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Animal models of Diamond-Blackfan anemia: updates and challenges

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Abstract

Diamond-Blackfan anemia (DBA) is a ribosomopathy that is characterized by macrocytic anemia, congenital malformations, and early onset during childhood. Genetic studies have demonstrated that most patients carry mutations in one of the 20 related genes, most of which encode ribosomal proteins (RP). Treatment of DBA includes corticosteroid therapy, chronic red blood cell transfusion, and other forms of immunosuppression. Currently, hematopoietic stem cell transplantation is the only cure for DBA. Interestingly, spontaneous remissions occur in 10-20% of transfusion-dependent DBA patients. However, there is no consistent association between specific mutations and clinical manifestations. In the past decades, researchers have made significant progress in understanding the pathogenesis of DBA, but it remains unclear how the ubiquitous RP haploinsufficiency causes the erythroid-specific defect in hematopoiesis in DBA patients, and why there is a difference in penetrance and spontaneous remission among individuals who carry identical mutations. In this paper, we provide a comprehensive review of the development of DBA animal models and discuss the future research directions for these important experimental systems.

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Introduction

Diamond-Blackfan anemia (DBA) is a rare congenital hypoplastic anemia which manifests with moderate or severe macrocytic anemia associated with short stature, physical anomalies involving bone development, and predisposition for malignancies[1-5]. More than 90% of patients are diagnosed during their first year of life (median age 12 weeks).[6] Elevated erythrocyte adenosine deaminase (eADA) activity is found in more than 75% of DBA patients. Many DBA patients are dependent on corticosteroids or red blood cell transfusion. However, chronic life-long exposures to these treatments often lead to intolerance.[1, 7, 8] Currently, hematopoietic stem cell transplantation is the only curative therapy.[6, 9]

Erythroid failure in DBA patients is characterized by a significant reduction of erythroid precursor/progenitors in bone marrow (BM), specifically, a blockade between the BFU-E and CFU-E stages or between the EPO-independent and EPO-dependent stages of erythroid development.[10, 11] The mutant genes that encode ribosomal protein (RP) are responsible for the ribosomal biogenesis defect in most DBA patients, directly affecting the synthesis of hemoglobin and the process of erythropoiesis. As a consequence, erythrocyte maturation is arrested leading to a toxic elevation of heme.[7] In 2019, Ulirsch *et al*[12] reported the genetic landscape of DBA. Heterozygous mutations in one of the 16 RP genes or 3 non-RP genes are found in approximately 70-80% of DBA patients.[1, 7, 12-14] Mutations in *RPS19, RPL5, RPS26, RPL11, RPL35a, RSL10, RPS24, and RPS17* have been identified in 70% of DBA patients. Mutations on *GATA1* and *TSR2* (a chaperone protein of eS26/*RPS26*) were also detected in DBA patients.[15-17] HEATR3 was reported associated with uL5 (*RPL11*) and uL18 (*RPL5*).[18] Recently, O'Donohue *et al*[13] reported homozygous missense and splice site variants on *HEATR3* in 6 DBA patients

from 4 distinct families, in which all parents are heterozygous for the variants found in their symptomatic children. For a complete list of DBA-associated mutant genes which have been reported in patients, please see **Suppl Table 1**.

However, besides the unifying macrocytic anemia, DBA patients show a high degree of clinical heterogeneity, with various severity and responsiveness to therapies among patients. Notably, spontaneous remissions occur in 10-20% of transfusion-dependent DBA patients by age 25 years, even in those who never had transfusion independence.[7, 19-21] In addition, asymptomatic family members of DBA patients with *RPS7* or *RPL15* mutations were reported, and an identical mutant RP gene was detected in blood cells from a patient with DBA both prior to and post remission.[22-24] To date, there is no obvious link to specific genetic mutations, gender, and treatment for the patients who attain remission or those who remain symptomatic with asymptomatic family members.[25, 26] A major unresolved question in DBA remains how a ubiquitous RP deficiency is responsible for the erythroid-specific defect in hematopoiesis and why there is different penetrance among individuals or family members who carry the identical genetic mutation.

