK.
2. Treatment of RPS19 deficient zebrafish with the TNF-α inhibitor etanercept rescues their erythroid and developmental defects.

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Conflict of interest statement:
The authors have nothing to disclose.