Ribosomal protein deficiency causes Tp53-independent erythropoiesis failure in zebrafish

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ABSTRACT

Diamond-Blackfan anemia is an inherited genetic disease caused by mutations in ribosomal protein genes. The disease is characterized by bone marrow failure, congenital anomalies, and a severe erythroid defect. The activation of the TP53 pathway has been suggested to be critical for the pathophysiology of Diamond-Blackfan anemia. While this pathway plays a role in the morphological defects that associate with ribosomal protein loss-of-function in animal models, its role in the erythroid defects has not been clearly established. To understand the specificity of erythroid defects in Diamond-Blackfan anemia, we knocked down five RP genes (two Diamond-Blackfan anemia-associated and three non-Diamond-Blackfan anemia-associated) in zebrafish and analyzed the effects on the developmental and erythroid phenotypes in the presence and absence of Tp53. The co-inhibition of Tp53 activity rescued the morphological deformities but did not alleviate the erythroid aplasia indicating that ribosomal protein deficiency causes erythroid failure in a Tp53-independent manner. Interestingly, treatment with L-Leucine or L-Arginine, amino acids that augment mRNA translation via mTOR pathway, rescued the morphological defects and resulted in a substantial recovery of erythroid cells. Our results suggest that altered translation because of impaired ribosome function could be responsible for the morphological and erythroid defects in ribosomal protein-deficient zebrafish.

Keywords: Ribosomopathy, DBA, ribosomal proteins, Tp53, zebrafish

Abbreviations:

DBA, Diamond-Blackfan Anemia; RP, ribosomal protein; MO, morpholino antisense oligo nucleotide; mTOR, mammalian target of rapamycin.

1. Introduction

Mutations in genes that encode proteins involved in ribosome biogenesis can cause specific disease conditions in humans called ribosomopathies, which are a collection of rare genetic disorders that mainly affect the bone marrow (Narla and Ebert, 2010). Diamond-Blackfan anemia (DBA) is a ribosomopathy caused by the mutation of ribosomal protein (RP) genes. DBA patients typically display a prominent tissue-specific phenotype: a loss of erythroid cells in the bone marrow that results in severe anemia. Some patients also exhibit other pleiotropic anomalies, such as growth retardation, craniofacial deformities, upper limb malformations and heart and kidney dysfunction (Lipton and Ellis, 2010). Currently, mutations have been identified in 10 RP genes (DBA-associated RPs); *RPS19* is the most frequently mutated gene in DBA patients (25% of the patients). The other DBA-associated RPs, which are less frequently mutated in patients, include *RPL5* (7%), *RPL11* (5-10%), *RPL35A* (2-4%), *RPS24* (2%), *RPS7* (1%), *RPS10* (2-6%), *RPS17* (1%), *RPS26* (2-6%) and *RPL26* (<1%) (Gazda et al., 2012; Vlachos et al., 2013). The recent identification of a large deletion in *RPL15* in a DBA patient expanded the list of DBA-associated RPs (Landowski et al., 2013). These known RP mutations now account for approximately half (~55%) of the DBA patients. However, the genes mutated in the other DBA patients remain unknown. Although it has been confirmed that ribosome biogenesis is defective in DBA, it is unclear how impaired ribosome synthesis specifically affects erythrocyte maturation.

The disruption of ribosome biogenesis evokes a nucleolar stress response, which activates the TP53 signaling pathway (Chakraborty et al., 2011). Studies in a variety of cellular and animal models have highlighted the critical role of TP53 in the clinical

manifestation of DBA. Human haematopoietic progenitor cells harbouring an shRNA-mediated knockdown of the *RPS19* gene display increased TP53 activity specifically in the erythroid lineage (Dutt et al., 2011). In transgenic mice with RPS19 deficiency, activated TP53 was responsible for the anemia phenotype (Jaako et al., 2011). Similarly, in zebrafish, the loss of Rpl11 and Rps29 resulted in Tp53-mediated developmental defects and impaired erythropoiesis (Danilova et al., 2011; Taylor et al., 2012). In previous studies, we demonstrated that the knockdown of RPs caused numerous common anomalies in zebrafish (Uechi et al., 2006) and that the co-inhibition of Tp53 rescued the morphological abnormalities associated with Rpl11 and Rps19 deficiency (Chakraborty et al., 2009; Torihara et al., 2011). It is generally accepted that the TP53 pathway is responsible for the morphological phenotypes that are associated with DBA. However, the role of TP53 in the erythroid phenotype of DBA has not been clearly established. The knockdown of *RPS19* in erythroid cells resulted in cell cycle arrest and proliferation defects via TP53-independent pathways (Iadevaia et al., 2010). Similarly, we have demonstrated that Rps19 deficiency in zebrafish caused erythroid defects, even in the absence of Tp53 (Torihara et al., 2011). Interestingly, in Rps7-deficient zebrafish, the simultaneous suppression of Tp53 activity resulted in incomplete rescue of the erythroid and morphological abnormalities (Duan et al., 2011).

In this study, we knocked down five RP genes (two DBA-associated and three non-DBA-associated) in zebrafish and analyzed the consequences of RP deficiency on morphological and erythroid development in the presence and absence of Tp53. Our results indicated that any RP deficiency, regardless of its role in DBA, led to erythropoietic failure in zebrafish in a Tp53-independent manner. We further demonstrated that treatment of RP-deficient embryos with amino acids that enhance

translational efficiency via the mTOR pathway rescued the morphological abnormalities and the erythroid defects. Our results support the hypothesis that altered translation because of impaired ribosome function is responsible for the developmental and erythroid phenotypes that are associated with RP deficiency.

