Defects of protein production in erythroid cells revealed in a zebrafish Diamond–Blackfan anemia model for mutation in RPS19

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Diamond–Blackfan anemia (DBA) is a rare congenital red cell aplasia that classically presents during early infancy in DBA patients. Approximately, 25% of patients carry a mutation in the ribosomal protein (RP) S19 gene; mutations in RPS24, RPS17, RPL35A, RPL11, and RPL5 have been reported. How ribosome protein deficiency causes defects specifically to red blood cells in DBA has not been well elucidated. To genetically model the predominant ribosome defect in DBA, we generated an rps19 null mutant through the use of TALEN-mediated gene targeting in zebrafish. Molecular characterization of this mutant line demonstrated that rps19 deficiency reproduced the erythroid defects of DBA, including a lack of mature red blood cells and p53 activation. Notably, we found that rps19 mutants’ production of globin proteins was significantly inhibited; however, globin transcript level was either increased or unaffected in rps19 mutant embryos. This dissociation of RNA/protein levels of globin genes was confirmed in another zebrafish DBA model with defects in rpl11. Using transgenic zebrafish with specific expression of mCherry in erythroid cells, we showed that protein production in erythroid cells was decreased when either rps19 or rpl11 was mutated. L-Leucine treatment alleviated the defects of protein production in erythroid cells and partially rescued the anemic phenotype in both rps19 and rpl11 mutants. Analysis of this model suggests that the decreased protein production in erythroid cells likely contributes to the blood-specific phenotype of DBA. Furthermore, the newly generated rps19 zebrafish mutant should serve as a useful animal model to study DBA. Our in vivo findings may provide clues for the future therapy strategy for DBA.

Received 21.2.14; revised 12.5.14; accepted 14.5.14; Edited by E Baehrecke

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Abbreviations: DBA, Diamond–Blackfan anemia; RP, ribosomal protein; TALEN, transcription activator-like effector nuclease; dpf, days post fertilization; HSC, hematopoietic stem cell; hbbe1, hemoglobin beta e1; hbbe3, hemoglobin beta e3

Citation: Cell Death and Disease (2014) 5, e1352; doi:10.1038/cddis.2014.318; published online 24 July 2014
differentiating nature. Protein synthesis rate was reported to be reduced in lymphocytes from DBA patients irrespective of RPS19 mutations.22 Studies of mouse erythroblasts with ribosomal protein haploinsufficiency revealed impaired translation of specific transcripts essential for erythropoiesis involving internal ribosomal entry site (IRES).23 In addition, human K562 cells were shown to express alternatively spliced isoforms of FLVCR1 transcripts, which were affected by expressing shRNA against RPS19.24 Dysfunction of FLVCR1 as a heme exporter in erythroblasts25 might also play a role in the erythroid defects of DBA in addition to defective translation rate. Furthermore, decreased expression of the key erythroid signaling protein KIT was detected in fetal liver cells of a RPS19-deficient mouse, which may contribute to the erythroid failure in DBA.26 Although much has been discovered utilizing various models of ribosome deficiency, the exact mechanism of erythroid failure in DBA remains largely unknown. More in vivo experiments in various models are needed to elucidate the pathogenesis of DBA.

To gain a better understanding of the mechanism leading to the erythroid-specific defects in DBA, we generated two independent lines of rps19 mutants using transcription activator-like effector nuclease (TALEN)-mediated gene targeting in zebrafish.27 Genetic knockout of rps19 resulted in the characteristic erythroid defects similar to DBA including a lack of mature red blood cells and p53 activation. Interestingly, we observed that rps19 mutants had significantly reduced production of globin proteins accompanied by either increased or unaffected level of mRNA transcripts. Similarly, this phenomenon was observed in rpl11 mutant zebrafish. Furthermore, we observed that protein production in erythroid cells was decreased with either a mutation in rps19 or rpl11. Treatment with l-leucine improved the defect of protein production and partially rescued the anemic phenotype in both rps19 and rpl11 mutants. These findings suggest that decreased protein production in red blood cells is likely a key contributing factor to erythroid-specific defect in DBA.