The molecular mechanism underlying the association between ribosome insufficiency and erythroid failure in DBA patients is not fully understood to date. It is well accepted that activation of p53 and dysregulated GATA1 contribute to the pathogenesis in DBA.[15, 16, 27-30] Dysregulation of cell cycle progression, apoptosis, and heme have also been linked to DBA.[31-33] Nemo-like kinase (NLK), a serine-threonine protein kinase, was reported hyperactive in erythroid progenitors from *RPS19* and *RPL11* knockdown human hematopoietic stem and progenitor cells (HSPCs).[34] Recently, Wilkes *et al*[35] reported that upregulated microRNA

(miRNA), miR-34 and miR-30, are associated with the downregulation of SATB1, a global chromatin organizer and transcriptional factor, in a preclinical model of DBA, suggesting that epigenetics may play an important role in DBA pathogenesis.

DBA provides a unique disease model for studying how ribosomal protein deficiency impacts ribosome biogenesis and subsequent protein translation. However, because of their essential role in those fundamental cellular processes, it has been historically challenging to develop animal models that faithfully recapitulate the complete pathogenesis of DBA. Despite these challenges, since the first reviews on murine and Zebrafish models of DBA, which ware published in 2011 by McGowan and Taylor,[36, 37] research has advanced rapidly in unraveling the pathogenesis of DBA. In this review, we focus on the DBA murine models that recapitulate the hematological features of DBA patients. We provide a comprehensive picture of recent progress and challenges in the development of animal models for DBA.

Zebrafish models

Several valuable zebrafish mutant lines have been developed, with data from these models providing many important insights into the role of RP defect in the regulation of erythropoiesis in DBA patients. Specifically, knockdown of *rps19* in zebrafish recapitulates the hematopoietic and developmental phenotypes of DBA, including erythropoietic failure with severe anemia, cell cycle arrest, and increased apoptosis, as well as upregulation of p53.[28, 38, 39] Bibikova *et al*[40] reported that *gata1* expression was downregulated in *rps19* deficient zebrafish, and that a TNF-α inhibitor, etanercept, could rescue the erythroid and development defects. Knockdown of *rpl11* in zebrafish leads to morphological defects in brain, head, and eyes, and pericardial edema.[29] Danilova *et al*[41] characterized the molecular and cellular impact of uL5 (*rpl11*) deficiency. The

rpl11 mutant zebrafish had anemia, decreased hematopoietic stem cells (HSCs), and activation of the Tp53 pathway with altered expression of genes involved in cell cycle arrest (*cdkn1a* and *ccng1*) and apoptosis (*bax* and *puma*). Taylor *et al*[42] also reported that the hematopoietic defects caused by mutant *rps29* depend on p53 activation. Moreover, they observed abnormal regulation of metabolic pathways with a shift from glycolysis to aerobic respiration, including upregulation of genes involved in gluconeogenesis, decreased biosynthesis, and increased catabolism. Nucleotide metabolism was also affected in the *rps29*-knockdown model because of upregulation of adenosine deaminase (ADA) and xanthine dehydrogenase/oxidase (xdh).[38, 43] Although there is no *RPL18* mutation reported in DBA patients, mutant *rpl18* causes maturation arrest of red blood cells in zebrafish, due to increased p53 activation and JAK2-STAT3 activation.[44] Thus, in zebrafish models, researchers have successfully recapitulated maturation arrest of red blood cells, growth retardation, decreased globin synthesis, increased ADA and apoptosis, and p53 activation, which are observed in DBA patients.[28] The advantage of zebrafish models is that they are relatively easier to establish and can be used for studying developmental hematopoiesis, which is difficult in mouse models. Zebrafish models provide a useful tool for *in vivo* drug screening to identify possible DBA therapeutics. Oyarbide *et al*[45] published an excellent review in 2019.

Mouse models harboring the mutations found in DBA patients

Rps19 (eS19)

RPS19 (eS19) is the most frequently mutated gene in DBA patients, accounting for 25% of DBA patients. It has been intensively studied in both human cells and mouse models, allowing valuable insights into all aspects of DBA caused by *RPS19* mutation.