2. Materials and methods

2.1. Zebrafish maintenance

The fish were maintained in optimum conditions as per standard guidelines. The embryos were raised in E3 embryo medium at 28.5°C. Wild-type embryos were obtained from AB lines. The *tp53* homozygous mutant line (*tp53*$^{m214k/m214k}$), which has a point mutation in the DNA-binding domain (Berghmans et al., 2005), was purchased from Zebrafish International Resource Center (ZIRC; http://zebrafish.org/zirc/home/guide.php).

2.2. Morpholino injections

Morpholinos (MOs) to knockdown RP genes were obtained from Gene Tools, LLC. For individual RPs, MOs were injected at 0.5 μg/μl using an IM-30 Electric Micro-injector (Narishige, Tokyo, Japan). In double-knockdown embryos, MOs for the RP and *tp53* were injected as a mixture at 0.5 μg/μl. The MO sequences targeting the RP genes were described previously by Uechi et al. (2006), and the p53 MO sequence was reported by Langheinrich et al. (2002).

2.3. In vitro mRNA synthesis and rescue experiments

To generate MO-resistant mRNA, five nucleotides were changed at the MO target site in the 5'UTR of *rpl35a* cDNA without altering the encoded amino acids (GenBank accession no. NM_001002487.1). The full-length *rpl35a* cDNA was PCR amplified, inserted into the pCS2+ vector and transformed in DH5α competent cells (Toyobo, Osaka, Japan). Capped *rpl35a* mRNA was generated *in vitro* from linearized pCS2+/*rpl35a* using SP6 polymerase in the mMESSAGE mMACHINE kit (Ambion, Foster City, CA, USA). Rescue experiments were performed by injecting a mixture of *rpl35a* mRNA (350 ng/μl) and *rpl35a* MO (0.5 μg/μl).

2.4. Hemoglobin staining

The density of blood cells around the cardial vein at 48 hours post fertilization (hpf) was detected by hemoglobin staining using *o*-dianisidine as previously described (Detrich et al., 1995).

2.5. Amino acid and rapamycin treatment

L-Leucine (Sigma-Aldrich, St. Louis, MO, USA) was utilized at a final concentration of 100 mM as previously described (Payne et al., 2012). L-Arginine monohydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was utilized at a final concentration of 50 mM. L-Alanine and L-Glycine (Wako, Osaka, Japan) were

utilized at a final concentration of 100 mM, respectively. The amino acids were directly added to the E3 embryo media, and 1-day-old embryos were treated for 24 hours. Rapamycin (LC Laboratories, Woburn, MA, USA) was prepared in DMSO and added to the E3 embryo media at a final concentration of 0.25 μM, 0.5 μM and 1.0 μM. To prevent pigmentation in the treated embryos, PTU was added at a final concentration of 0.2 mM.

3. Results

3.1. Developmental abnormalities in RP-deficient zebrafish

Previously, we demonstrated that the systematic knockdown of 21 RPs caused developmental defects in zebrafish and that increased Tp53 activity was responsible for the morphological abnormalities that are associated with the deficiency of two DBA-associated RPs (Rpl11 and Rps19) (Uechi et al., 2006; Chakraborty et al., 2009; Torihara et al., 2011). To determine whether Tp53 is responsible for the common morphological defects that are observed in other RP-deficient zebrafish (Uechi et al., 2006), we knocked down five RP genes, including two DBA-associated genes and three non-DBA-associated genes, and analyzed the consequences of RP depletion on embryonic development. For the DBA-associated RPs, we chose to study *rpl35a* and *rps24* because loss-of-function analyses of these two RPs have not yet been performed in animal models. For the non-DBA-associated RPs, we selected *rps3, rpl35* and *rplp1* because no mutations have been identified in these RP genes in DBA patients.

At 24 hpf, a stage when many organs are already recognizable, all of the RP knockdown zebrafish displayed morphological abnormalities, such as aplasia in the brain and a bent tail (Fig. 1A and 1B). Other anomalies commonly associated with RP knockdown embryos, such as a thin yolk sac extension, a thin body trunk and reduced body length, were also observed in the RP-deficient embryos (Fig. 1A and 1B). By 48 hpf, when morphogenesis is almost complete and the blood cells are in circulation, the RP knockdown embryos had significantly smaller heads and eyes, a defective heart with pericardial edema and reduced pigmentation (Fig. 1C and Supplementary Fig. 1). The control MO-injected embryos did not exhibit any morphological defects (Fig. 1C and Supplementary Fig. 2). The co-injection of MO-resistant mRNA completely rescued the developmental defects, indicating that the phenotypes specifically resulted from RP deficiency (data shown for *rpl35a*, Fig. 1A and 1C).

3.2. Rescuing the developmental abnormalities in RP-deficient zebrafish via the simultaneous loss of functional Tp53

The morphological defects in RP-deficient zebrafish are Tp53-dependent. To determine whether this was true for the five RP-deficient zebrafish studied here, we knocked down the five RPs in *tp53* homozygous mutant ($tp53^{Mut}$) embryos and observed the phenotypes at 24 hpf. As expected, none of the RP-deficient/*tp53*-mutant embryos displayed any abnormalities, indicating that the simultaneous loss of Tp53 rescued the developmental defects in the RP-deficient zebrafish (Fig. 1A and 1B). The double knockdown of RP and Tp53 using MOs in a wild-type background ($tp53^{WT}$) resulted in embryos that were indistinguishable from uninjected controls,

further confirming the role of Tp53 in the morphological abnormalities that are associated with RP deficiency (data not shown).