Results

Generation of rps19 mutant with TALENs. Rps19 was the first mutated gene identified in human DBA patients.2 Zebrafish have a single copy of the rps19 gene that shares over 88% amino acid sequence identity with the human RPS19 gene. To generate a zebrafish DBA model carrying rps19 mutation, TALEN target sites were designed at the boundary of the second intron and the third exon of the rps19 gene (Figure 1a). The left and right TALENs were co-injected in zebrafish embryos at the one-cell stage to induce insertions or deletions (indels) into the rps19 gene (Figure 1b). This resulted in premature stop codons in the mRNA (Figure 1c). Two independent stable germline mutations of rps19 were established (Figures 1b and c). Both rps19 mutant lines displayed a similar phenotype and we chose the first mutant line (noted as ‘Mu1’ in Figure 1c) for further investigation. Expression analysis of rps19 mRNA showed 490% reduction in the rps19 homozygous mutants (Figure 1d) likely due to nonsense-mediated decay of mRNA. Because mutations of RPS19 in DBA patients include allele

Figure 1  The rps19 gene in zebrafish is targeted using TALENs. (a) Partial structure and sequence of the zebrafish rps19 gene. The binding sites of the left and right TALENs were underlined. The intron sequence was shown in lowercase and the exon in uppercase. P1 and P2 showed the primer sites for amplification. E2, E3, and E4 represent the second, third, and fourth exon of the rps19 gene, respectively. (b) Identification of mutations by enzyme digestion. PCR products (~305 bp) were processed with an enzyme that can recognize and cut at mismatch region. The presence of lower bands (~135 bp and ~170 bp) suggested a mutation. (c) Genomic sequences of the rps19 mutations. Deletions and insertions were shown in dashes and red letters, respectively. The premature stop codons produced in the two independent mutations were underlined. (d) Real-time PCR result showed rps19 gene expression was reduced by more than 99% in mutant 1. ***P<0.001; Student’s t-test). W, WT control; M, mutant; IC, internal control; L, DNA Ladder; bp, base pair
deletion, missense mutations, premature stop codons, and internal deletions, we reason that the rps19 mutant zebrafish would mimic the premature stop codon mutations found in DBA patients.

**Phenotypic analysis of rps19 mutants.** Rps19 homozygous mutants exhibited a similar phenotype as that of rps19 morphants.28 At 24 h post fertilization (hpf), rps19 homozygous mutants showed abnormal development of midbrain–hindbrain boundary (Figure 2a). A curved tail was observed in the mutant embryos at 48 hpf. Compared with siblings, rps19 mutants exhibited smaller heads and smaller eyes at 3 days post fertilization (dpf). The mutants developed edema (Figure 2a) and died ~5 dpf.

To determine if our rps19 mutants displayed a DBA-like hematopoietic phenotype, we investigated the expression of multiple primitive and definitive hematopoietic markers using RNA whole-mount in situ hybridization. The initial...
specification of the primitive hematopoietic cells located in the intermediate cell mass of zebrafish as marked by scl expression was not highly affected in rps19 homozygous mutants at 24 hpf (Figure 2b). However, compared to siblings, expression of the definitive hematopoietic stem cells (HSCs) marker c-myb was nearly absent in rps19 mutants at 3 dpf. O-Dianisidine staining revealed that rps19 mutants had greatly decreased hemoglobin levels at 4 dpf (Figure 2b). This indicated that the number of red blood cells was probably reduced in rps19 mutants. The reduction of red blood cells may be caused by a decreased cell survival and/or production of definitive HSCs. This is supported by the observation that lymphoid T cells in the thymus, marked by rag1 expression, were also nearly lacking in rps19 mutants at 4 dpf (Figure 2b).

Upregulation of the p53 signaling pathway was reported in the majority of DBA patients, DBA cell culture models, and animal models. As expected, rps19 deficiency in our mutants led to activation of the p53 signaling pathway (Figure 2c), including p53 and its target genes such as the cell-cycle arrest gene, p21 (Figure 2d), and the apoptotic gene, bax (Figure 2e). In addition, similar with the results previously found in rps29 mutants and other ribosomal protein-deficient models,29,30 knockdown of p53 expression could partially rescue mutant phenotype observed in rps19 homozygous mutants, including the morphological phenotype. Anemic phenotype observed in rps19 mutants was also partially improved by downregulation of p53 (Supplementary Figure S1). In conclusion, the rps19 mutants we generated with TALENs exhibited the characteristic phenotype of DBA patients and should be useful as a model to study pathogenesis of this genetic disease.