RNA interference (RNAi) exploits an endogenous mechanism of gene regulation that can be adapted to target and suppress specific genes *in vivo*.[46] Short hairpin RNAs (*shRNAs*) containing a specific sequence homologous to a target RNA transcript are embedded within a natural miRNA backbone; upon delivery to the cell, *shRNA* is processed to form siRNA which can bind to a target RNA and functionally inactivate it.[47] shRNA can silence transcripts without modifying the genomic locus, thus they can be reversible. Using transgenic RNAi, Jaako *et al*[48] engineered a mouse model containing doxycycline-regulatable *shRNAs* targeting the *Rps19* transcript, creating an inducible regulation of *Rps19* expression in adult mice. They demonstrated that *Rps19*-deficient mice developed macrocytic anemia along with leukocytopenia and viable platelet counts. The severity of the disease phenotype was correlated with loss of *Rps19*-expression and could be rescued by overexpression of *Rps19* or inactivation of *Trp53*. They also demonstrated that chronic eS19 deficiency caused irreversible exhaustion of HSCs and impaired proliferation and apoptosis in hematopoietic progenitors, resulting in BM failure.

Matsson *et al*[49] reported mice in which they engineered constitutive disruption of *Rps19* gene by deletion of the 5'UTR and exons 1 to 4. Whereas zygotes homozygous for the knockout ($Rps19^{-/-}$) could not develop to normal blastocysts leading to embryonic lethality, heterozygous mice with a single copy of wildtype *Rps19* ($Rps19^{+/-}$) could maintain normal ribosomal/extra-ribosomal functions. Interestingly, no erythrocyte abnormality was detected in the $Rps19^{+/-}$ mice. Kubik-Zahorodna *et al*[50] reported that homozygous mice with an Arginine 67 deletion (Arg67del) in eS19 presented with growth retardation, macroscopic skeleton deformation, hydrocephalus, and behavioral defects without obvious hematological abnormality, whereas heterozygous mice also had no phenotype. Devlin *et al*[51] reported transgenic mouse models with either a constitutive

or *Prion-Cre*-mediated conditional missense mutation at the highly conserved amino acid 62 of eS19 (RPS19R62W). They demonstrated that the constitutive mutation was embryonic lethal, but the conditional RPS19R62W mutation exerted a dominant negative effective which manifested with many, but not all, of the pathogenic features of human DBA, including growth retardation, mild anemia with reduced numbers of erythroid progenitors, and significant inhibition of terminal erythroid maturation.

Xenotransplantation of CD34+ cells from DBA patients with *RPS19* haploinsufficiency into sub-lethally irradiated immunocompromised nonobese diabetic/severe combined immunodeficient-β2 microglobulin null mice provided conclusive evidence that *RPS19* mutation is the cause of the disease phenotype in DBA patients.[43] Additionally, Flygare *et al* and Zivny *et al*[52, 53] reported that over-expression of *RPS19* in DBA CD34+ cells with *RPL19* mutation improved engraftment and erythropoiesis.

***Rpl11* (uL5)**

Heterozygous loss-of-function mutations in *RPL11*(uL5) gene are found in 5-20% of DBA patients.[1] Morgado-Palacin *et al*[54] reported transgenic mouse models with a Cre-mediated conditional knockout of exon 3 and 4 of *Rpl11*. When using a ubiquitous Cre recombinase (Tg.pCAG-Cre) which was constitutively expressed at early stages of development, they could not produce any $Rpl11^{+/\Delta}$ pups, suggesting a single copy of *Rpl11* is not sufficient to support embryonic development. When they induced *Rpl11*deletion in adult mice with $Rpl11^{loxp/loxp}$ and a ubiquitous tamoxifen-inducible Cre recombinase (*Tx-Cre*), they found that complete deletion of *Rpl11* ($Rpl11^{\Delta/\Delta}$) in adult mice (1.5 months old) was lethal (survival time < 8 weeks post-deletion). Further, adult mice with *Rpl11* haploinsufficiency ($Rpl11^{+/\Delta}$) had lower red blood cell

counts and macrocytosis because of a significant decrease in erythroblasts in BM, when compared to their wild-type littermates. They demonstrated that partial loss of *Rpl11* impairs erythroid maturation, reduces p53 responses, and increases cMYC levels. Their data also recapitulated the *Cdkn1a* and *Bax* molecular defects observed in some DBA patients. They concluded that diploid *Rpl11* is required for embryonic development; and that partial loss of *Rpl11* in adult mice causes non-lethal DBA-like anemia and increase cancer susceptibility.[54]