3.3. The incomplete recovery of erythroid cells in RP-deficient zebrafish by the simultaneous loss of functional Tp53

The most conspicuous phenotype of DBA is the loss of erythroid cells in the bone marrow, which results in severe anemia. In previous studies, we demonstrated that the loss of Rps19 resulted in a drastic reduction of erythrocytes in zebrafish in a Tp53-independent manner (Uechi et al., 2008, Torihara et al., 2011). To determine whether the loss of Rpl35a recapitulates the erythroid phenotype of DBA in zebrafish, we performed hemoglobin staining of Rpl35a-deficient wild-type (*tp53*^{WT}) and *tp53*-mutant (*tp53*^{Mut}) embryos. The number of circulating erythrocytes was significantly reduced in the cardial vein of Rpl35a-deficient embryos compared with the uninjected controls (Fig. 2A). The simultaneous knockdown of *tp53* failed to restore the erythrocyte count in Rpl35a-deficient embryos, whereas the coinjection of MO-resistant *rpl35a* mRNA resulted in a nearly complete recovery of blood cells (Fig. 2A). The knockdown of *rpl35a* in *tp53*-mutant embryos significantly decreased the erythrocyte count compared with uninjected and control MO-injected embryos (Fig. 2A). Similarly, the knockdown of another DBA-associated RP, *rps24*, and three other non-DBA-associated RPs (*rps3*, *rpl35* and *rplp1*) in *tp53*^{Mut} embryos resulted in severe erythroid failure, as evidenced by the significant reduction in hemoglobin staining intensity in the cardial vein (Fig. 2B). The co-inhibition of these four RPs and Tp53 in *tp53*^{WT} embryos also resulted in similar erythroid defects (Fig. 2B). The co-injection of Control MO and Tp53 MO in *tp53*^{WT} embryos did not result in any

erythroid defects (Fig. 2B). We further evaluated the decreased erythrocyte density in Rpl35a- and Rps24-deficient embryos compared with uninjected and mRNA-rescued embryos and categorized them into three levels: normal, moderate and severely reduced. The majority of the Rpl35a-deficient embryos (91%) exhibited a severely reduced erythrocyte density in the *tp53*-mutant background (*rpl35a*^{MO}/*tp53*^{Mut}), which was comparable to the percentage of double knockdown embryos with a severely reduced erythrocyte density (88%) in the wild-type background (*rpl35a*^{MO}/*tp53*^{MO}) (Fig. 2C). Only a small percentage of the embryos (9-12%) displayed a moderate decrease in erythrocytes in both *tp53*-mutant and the Tp53-deficient wild-type backgrounds. In contrast, the majority of the *rpl35a* mRNA-rescued embryos (88%) had a normal erythrocyte density. As expected, 100% of the Rpl35A-deficient embryos displayed severely reduced erythrocyte density in the presence of Tp53 (*rpl35a*^{MO}/*tp53*^{WT}), whereas none of the uninjected *tp53*^{WT} or *tp53*^{Mut} embryos exhibited severe or moderate decreases in erythrocyte density. Similar reductions in erythrocyte density were observed when *rps24* was knocked down in the *tp53*-mutant and the Tp53-deficient wild-type backgrounds (Fig. 2C). These results indicated that the deficiency of any RP, regardless of its role in DBA, led to erythroid failure and that the simultaneous suppression of Tp53 function did not rescue the erythroid defects in RP-deficient zebrafish.

3.4. Rescuing the morphological and erythroid defects in RP-deficient zebrafish with L-Leucine or L-Arginine treatment

Certain amino acids, such as L-Leucine and L-Arginine, stimulate cell proliferation by augmenting cap-dependent mRNA translation through the mTOR-

RPS6K-RPS6-EIF4EBP1 signaling pathway (Kim et al., 2013). Previously, Payne et al. (2012) demonstrated that L-Leucine improved the developmental and anemia phenotypes in Rps19- and Rps14-deficient zebrafish by activating the mammalian target of rapamycin complex 1 (mTORC1), which is an important component of the mTOR pathway. To determine whether L-Leucine or L-Arginine could alleviate the morphological and erythroid defects in Rpl35a-deficient embryos, we treated $rpl35a^{MO}/tp53^{WT}$ embryos with L-Leucine or L-Arginine for 24 hours and analyzed the effects on the morphological and erythroid defects. Compared with untreated knockdown embryos, the morphological abnormalities, such as the small eyes and bent tail that are associated with Rpl35a deficiency, were almost completely reversed in L-Leucine- or L-Arginine-treated embryos (Fig. 3). We also observed a substantial recovery of the anemia phenotype in L-Leucine- or L-Arginine-treated Rpl35a-deficient embryos, as evidenced by the significant increase in hemoglobin staining in the cardial vein (Fig. 4A). Treatment with L-Leucine or L-Arginine returned the erythrocyte density to normal in 48% or 59%, respectively, of the Rpl35a-deficient embryos (Fig. 4B). To determine if the phenotypic rescue in Rpl35a-deficient embryos by L-Leucine and L-Arginine was due to the activation of the mTOR pathway, we treated the embryos with L-Alanine or L-Glycine, amino acids not known to stimulate the mTOR signaling. Treatments with L-Alanine and L-Glycine did not improve the morphological defects or anemia in Rpl35a-deficient embryos, suggesting an involvement of the mTOR pathway (Supplementary Fig. 3 and 4). To further confirm the role of mTOR, we assessed the effects of rapamycin, a specific inhibitor of the mTORC1 complex, on the phenotypic rescue of amino acid-treated Rpl35a-deficient embryos. The presence of rapamycin prevented the morphological and erythroid recovery in L-Leucine and L-Arginine treated Rpl35a-deficient

embryos (Supplementary Fig. 3 and 4). Treatment with rapamycin alone did not result in any morphological or erythroid phenotypes in the wild-type embryos (Supplementary Fig. 3 and 4). These results indicated that L-Leucine or L-Arginine improved the developmental and erythroid abnormalities of RP-deficient zebrafish via activation of the mTOR pathway.