RNA and protein levels of globin genes are dissociated in rps19 mutants. The primary phenotype of DBA is anemia. In the course of characterizing embryonic hemoglobin gene expression in rps19 mutants we noted that mRNA for hemoglobin beta e1 (hbbe1) was not reduced whereas hemoglobin gene hbbe3 mRNA was increased by >50-fold (Figures 3a and b). This was somewhat unexpected given that rps19 mutants lacked globin proteins as revealed by o-dianisidine staining. To reconcile this issue, we obtained antibodies specifically recognizing hbbe1 or hbbe3 proteins and quantified globin protein levels by western blot analysis. As expected, we observed significant reduction of protein for both hbbe1 and hbbe3 (Figure 3c), suggesting that there is dissociation of RNA and protein levels for globin gene expression in rps19 mutants. As a control, we analyzed both the mRNA and protein levels for neutrophil marker mpx. We detected ~50% less mpx mRNA in rps19 mutants, compared with control embryos (Figure 3d). However, no reduction in mpx protein was detected in rps19 mutants (Figure 3e). This indicates that the dissociation of RNA and protein production is not ubiquitous but at least partially erythroid cell-specific or globin gene-specific effect.

Protein production in erythroid cells is decreased in rps19 mutants. Globin is the most abundant protein in red blood cells. The dissociation of globin genes’ RNA and protein production could result from the deficient protein production in erythroid cells. Alternatively, the dissociation can be due to globin gene-specific pathogenesis. In order to address these questions, we generated transgenic zebrafish specifically labeling red blood cells with mCherry. As shown in Figure 4a, zebrafish erythroid-specific regulatory region—locus control region (LCR) as well as a portion of a/hbc, bidirectional proximal promoter31 was cloned into mCherry reporter constructs. Because the dissociation of RNA and protein production was more significant for hbbe3 than hbbe1, we included the 5’ untranslated region (UTR) or 3’UTR of hbbe3 in these constructs (Figure 4a). We observed robust erythroid-specific expression of mCherry in these transgenic embryos, especially in mCherry control line (Supplementary Figure S2). We examined if the globin gene structure has any effects on mCherry expression at the mRNA level and protein level in rps19 mutants. Real-time PCR results showed equal to more mCherry mRNA in mCherry-positive rps19 mutant embryos than the control embryos at 3 dpf (Figure 4b). However, less mCherry protein, measured through fluorescent intensity, was detected in rps19 mutants compared to siblings in all three transgenic lines (Figures 4c and d). Interestingly, this RNA/protein production dissociation of mCherry was more severe in mCherry control line. Therefore, we observed the dissociation of RNA/protein levels with erythroid-specific mCherry expression regardless of gene structure of hbbe3. This indicates that protein production in erythroid cells was inhibited in rps19 mutants.

RNA and protein levels of globin genes are also dissociated in rpl11 mutants. Previously, a rpl11 mutant identified with a retrovirus-mediated mutation screening library,32 has been established as a zebrafish DBA model for its anemic phenotype, p53 upregulation, and metabolic defects.30 To determine if the RNA/protein production dissociation for globin gene expression was specific to rps19 mutant or general to DBA-associated ribosomal protein deficiency, we analyzed mRNA expression and protein level in zebrafish rpl11 mutants. Real-time PCR results showed slightly increased expression of hbbe1 mRNA in rpl11 mutant (Figure 5a), while expression of hbbe3 mRNA was increased by ~50-folds in rpl11 mutants compared to control embryos (Figure 5b). Furthermore, slightly less mpx mRNA was detected in rpl11 mutants at 3 dpf (Figure 5d). Whole-mount in situ hybridization analysis confirmed the real-time PCR as equal to more hbbe1 mRNA was observed in rpl11 mutants compared with siblings at 3 dpf (Supplementary Figure S3a). Expression of hbbe3 mRNA was significantly increased in rpl11 mutant (Supplementary Figure S3b); mpx exhibited similar expression levels between the siblings and rpl11 mutants (Supplementary Figure S3c). As we discovered in rps19 mutants, less hbbe1 and hbbe3 proteins were also shown in rpl11 mutants by western blot analysis (Figure 5e), whereas protein level of mpx was comparable between control and rpl11 mutants (Figure 5e). These findings demonstrated that the RNA/protein production dissociation of globin genes without concurrent dissociation seen in the neutrophil cells is likely a common phenomenon shared between multiple ribosome mutations found in DBA.
Protein production in erythroid cells is also decreased in rpl11 mutants. To assess protein production in erythroid cells in rpl11 mutants, we bred the transgenic lines into the rpl11 mutant line. As expected, approximately two- to fivefold more mCherry mRNA was detected by real-time PCR in mCherry-positive rpl11 mutant embryos compared with siblings at 3 dpf (Figure 6a). However, less mCherry protein was produced in rpl11 mutants (Figures 6b and c). This suggests that protein production in erythroid cells was also inhibited in rpl11 mutants. Similar with rps19 mutants, the dissociation of RNA and protein levels of mCherry was more obvious in rpl11 mutants of mCherry control transgenic line.