Using mouse model with $Rpl11^{+/loxp}$ *Tx-Cre*, Doty *et al*[55] recently reported data from the single-cell analysis of erythropoiesis in adult mice with $Rpl11^{+/\Delta}$. They found that the transcriptional pathways regulated by GATA1, GATA1-target genes, and genes involved in mitotic spindle formation were significantly down-regulated in adult mice with $Rpl11^{\Delta/+}$, which are observed in DBA patients.[15] They also reported that *Rpl11* haploinsufficiency uniquely caused upregulation of several mitochondrial genes, p53 and CDKN1 pathway genes, as well as DNA damage checkpoint genes.[55]

***Rpl5* (uL18) *and Rps24* (eS24)**

RPL5 (uL18) is one of the most commonly mutated RP gene found in DBA patients (7%).[1] In 2016, Kazerounian *et al*[56] reported 2 mouse models with conditional knockout of *Rpl5* or *Rps24* (eS24). A pGK-gb2 Loxp/FRT-flanked neomycin cassette was inserted into either *Rpl5* or *Rps24*, resulting in deletion of exons 1-8 for *Rpl5* and exons 2-3 for *Rps24*, respectively. They demonstrated that homozygous deletion of *Rpl5* or *Rps24* was embryonic lethal. Heterozygous *Rpl5* and *Rps24* mice were born normal and did not develop any sign of disease, including anemia. Real-time qPCR and immunoblot analysis demonstrated no significant reduction of *Rpl5* and *Rps24* expression, suggesting that one copy of wild-type *Rpl5* and *Rps24* is sufficient to

prevent development of anemia. However, some of the aged mice with heterozygous deletion of *Rpl5* or *Rps24* developed soft tissue sarcoma after age of 17 months. Although it was low incidence, they concluded that prolonged RP deficiency may increase the risk of cancer, as observed in some DBA patients. Later, Kazerounian *et al*[57] reported a mouse model which used doxycycline-regulatable *Rpl5*-targeting *shRNAs* to induce downregulation of *Rpl5* expression. They suggested that mice with inducible *Rpl5*-targeting *shRNA* recapitulate the major features of DBA, including mild anemia, reticulocytopenia, and BM erythroblastopenia.

Recently, Yu *et al* [58] reported a mouse model with an intronic ENU-induced mutation in *Rpl5* gene ($Rpl5^{Skax23\text{-}Jus/+}$), which alters the 6th nucleotide in intron 1 (c.3+6C>T). $Rpl5^{Skax23\text{-}Jus/+}$ mice had a profound delay in erythroid maturation and increased mortality at embryonic day (E) 12.5, which improved by E14.5. Macrocytic anemia and cardiac defect were observed in newborn mice with $Rpl5^{+/-}$. The phenotype penetrance of kinky tail was 100%. Most of the diseased mice died within 3 weeks postnatal due to a severe pancytopenia or ventricular septal defect (VSD). However, the anemia observed at birth in $Rpl5^{+/-}$ mice was surprisingly resolved completely in adult mice which survived to 7 weeks,[58] suggesting that epigenetic regulation may play an important role in spontaneous remission observed in DBA patients.

***Rps7* (eS7)**

Watkins-Chow *et al*[59] reported a mouse model with an ENU-induced mutations in *Rps7* (eS7) gene ($Rps7^{Mtu}$ and $Rps7^{Zma}$). Although the mutant mice did not show any defect in hematopoiesis, they demonstrated that *Rps7* haploinsufficiency causes decreased mouse size, abnormal skeletal morphology, mid-ventral white spotting, and eye malformations, phenotypes that also occur in mice with haploinsufficiency for other ribosomal protein subunits. In addition, significant

apoptosis occurs in central nervous system (CNS) along with subtle behavioral phenotypes, suggesting eS7 is particularly important for CNS development. The data reported by Sato *et al*[60] also suggest that *RPS7* deficiency contributes to the pathogenesis of DBA indirectly through other mechanisms, such as protein-protein interaction in ribosome assembly.