4. Discussion

Over the last decade, remarkable progress has been made in understanding the molecular mechanisms of DBA pathophysiology. What was initially thought to be a disease involving extra-ribosomal functions of RPS19 has now been confirmed as a disease of ribosome dysfunction. Although *RPS19* remains the most commonly mutated gene in DBA, genome-wide exome sequencing of all RP genes in a large number of DBA patients without mutations in *RPS19* has revealed mutations in 9 other RP genes (reviewed in Chakraborty and Kenmochi, 2012; Gazda et al., 2012). Recently, an array-comparative genomic hybridization analysis identified large deletions in another novel RP gene, *RPL15* (Landowski et al., 2013). Currently, 11 RPs are known to be involved in DBA, but these identified mutations only account for approximately half (~55%) of all DBA cases; the genes that are mutated in the remaining DBA cases are unknown.

Several hypotheses have been proposed for the pathophysiology of DBA (Ball, 2011), but it is unclear how mutations in ubiquitously expressed RP genes specifically affect erythropoiesis. Ribosomal malfunction triggers nucleolar stress, which results in the translocation of several RPs to the nucleoplasm where they bind to and sequester MDM2 from TP53, leading to the activation of the TP53 signalling pathway

(reviewed in Chakraborty and Kenmochi, 2012). Several *in vitro* (Sieff et al., 2010; Dutt et al., 2011; Moniz et al., 2012) and *in vivo* (McGowan et al., 2008; Danilova et al., 2008; Danilova et al., 2011; Jaako et al., 2011) studies have highlighted the central role of TP53 in erythropoietic failure in DBA. However, *RPL11* and *RPL5*, two important RPs essential for ribosomal stress-mediated TP53 activation, are mutated in DBA (Gazda et al., 2008), indicating that TP53-independent pathways could also play a role in the erythroid phenotype of DBA. In this study, we observed that the individual knockdown of five different RP genes resulted in morphological abnormalities and erythroid defects in zebrafish embryos. Suppressing Tp53 activity in zebrafish embryos, either by genetic inactivation or antisense-mediated inhibition, rescued the morphological abnormalities, which is consistent with the results obtained in mouse and zebrafish models of DBA (McGowan et al., 2008; Chakraborty et al., 2009; Torihara et al., 2011), but failed to restore the erythroid cell count. Interestingly, erythroid insufficiency was a common phenotype in all the RP-deficient zebrafish, including the non-DBA-associated RPs.

We do not know whether erythroid defect is a general response to the deficiency of all the RPs, but our results suggested that the erythroid failure observed in our RP-deficient zebrafish is independent of Tp53 activity, which is in contrast to the widely accepted notion that the erythroid insufficiency in DBA is due to an activated TP53 response. It should be noted that despite the development of several cellular and animal models for DBA, the observations in these systems have not been consistent and the role of TP53 has not been clearly defined. For instance, RPS19-deficient human hematopoietic progenitor cells (Dutt et al., 2011) or *RPS19* mutated primary hematopoietic cells from DBA patients (Moniz et al., 2012) exhibited cell cycle arrest and apoptosis in the erythroid lineage, which were reverted upon TP53 depletion.

However, loss of RPS19 resulted in cell cycle arrest and block of cell proliferation even in TP53 mutated erythroid cell lines (Iadevaia et al., 2010), suggesting that the erythroid defect upon RP deficiency is not necessarily dependent on functional TP53 alone. Similarly, in animal models of DBA, the severity of erythroid defects has been a matter of concern. RPS19 heterozygous mice displayed either a normal phenotype (Mattson et al., 2004) or mild anemia (McGowan et al., 2008; Jaako et al., 2011), whereas RPS7 heterozygous mice showed no apparent erythroid defects (Watkins-Chow et al., 2013). Although the suppression of TP53 rescued the erythroid defects in RPS19-deficient mice, it is not known if the same could be recapitulated in mouse models with severe anemic conditions as seen in DBA patients. On the other hand, in zebrafish, deficiency of DBA-associated RPs (Rps19, Rpl11, and Rps7) resulted in severe anemia (Uechi et al., 2008; Danilova et al., 2008; Duan et al., 2011; Danilova et al., 2011), which is comparable to the patient phenotype, but the role of Tp53 has been controversial. For instance, when Rps19 was knocked down in *tp53* $^{-/-}$ mutants the morphological and developmental defects were rescued (Danilova et al., 2008), but the erythroid defects were not rescued when Tp53 was co-inhibited by MO (Torihara et al., 2011). In contrast, MO-mediated tp53 depletion in *rpl11*$^{-/-}$ mutants resulted in increased erythrocyte counts compared to un-injected *rpl11*$^{-/-}$ mutants (Danilova et al., 2011). We speculate that the variation in Tp53 response could be caused by differences between homozygous mutants and knocked down animals. The maternal mRNA deposited in homozygous mutant embryos from the heterozygous parent might compensate in part for the loss of an RP. On the other hand, the antisense morpholino would be expected to knockdown any target mRNA, including the maternally derived ones. In this study, we used both *tp53* MO and *tp53* $^{-/-}$ mutants in order to avoid discrepancies that might arise due to differences in experimental

approach (antisense-mediated inhibition vs. genetic inactivation) as seen in previous studies.

In this study, treatment with amino acids, such as L-Leucine or L-Arginine, rescued the anemia and morphological abnormalities in Rpl35a-deficient embryos. L-Leucine was previously reported to improve the anemia phenotype in zebrafish and mouse models of DBA (Payne et al., 2012; Jaako et al., 2012), and our data corroborate these previous observations. Studies of DBA patient-derived lymphoblasts and fibroblasts have demonstrated that haploinsufficiency of an RP leads to a reduction in global translation (Cmejlova et al., 2006; Avondo et al., 2009). Our RP-deficient zebrafish recovered from the morphological and erythroid defects when they were treated with amino acids, which stimulate cap-dependent mRNA translation via mTOR activation (Kim et al., 2013). Treatment with rapamycin, an inhibitor of mTORC1, abrogated the morphological and erythroid recovery in amino acid-treated RP-deficient embryos, further confirming the involvement of the mTOR pathway. These findings strongly indicated that the morphological and erythroid defects in RP-deficient zebrafish embryos might be due to a decrease in overall translation. The haploinsufficiency of ribosomal proteins results in defective ribosome biogenesis, which may limit the number of functionally active ribosomes or increase the abundance of defective ribosomes. A quantitative or qualitative change in ribosome production may alter the translational output, resulting in decreased overall translation or in the selective inhibition of the translation of specific mRNAs. Recently, it was reported that the loss of RPS19 and RPL11 in mouse erythroblasts impaired the translation of erythroid-specific transcripts, such as *BAG1* and *CSDE,* although the role of these proteins in erythropoiesis is not clearly defined (Horos et al., 2012).