To exclude the possibility that general protein translational defect led to erythroid protein production deficit, we examined the translational rates in both models. Cells from wild-type control embryos and rps19 mutants were respectively starved by culturing in methionine and cysteine-free medium. Following starvation, 35S-labeled methionine and cysteine were incorporated into those cells and the radioactivity was measured. As shown in Supplementary Figure S4a, the global translation rate was reduced only ~40% in rps19

Figure 3  RNA and protein levels of globin genes are dissociated in rps19 mutants. (a) mRNA expression of globin gene hemoglobin beta e1 (hbbe1) was not affected or slightly decreased in rps19 mutant embryos at 3 dpf by real-time PCR analysis. (b) Real-time PCR results showed ~70-fold more hbbe3 mRNA was accumulated in rps19 mutant embryos than control embryos. (c) Western blot results indicated protein levels of both hbbe1 and hbbe3 were dramatically decreased in rps19 mutant embryos at 3 dpf. Histograms represented quantification of western blots. (d) Decreased mpx mRNA was detected in rps19 mutants compared with control embryos. (e) Equal to more mpx protein was produced in rps19 mutants than control embryos at 3 dpf. Histograms represented quantification of western blots. Actin acts as a loading control. The results are representative for three independent experiments. *P < 0.05; **P < 0.001; Student’s t-test
mutants compared to the control group, which was much less significant than the reduction of erythroid-specific mCherry protein levels in *rps19* transgenic mutants (~70–85%). We performed the same experiments with *rpl11* mutants. Results showed that general translation rate was comparable between control embryos and *rpl11* mutants.
(Supplementary Figure S4b). These findings suggest that the erythroid protein production defect we observed in both rps19 mutants and rpl11 mutants was not simply contributed by general translational defect. Meanwhile, expression of gata1, which is essential for differentiation of erythroid progenitor cells, was unaffected or slightly reduced in both rps19 mutants and rpl11 mutants, with more gata1 mRNA was detected in both mutant models at

**Figure 5**  RNA and protein levels of globin genes are also dissociated in rpl11 mutants. (a) mRNA expression of hbbe1 was increased to 1.5-fold in rpl11 mutant embryos at 3 dpf by real-time PCR analysis. (b) Real-time PCR results showed ~ 50-fold more hbbe3 mRNA was accumulated in rpl11 mutant embryos compared to control embryos. (c) Western blot results indicated dramatic decrease of hbbe1 and hbbe3 protein levels in rpl11 mutants at 3 dpf. Histograms represented quantification of western blots. (d) Slightly decreased mpx mRNA was detected in rpl11 mutants compared with control embryos. RNA was pooled from 10 embryos. (e) Mpx protein was detected by western blot. Expression of mpx protein was comparable between rpl11 mutant embryos and siblings, or more mpx protein was detected in rpl11 mutant embryos than siblings. Histograms represented quantification of western blots. Actin acts as a loading control. The results are representative for three independent experiments. *P<0.05; **P<0.01; Student’s t-test