Rps20 **(uS10)**

Recently, a missense mutation in *RPS20* (uS10) was reported in patients with DBA.[14] However, mice with heterozygous *Rps20* mutation showed only dark skin.[61]

Gata1

GATA1 is an essential hematopoietic transcription factor in early HSPCs for the specification of erythropoiesis, as well as megakaryocytes and eosinophils.[62] Although *GATA1* mutation is found in <1% of DBA patients, it has also been reported that a reduction of GATA1 expression is observed at the early HSPC stages in the uncultured BM cells from DBA patients with diverse RP gene mutations,[15, 16, 40, 63] suggesting that GATA1 deficiency may be a second hit in some patients with RP gene mutations. In humans and mice, *GATA1* and *Gata1* are located on the X chromosome, and RP haploinsufficiency impairs the translation of *GATA1* mRNA.[15, 16] Fujiwara *et al*[64] reported that mouse embryos lacking *Gata1* died at approximately E11.5 because of maturation arrest of erythroid progenitors. Female heterozygotes ($Gata1^{+/-}$) were born pale due to random X chromosome inactivation. Interestingly, those mice recovered during the neonatal period, presumably a result of *in vivo* selection for progenitors able to express *Gata1*. Later Gutiérrez *et al*[65] reported mouse models with inducible *Gata1*-deletion using conditional *Gata1* knockout mediated by either interferon-Cre recombinase (*Mx-Cre*) or *Tx-Cre*. They found that Mx-Cre-mediated *Gata1* deletion, although anemia was not observed, caused

maturation arrest of erythroid cells at the proerythroblast stage, thrombocytopenia, and excessive proliferation of megakaryocytes in spleens from adult *Gata1*-null mice; Tx-Cre-mediated *Gata1* deletion caused depletion of the erythroid compartment in both BM and spleen, resulting in severe anemia in adult mice. Furthermore, formation of both early and late erythroid progenitors was also found significantly reduced in adult BM in the absence of *Gata1*.[65]

Mouse models with molecular defects not found in DBA patients but relevant to DBA pathogenesis

Despite extensive efforts to sequence all genes coding ribosomal proteins in affected individuals, molecular lesions remain elusive in approximately 20-25% of DBA cases, and the correlation between genotype and phenotype is not clear in DBA patients.[1] Therefore, in efforts to develop mouse models mimicking the clinical features or pathogenesis which are observed in DBA patients, researchers have explored the impact of other proteins with known roles in maturation of erythrocytes, accumulation of heme, and bone development.

Rps14 (uS11)

Rps14 (uS11) mutation has never been reported in DBA patients, neither 5q deletion. In an attempt to mimic 5q deletion in mice, Barlow *et al*[66] investigated mice engineered with LIM domain only 2 (*Lmo2*)-Cre and $Cd74^{+/lox}Nid67^{+/lox}$ (containing *Rps14* gene for uS11, $Cd74^{+/\Delta}$ $Nid67^{+/\Delta}$) and reported that haploinsufficiency of the *CD74-Nid67 interval* caused DBA-like phenotype, including macrocytic anemia, thrombocytopenia, and hypocellularity in BM, which was associated with increased p53 expression and apoptosis as observed in DBA samples.[27] They

also demonstrated that deletion of *Trp53* completely restored the populations of HSCs, CMP/MEP, and GMP, and reversed the dysplasia in BM of mice carrying $Cd74^{+/\Delta}$ $Nid67^{+/\Delta}$.

Flvcr

Feline leukemia virus subgroup C receptor (FLVCR) is a heme export protein, and delayed globin synthesis leads to excess heme in DBA patients.[67] Although DBA-causative mutation in the *FLVCR1* gene has never been identified in DBA families,[68] Keel *et al*[69] reported mouse models with constitutive $Flvcr^{+/-}$ and inducible *Flvcr* mutations ($Flvcr^{+/flox}$*;Mx-cre*). They demonstrated that *Flvcr*-null mice lack definitive erythropoiesis and have craniofacial and limb deformities; while deletion of *Flvcr* at neonatal causes severe macrocytic anemia with proerythroblast maturation arrest, resembling the clinical features of DBA patients. Later, the same group reported the single cell analyses data from the mice with *Flvcr* deletion.[70] They demonstrated that heme-GATA1 feedback loop regulates red cell differentiation; and deletion of *Flvcr1* causes high levels of intracellular heme, which decreases *GATA1* and expression of GATA1-target genes, as well as mitotic spindle genes in late stage erythroid cells ($CD71^{+}Ter119^{lo\text{-}hi}$). Their data suggest that the excessive heme may be responsible for the progression of RP imbalance, prematurely lower GATA1, and impede mitosis in late stage of DBA patients.