The identification of mutations in several RP genes in DBA patients has made it clear that DBA is not a disease caused by the loss of function of a specific RP but is instead a disease caused by defects in overall ribosome production. Ribosome biogenesis defects lead to two different outcomes: a cellular stress response that involves TP53 and impaired translation that alters the translational output. In this study, the morphological abnormalities associated with RP deficiency were dependent on Tp53, but the erythroid defects were Tp53-independent. Interestingly, the morphological and erythroid abnormalities improved when the RP-deficient zebrafish were treated with amino acids, which augment mRNA translation via mTOR activation. Therefore, we hypothesized that the reduced expression of RPs decreased the translational efficiency of the ribosomes, which impacted global translation. However, the specificity of erythroid defects in DBA suggests that the effects of decreased translation may be more pronounced on particular mRNAs that are specifically expressed in erythroid cells. Identifying these mRNAs will be crucial for developing efficient diagnostic and treatment strategies for DBA.

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Figure legends

Fig. 1. Morphological abnormalities in RP-deficient zebrafish. Lateral images of embryos at 24 and 48 hpf. (A) Knockdown of *rpl35a* (*rpl35a*^{MO}) in wild-type embryos (*tp53*^{WT}) caused aplasia in the brain (indicated with a triangle), small eyes, a thin yolk extension (black solid line) and trunk, reduced body length and a bent tail (black dotted curve) at 24 hpf. The co-injection of *rpl35a* mRNA and *rpl35a* MO (*rpl35a*^{MO}*/mRNA*) or the knockdown of *rpl35a* in *tp53* mutants (*tp53*^{Mut}) completely rescued these morphological phenotypes. (B) The individual knockdown of RP genes (*rps24, rps3, rpl35 or rplp1*) resulted in similar morphological phenotypes in *tp53*^{WT}, but not *tp53*^{Mut}, embryos. (C) The co-injection of exogenous *rpl35a* mRNA rescued the morphological defects in Rpl35a-deficient zebrafish, even at later stages of development (48 hpf). Scale bar: 500 μm.

Fig. 2. Erythropoiesis failure in RP-deficient zebrafish. (A-B) Hemoglobin staining of cardial veins at 48 hpf. (A) Compared with the uninjected embryos, the wild-type (*tp53*^{WT}) and *tp53* mutant (*tp53*^{Mut}) embryos injected with *rpl35a* MO (*rpl35a*^{MO}) exhibited a significant reduction in the number of hemoglobin-stained cells (orange dots). *tp53*^{WT} embryos co-injected with *rpl35a* mRNA and *rpl35a* MO (*rpl35a*^{MO}*/mRNA*) had a nearly complete recovery of blood cells, whereas only a few blood cells were observed in double-knockdown *tp53*^{WT} embryos (*rpl35a*^{MO}*/tp53*^{MO}) or in *rpl35a*-knockdown *tp53*^{Mut} embryos (*rpl35a*^{MO}*/tp53*^{Mut}). Control MO-injected embryos did not exhibit any differences in the hemoglobin staining pattern compared with uninjected embryos. (B) The double-knockdown of RPs (*rps24*^{MO}, *rps3*^{MO}, *rpl35*^{MO} or *rplp1*^{MO}) and *tp53* (*tp53*^{MO}) in *tp53*^{WT} embryos (upper panel) or the single-

knockdown in *tp53*^{Mut} embryos (lower panel) resulted in very weak hemoglobin staining, suggesting a decrease in the number of blood cells. Control MO and tp53 MO-injected embryos exhibited a normal hemoglobin staining pattern. (C) The density of circulating erythrocytes was scored at three levels: normal, moderate and severe deficiency, relative to uninjected embryos. The percentage of embryos in each level was calculated. The highlighted values represent the most frequently observed level of the erythrocyte density in each group.

Fig. 3. Amino acid treatment of Rpl35a-deficient embryos. (A-B) Lateral images of embryos at 48 hpf showing an almost complete recovery of the small eye (arrow), pericardial edema (black triangle) and bent tail (dotted line) phenotypes in Rpl35a-deficient embryos (*rpl35a*^{MO}) after treatment with L-Leucine (*rpl35a*^{MO}/L-Leu+) or L-Arginine (*rpl35a*^{MO}/L-Arg+). Scale bar: A, 100 μm and B, 500 μm.

Fig. 4. Erythrocyte recovery in Rpl35a-deficient embryos treated with amino acids. (A) Hemoglobin staining of cardial veins at day 3 post-fertilization. Compared with the control MO-injected group, Rpl35a-deficient embryos (*rpl35a*^{MO}) exhibited severe anemia, but treatment with L-Leucine (*rpl35a*^{MO}/L-Leu+) or L-Arginine (*rpl35a*^{MO}/L-Arg+) significantly reversed this phenotype. (B) The density of circulating erythrocytes was scored at three levels: normal, moderate and severe deficiency, relative to uninjected embryos. The percentage of embryos in each level was calculated. The highlighted values represent the most frequently observed level of the erythrocyte density in each group.