**Figure 4**  Protein production in erythroid cells is decreased in rps19 mutants. (a) Representation of the constructs for generating transgenic zebrafish lines. Zebrafish erythroid-specific regulatory region – locus control region (LCR) as well as a portion of α/β α2 bidirectional proximal promoter was cloned into mCherry reporter constructs. 5'UTR or 3'UTR of hbbe3 was also included into one of the constructs. (b) Real-time PCR results showed more or the same amount of mCherry RNA in mCherry-positive rps19 mutant embryos compared with control group at 3 dpf. (c) Representative images of mCherry-positive embryos of different transgenic lines at 3 dpf. (d) Quantification of intensity of mCherry fluorescence in the embryos shown in c. Intensity of mCherry fluorescence shown in rps19 mutant mCherry control transgenic embryos was only 27% of the siblings. Approximately, 30% of mCherry fluorescence was exhibited by rps19 mutant hbbe3 5'UTR mCherry transgenic embryos, compared with the control embryos. The results are representative for three independent experiments. ***P<0.001; **P<0.01; Student’s t-test
Protein production in erythroid cells is also decreased in rpl11 mutants. (a) Real-time PCR results showed more than twofolds of mCherry RNA accumulated in mCherry-positive rpl11 mutant embryos compared with control groups at 3 dpf. (b) Representative images of mCherry-positive embryos of different transgenic lines at 3 dpf. Less mCherry expression was observed in rpl11 mutants than control embryos. Lateral view shown with anterior to the left. (c) Quantification of intensity of mCherry fluorescence in the embryos shown in b. Intensity of mCherry fluorescence shown in rpl11 mutant mCherry control transgenic embryos was ~50% of the siblings. Approximately, 40% of mCherry fluorescence was exhibited by rpl11 mutant hbbe3 3’UTR mCherry transgenic embryos, compared with the control embryos. The results are representative for three independent experiments. **P < 0.01; *P < 0.05; Student’s t-test.
This indicates the production of erythroid progenitors is probably unaffected. In addition, no significant difference was detected in the percentage of mCherry-positive cells in both rps19 and rpl11 mutant transgenic embryos from Tg (LCR: mCherry) at 3 dpf (Supplementary Figure S5e and f), suggesting that the defects of protein production in erythroid cells were not simply due to reduction of erythroid cell numbers.

Furthermore, to exclude any adverse effect of blood circulation on the defects of gene expression in erythroid cells in both ribosomal mutation models, we injected rhodamine fluorescent dye into heart and analyzed the status of circulation at 3 dpf. Results showed that circulation was intact in both rps19 and rpl11 mutants (Supplementary Figure S6). This indicates that deficits of protein production in erythroid cells observed in both ribosomal mutation models were not caused by impaired circulation.

Taken together, for the first time we uncovered an RNA/protein production dissociation of globin genes in both rps19 and rpl11 mutants. Furthermore, defective protein synthesis in erythroid cells was seen in both rps19 and rpl11 mutants. This indicates that accumulative defects of protein production in erythroid cells probably contribute to the reduction of globin proteins, which leads to the blood-specific defects in DBA patients.

L-leucine treatment alleviates the decreased protein production of globin genes and partially rescues the anemic phenotype in both rps19 and rpl11 mutants. L-Leucine is known to increase protein synthesis in skeletal muscle.33 Protein synthesis was increased in DBA-derived cells after treatment with L-leucine.22 L-Leucine treatment improved anemia in several animal models with ribosomal protein deficiency.34,35 To evaluate the effect of L-leucine treatment on our disease models, we treated both rps19 and rpl11 mutants with L-leucine. Compared to control, addition of L-leucine improved part of the developmental defects, including edema, in both rps19 and rpl11 mutants (Figures 7a and 8a). To assess if L-leucine treatment plays a role in the protein synthesis defect of globin genes described above, we analyzed expression of hbbe3. Real-time PCR results showed that expression of hbbe3 at mRNA level was comparable between control mutant embryos and mutant embryos raised in L-leucine in both rps19 and rpl11 mutants at 3 dpf (Figures 7b and 8b). However, protein level of hbbe3 was significantly increased in mutant embryos.
treated with L-leucine compared to control regardless of rps19 mutation or rpl11 mutation. Meanwhile, protein level of hbbe3 in wild-type and heterozygous siblings was not altered by the addition of L-leucine (Figures 7c and 8c). To address if increasing the protein production of globin gene could lead to the improvement of anemia observed in mutants, we performed o-dianisidine staining for both rps19 and rpl11 mutants. We found that L-leucine treatment partially improved the anemic phenotype in both rps19 and rpl11 mutants (Figures 7d and 8d). Overall, our rps19 model, as well as rpl11 mutants is responsive to L-leucine treatment. Anemia was improved in both rps19 and rpl11 mutants with L-leucine treatment, similar to human DBA patients following L-leucine treatment.36 Our zebrafish rps19 mutant line appears promising as a useful model towards better understanding the mechanistic defects in DBA and also aids in the discovery of novel therapeutics to treat the disease.