Supplementary data

Supplementary Fig. 1. Morphological abnormalities in RP-deficient zebrafish at later developmental stages (48 hpf). Lateral images of *rps24, rps3, rpl35 and rplp1* knockdown embryos illustrate the anomalies, such as a smaller head and eyes, pericardial edema, and reduced pigmentation. Scale bar: 500 μm.

Supplementary Fig. 2. Morphology of the control MO-injected embryos. Lateral views of the embryos at 24 hpf are presented. No morphological defects were observed in control MO-injected *tp53* wild-type and *tp53* mutant embryos at 24 hpf. Scale bar: 500 μm.

Supplementary Fig. 3. Morphology of Rpl35a-deficient embryos treated with amino acids and rapamycin. Lateral images of embryos at 48 hpf. Uninjected wild-type embryos treated with rapamycin (Rap+) at a concentration of 0.5 μM and 1.0 μM did not develop any significant morphological defects at 48 hpf. Rpl35a-deficient embryos ($rpl35a^{MO}$) displayed morphological defects, which were alleviated by L-Leucine (L-Leu+) or L-Arginine (L-Arg+) treatment. Rapamycin at a concentration of 1.0 μM inhibited the recovery of morphological defects in Rpl35a-deficient embryos treated with L-Leucine (L-Leu+/Rap+) or L-Arginine (L-Arg+/Rap+), respectively. Treatment with L-Alanine (L-Ala+) or L-Glycine (L-Gly+) failed to improve the morphological defects in Rpl35a-deficient embryos. Scale bar: 500 μm.

Supplementary Fig. 4. Erythropoiesis in Rpl35a-deficient embryos treated with amino acids and rapamycin. Uninjected wild-type embryos treated with rapamycin

(Rap) at a concentration of 0.25 μM, 0.5 μM and 1.0 μM displayed normal haemoglobin staining. Rpl35a-deficient embryos (*rpl35a*^{MO}) displayed severely decreased erythrocyte density, which was significantly recovered by L-Leucine (*rpl35a*^{MO}/L-Leu+) or L-Arginine (*rpl35a*^{MO}/L-Arg+) treatment. Rapamycin (Rap) at a concentration of 0.5 μM and 1.0 μM inhibited the erythroid recovery in Rpl35a-deficient embryos treated with L-Leucine and L-Arginine, respectively. L-Alanine (*rpl35a*^{MO}/L-Ala+) or L-Glycine (*rpl35a*^{MO}/L-Gly+) treatment failed to reverse the erythroid defect in Rpl35a-deficient embryos.

Fig. 1

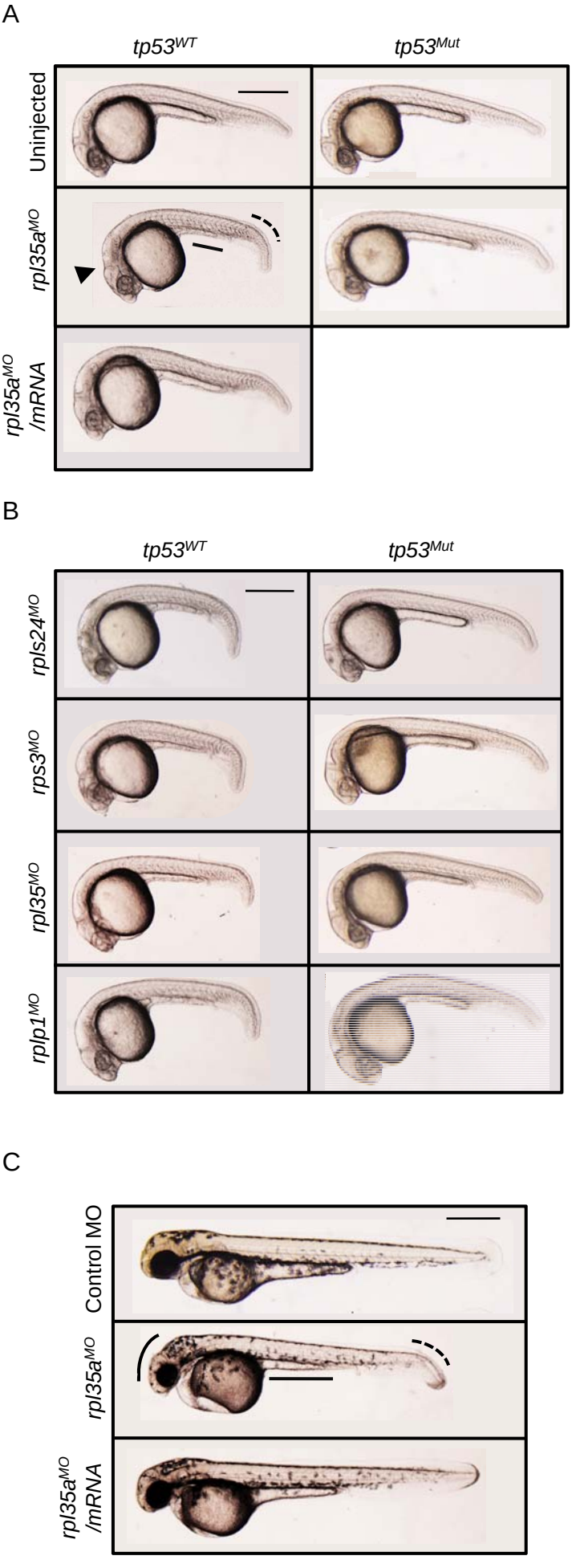
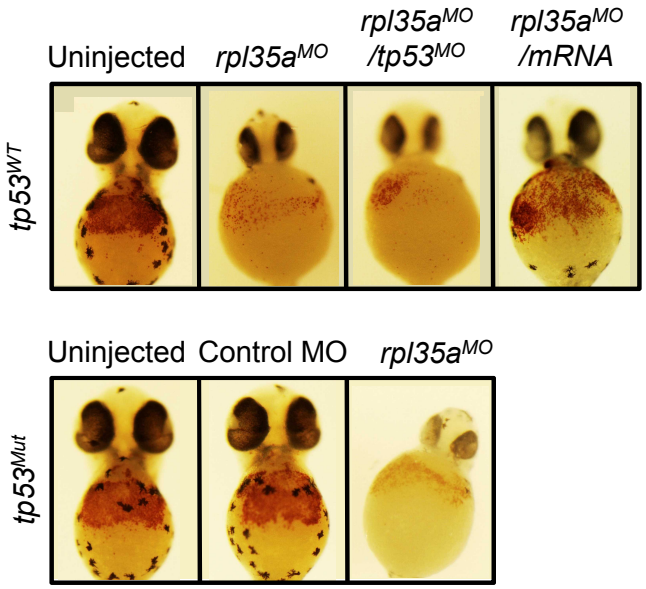
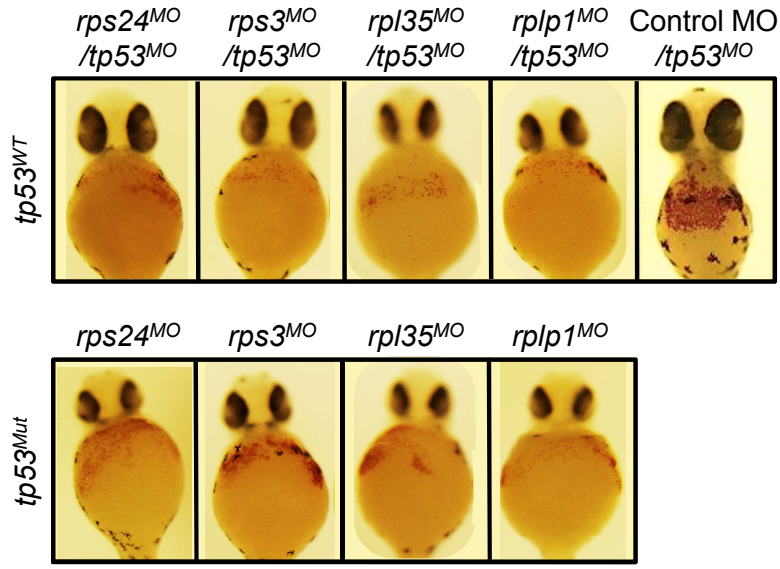