Discussion

In this study, we generated rps19 mutant zebrafish lines using TALENs. As reported in DBA patients, rps19 mutants exhibited hematopoietic phenotypes and developed anemia associated with activation of the p53 signaling pathway. We therefore believe that this rps19 mutant zebrafish should serve as a useful animal model to study the pathogenesis of DBA.

Mechanism of erythroid failure in DBA. RPS19 was the first identified gene whose mutation is currently identified in 25% of DBA patients. To date, mutations in at least other nine ribosomal genes, including RPL11, have been reported in DBA patients.12 The ribosomal stress and upregulation of the P53 signaling pathway caused by haploinsufficiency of ribosomal proteins are thought to lead to the clinical features of DBA.17,18 According to data from in vitro experiments, the specific erythropoietic defects in DBA were thought to be attributed to the hypersensitivity of erythroblasts to the upregulation of TP5317 and/or the high demand for ribosome synthetic rates of rapidly proliferative erythroid cells.26 Other hypotheses, including reduced translation of some specific transcripts essential for erythropoiesis23 also exist. Recently, a mutation of GATA1, an erythroid-specific transcription factor, was identified in two siblings and another unrelated DBA patients.37 Splicing of GATA1 was impaired by the
mutation. Mutation of the erythroid-specific gene may contribute to the erythroid-specific phenotype of DBA. However, it remains controversial whether these patients are real DBA cases, because their clinical manifestations did not meet all of the clinical diagnostic criteria for DBA.1

For the first time, our in vivo data demonstrated translational defects with globin genes in both rps19 and rpl11 mutants, despite increased or similar transcriptional levels. More importantly, we found that translation was disrupted specifically in erythrocytes in both rps19- and rpl11-deficient zebrafish. This is further supported by observations showing translation of a neutrophil-specific gene was largely unaffected in both mutant zebrafish. We speculate that protein synthesis defects may affect the survival of erythrocytes, for instance, through the activation of cell-cycle arrest and apoptosis in erythrocytes.38 These findings will advance our understanding toward the mechanisms of erythroid failure in DBA.

**Extra-ribosomal functions of ribosomal proteins.** Some ribosomal proteins have been reported to have unexpected functions outside of the ribosome (extra-ribosomal functions). In eukaryotic cells, RPL2, RPL30, RPS14, and RPS28 were reported to function by binding to their own transcripts or some other mechanisms to perform regulation of ribosomal protein production.39–42 RPL5, RPL11 and 5S rRNA were found to interact with MD2M, which is responsible for regulating the p53 signaling pathway via the ubiquitination and degradation of p53.43–46 RPL13a was reported to be involved in a transcript-specific translational control mechanism. RPL13a can be phosphorylated and released from the 60S ribosomal subunit in response to interferon-γ.47 Released RPL13a specifically binds the 3'UTR interferon-gamma-activated inhibitor of translation element of its target mRNAs and inhibits translation.48

Recently, analysis of RPL38's function provided insight into the specialized ribosome activity controlled by ribosomal proteins. Rpl38 was found to be deleted in tall short (Ts) mice. Rpl38 had higher expression in somites and neural tube, where Ts mice showed tissue-specific phenotype. Interestingly, translation of 8 of the 39 homeobox (Hox) genes, which are essential for the morphological development along the axial skeleton was reduced in Ts mice. The tissue-specific expression pattern of Rpl38 correlated with the specific phenotype observed in Ts mice.48 These findings suggested the possibility that increased expression of specific ribosomal proteins in unique tissues might lead to the heterogeneous ribosomes in the distinguished cell tissues by specificities in translating distinct classes of mRNAs. However, this is not the case for rps19 or rpl11. We checked the expression patterns of rps19, rpl11 and rpl38 in zebrafish using RNA whole-mount in situ hybridization. All of them had ubiquitous expression during embryonic development (data not shown). This suggested that the erythroid-specific translational defect exhibited by rps19- and rpl11-deficient zebrafish was not attributed to the tissue-specific expression of rps19 and rpl11. It is possible that some erythroid-specific ribosome-associated factors, which have interactions with rps19 and rpl11 may mediate this event by inhibiting the translation of erythroid genes. Application of ribosomal profiling strategy will put forward some candidates for this issue.

In summary, utilizing TALENs we generated rps19 mutants in zebrafish. Similar with DBA patients, rps19 mutants had hematopoietic defects and developed anemia; thus, serving as a novel DBA model. Using the newly generated line, we reported the RNA and protein production dissociation of globin genes in rps19 mutant and confirmed similar findings in rpl11 mutant. Further analysis demonstrates that the dissociation may result from reduced protein production in erythrocyte cells in rps19- or rpl11-deficient zebrafish. Expression, however, of neutrophil gene was unaffected. For the first time, our in vivo data suggested that the erythroid defects in DBA may be caused by the decreased protein production of erythrocyte cells in patients. Recently, L-leucine was found to improve the anemia of rps19-deficient zebrafish by activating the mTOR pathway;34 however, the underlying mechanism is still under investigation. Our results showed L-leucine treatment alleviated the protein production defects of globin gene and partially rescued the anemic phenotype in both rps19 and rpl11 mutants. Based on our findings, L-leucine may work through activating translation of erythroid cells. Our findings will aid in the understanding of clinical manifestation of DBA. Additionally, the newly derived rps19 mutants promise to be useful for investigating the basis for erythroid defects and serve as a tool to carry out therapeutic screens to develop much-needed treatments toward DBA.

**Materials and Methods**

**Zebrafish lines and maintenance.** Zebrafish (Danio rerio) was raised and maintained under standard laboratory conditions at 28.5 °C. All the three constructs for transgenic fish were generated utilizing the Gateway system (Invitrogen, Carlsbad, CA, USA). Approximately, 30 μg of the constructs together with 15 μg Tol2 mRNA were injected into the embryos at one-cell stage; 5 ng of pST ATG morpholino49 was injected into the embryos at one-cell stage for pST rescue experiment.

**Generating rps19 mutant by TALENs.** Design of TALENs target site using the online service at: https://talet-nct.cacornell.edu/node/add/talen. The sequence-specific TAL effector repeats against the right and left arms (Figure 1a) were constructed using the ‘unit assembly’ method.52 mRNAs encoding left and right TALENs were synthesized in vitro by using the T7 mMessage Machine Kit (Ambion, Austin, TX, USA) and purified using a kit from Invitrogen according to the manufacturer’s instructions. mRNAs were mixed at the ratio of 1:1 and co-injected into wild-type zebrafish embryos at one-cell stage at 100 pg mRNA/embryo as the final dose. Every three injected and morphologically normal embryos were mixed at 24 hpf to isolate genomic DNA followed by PCR amplification. Total of 24 embryos from each injection group were used to evaluate the efficiency of the generated TALENs. A 305 bp genomic DNA fragment containing the target site was PCR amplified from injected and/or uninjected control embryos. Primers used for this PCR were: forward: 5'-AAGTCCTGTCTTGGGACAA-3'; reverse: 5'-GAATTGACAGAAATTTACAGT-3'. The PCR products were denatured, annealed and digested with Transgenic Surveyor Mutation Detection Kit (Transgenic, Omaha, NE, USA) for Standard Gel Electrophoresis (catalog number: 706020) by following the manufacturer’s instruction. The digested PCR products were separated by electrophoresis on 2% agarose gel and/or 0.8% PAGE gel.