A
tp53WT
tp53Mut
Uninjected
rpl35aMO
rpl35aMO /mRNA
B
tp53WT
tp53Mut
rpls24MO
rps3MO
rpl35MO
rplp1MO
C
Control MO
rpl35aMO
rpl35aMO /mRNA

Fig. 2

A



B

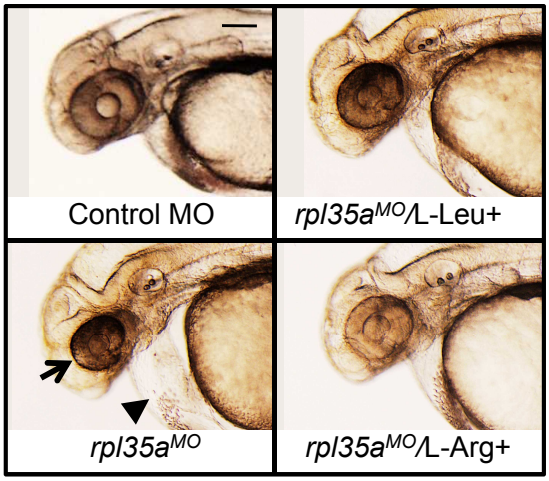


C

Erythrocyte density	*rpl35a*^{MO} /*tp53*^{WT} n=32	*rpl35a*^{MO} /*tp53*^{MO} n=36	*rpl35a*^{MO} /*tp53*^{Mut} n=34	*rpl35a*^{MO} /*mRNA* n=32	*rps24*^{MO} /*tp53*^{WT} n=27	*rps24*^{MO} /*tp53*^{MO} n=31	*rps24*^{MO} /*tp53*^{Mut} n=31	*tp53*^{WT} n=33	*tp53*^{Mut} n=34	
Normal	0	0	0	88	0	0	0	100	100	(%)
Moderate	0	12	9	12	0	6	10	0	0	
Severe	100	88	91	0	100	94	90	0	0	

Fig. 3

A


Control MO
rpl35a^MO/L-Leu+
rpl35a^MO
rpl35a^MO/L-Arg+

B

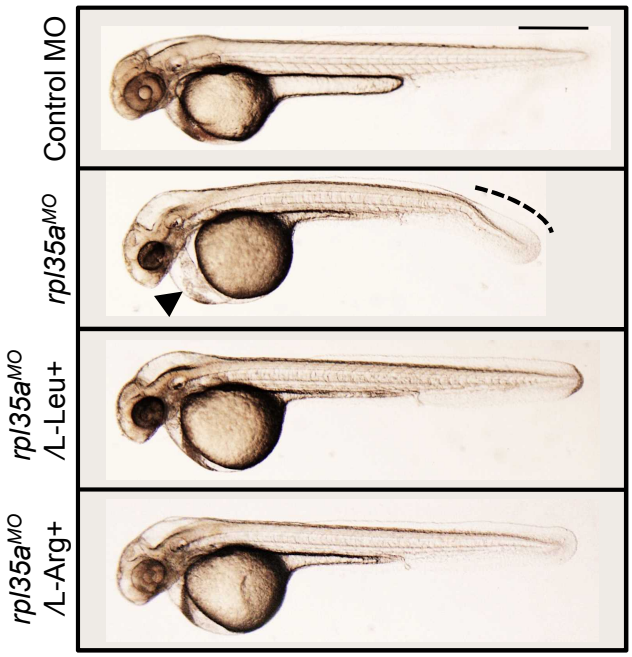
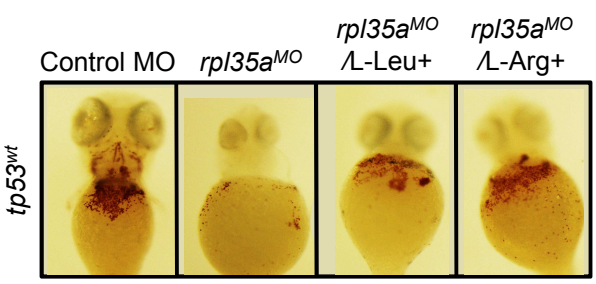