**Real-time PCR.** RNA was isolated from 7–15 embryos at 3 dph using Trizol (Invitrogen) according to the manufacturer’s protocol; 2 μg of total RNA was used for cDNA synthesis by reverse transcription using Oligo(dT)20–24 (Invitrogen). Real-time PCR was performed using FastStart Universal SYBR Green MasterMix (Roche Diagnostics, Indianapolis, IN, USA) and a MyQ Single-Color PCR thermal cycler (BioRad, Hercules, CA, USA). Real-time PCR primers used for p35 signaling pathway were from previous literature.12 Sequences of real-time PCR primers for ββεε: RTF: 5'-TTGGTGAGGACAGACTCTGGAA-3' and RTR: 5'-TCAGGGATTATAT-3'; for βεεε: RTF: 5'-ATGCTGTGTGAGAACTG-3' and RTR: 5'-ATTATCCATGTGAGAAGC-3'; for mpɛ: RTF3: 5'-GGGGAGGAGAA-3';
Whole-mount in situ hybridization. Whole-mount in situ hybridization was conducted as described using c-myb, mpx, rag1, hbbe1, and hbbe5 rhibprobes.

Whole-mount o-dianisidine staining. Hemoglobin was measured by whole-mount o-dianisidine as described previously.

Western blot analysis. Approximately, 15–20 embryos were rinsed and lysed in lysis buffer. Protein concentration was measured with bichonichinic acid Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) or NanoDrop 1000 Spectrophotometer (Thermo Scientific). 20–40 mg of total protein was employed for western blot analysis. Procedure of western blot was described previously. Primary antibodies for hbbe1 (AnaSpec, Fremont, CA, USA), mpx (AnaSpec), gata1a (AnaSpec), and anti (Sigma, St. Louis, MO, USA) and horseradish peroxidase-linked secondary antibodies (GE Healthcare, Upssala, Sweden) were used. Synthetic peptide corresponding to the N-terminal region of zebrafish hbbe3 was produced for generating customized primary antibody for hbbe3 (AnaSpec). Western blot results were quantified using ImageJ which was downloaded from NIH website.

Analysis of mCherry fluorescence. For each group, 28–30 embryos were photoed under fluorescent microscope (Zeiss, Oberkochen, Germany) at 3 dpf. The fluorescence of mCherry was analyzed using ImageJ. Images were opened using ImageJ software. The same thresholds for both control and mutant embryos were set up to reflect the expression of mCherry throughout the whole embryo. Area, minimum and maximum gray value, integrated density, and mean gray value were measured. Measurements were set as limit to threshold. The whole embryo was measured. The value of area and mean intensity was multiplied for the intensity of mCherry fluorescence for each embryo.

L-Leucine treatment. Embryos were divided into two groups at 10 hpf. One group was raised in fish water with 100 mM L-leucine (Sigma), and the other group was raised in fish water. Embryos were collected for the following experiments at 3 and 4 dpf.

Analysis of general translation rate. Overall, 40–80 embryos at 3 dpf were homogenized and dissociated into single cells with 0.25% Trypsin-EDTA (Invitrogen). Cells were counted. Comparable amount of cells (40–80 embryos at 3 dpf) were resuspended in 100 μl lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and proteinase inhibitor cocktail (Roche Diagnostics). Proteins in the lysate were precipitated by adding trichloroacetic acid (Sigma-Aldrich) and incubated for 30 min on ice. Proteins were recovered by vacuum filtration on cellulose filters (Whatman, Upppsala, Sweden) and dried out. The filters were dissolved with trichloroacetic acid, and radioactivity from samples were measured with Liquid Scintillation Analyzer (Perkin Elmer). Meanwhile, protein concentration was measured using NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Examination of circulation by angiocardiography. Embryos at 3 dpf were treated with 0.1% tricaine. After injecting rhodamine fluorescent dye into their hearts, embryos were immediately observed and pictured with fluorescent microscope (Zeiss); 8–10 embryos for each group were injected and observed.

Analysis of percentage of mCherry-positive cells. Equal number (30–50) of transgenic embryos at 3 dpf were homogenized and dissociated into single cells with 0.25% Trypsin-EDTA (Invitrogen). Cells were rinsed twice and resuspended in 100 μl PBS. The percentage of mCherry-positive cells was analyzed using BD LSRRFortessa cell analyzer (BD Biosciences, San Jose, CA, USA).
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