Control MO
rpl35a^MO
rpl35a^MO /L-Leu+
rpl35a^MO /L-Arg+

Fig. 4

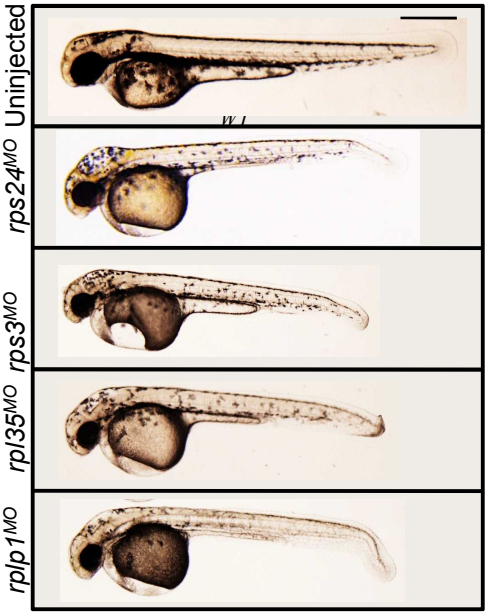
A



B

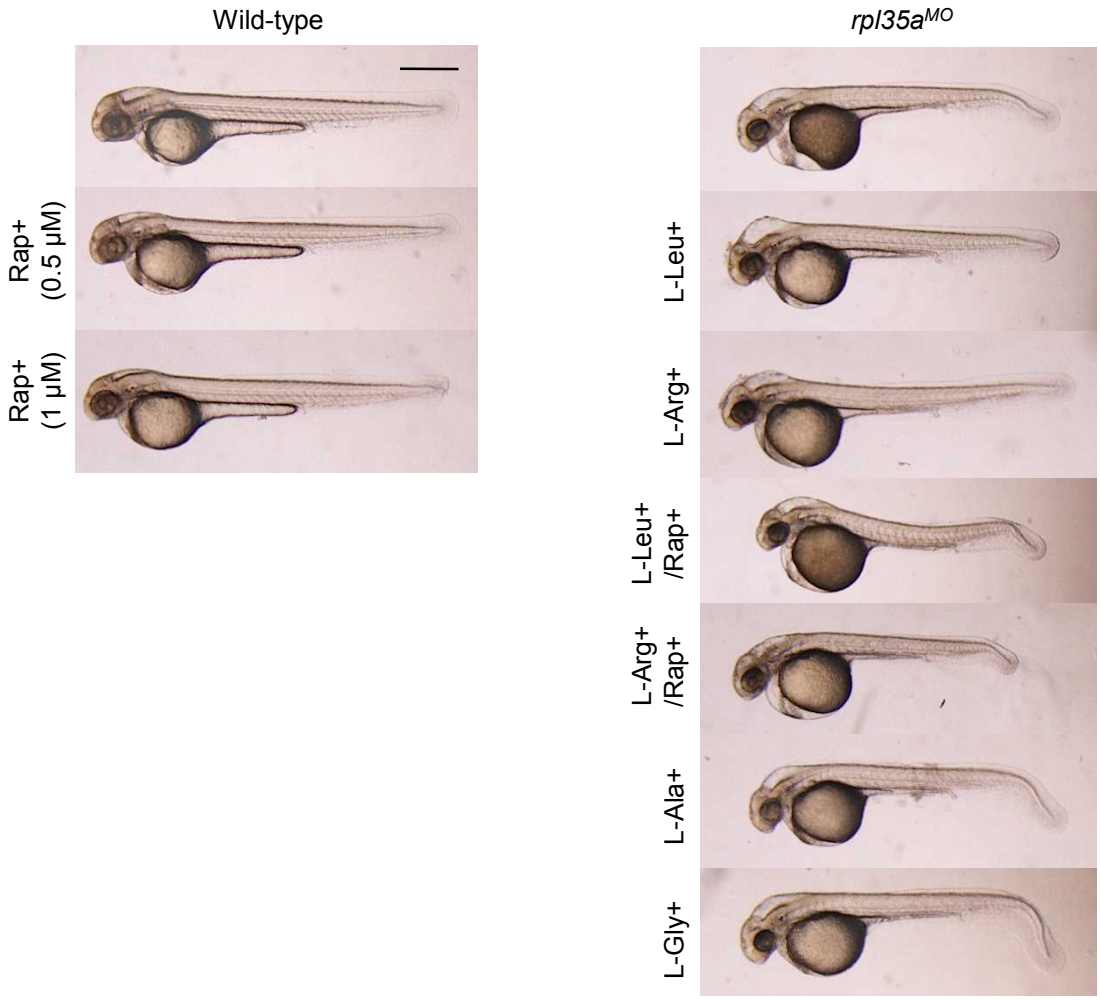
Erythrocyte density	*rpl35a*^{MO} n=24	*rpl35a*^{MO} /L-Leu+ n=23	*rpl35a*^{MO} /L-Arg+ n=22	Control MO n=23	
Normal	0	48	59	100	(%)
Moderate	0	17	14	0	
Severe	100	35	27	0	

Supplementary Fig. 1



Supplementary Fig. 2

Supplementary Fig.3



Supplementary Fig.4