***Rps6* (eS6)**

Mouse embryos with haploinsufficiency of *Rps6* ($Rps6^{+/\Delta}$) are small and die at gastrulation; genetic inactivation of p53 bypasses this checkpoint, prolonging embryos development until E12.5, at which point the embryos likely die from anemia.[71] Two groups reported their observations in mice with inducible *Rps6*-deletion using conditional *Rps6* knockout mediated by

Mx-Cre.[72, 73] Regardless of the timing of deletion, induced at either neonatal (PND5-7) or adult ages (7-9 weeks), mice with *Rps6*$^{+/\Delta}$ exhibited robust macrocytic anemia, granulocytopenia, lymphopenia, and progressive thrombocytosis. Elevated erythroid ADA (eADA) was also observed in those mice, which occurs in >80% of DBA patients. McGowan *et al*[72] also demonstrated that deletion of *Trp53* rescued the red blood cell counts and BFU-E or mature erythroid precursors in *Rps6*-deficient mice, a finding that was also reported by Tiu *et al,*[30] collectively confirming that p53 plays an important role in the pathogenesis of DBA. In particular, Tiu *et al*[30] characterized limb development defects upon *Rps6*-deletion during developing limb bud mesenchyme, which are similar to the congenital birth defects found in DBA patients, including radial hypoplasia and defects in digit formation. They also observed a decrease in translation, specifically of transcripts controlling limb development upon *Rps6*-deletion. This decrease could be rescued by knockdown of either p53 and/or the repressor of cap binding protein, 4E-BP1. 4E-BP1 represses the eukaryotic initiation factor eIF4E, an essential eukaryotic initiation factor. Strikingly, it was also observed in cells with *Rps19*- and *Rps14*-knockdown that the selective translation phenomena was driven by upregulation of p53 and 4E-BP1, suggesting a common pathway via the p53-4EBP1-eIF4E axis by which selective changes in translation occur upon ribosome perturbation.[30]

Other mutations

Mouse models with mutations on *Rpl24* (eL24)[74] *and Rpl27a* (eL27)[75] were reported with none-hematological phenotype (see more in **Table 1)**

Conclusions and future directions

In DBA patients, most molecular defects are caused by haploinsufficiency in one of the RP genes.[1, 12] In 2005, DBA was recognized as the first ribosomopathy in humans.[76] Ribosomopathies broadly comprise two categories: disorders caused by single-copy mutations in specific ribosomal proteins, and disorders associated with defects in ribosome biogenesis factors. The phenotypic patterns among different ribosomopathies are divergent but often share some overlapping features, including effects on BM-derived cell lineages and skeletal tissues. It is unknown how the phenotypic diversity is regulated among the ribosomopathies. These common tissue specificities of the different ribosomopathies are very challenging to reconcile with the ubiquitous requirement for ribosomes in all cells.[77]

Due to the limited numbers of BM cells in DBA patients, studies on the critical roles of ribosomes in protein biosynthesis has been very challenging. Researchers have successfully recapitulated the macrocytic anemia and growth retardation of DBA patients in zebrafish and mouse models with engineered mutations of *Rps19, Rpl11, Rpl5, Rps24, Rps7, Rps14,* and *Gata1* (**Table 1**). Other animal models with mutant genes not found in DBA patients, such as *Rps6* and *Flvcr,* have advanced our understanding of how ribosome dysfunction impacts erythropoiesis and contributes to the pathogenesis of DBA. Haploinsufficiency of *Rpl11* and *Rpl5* in mice also resemble the clinical features of DBA. While in some models the RP composition of ribosomes varies,[78] Khajuria *et al*[63] recently reported that in hematopoietic cells, RP haploinsufficiency did not demonstrate an altered ribosome composition. Rather, the impaired lineage commitment, a characteristic of hematopoiesis in DBA patients, arises from the reduced cellular levels of ribosomes, suggesting that ribosome levels are rate-limiting and selectively regulate the translation and lineage commitment in human hematopoiesis.

DBA patients with RP gene mutations were identified as being exclusively heterozygous. From mouse models, we learned that homozygous RP gene mutations are embryonic lethal, and fetuses with heterozygous deletions occurring in early development cannot survival to birth, such as homozygous *Rps19* fetuses and heterozygous *Rpl11* fetuses.[49, 54] Therefore, it suggests that gene dosage and the timing of mutation hits play a critical role in recapitulating the clinical manifestation of DBA patients. Although each of the reported mouse models has various limitation in recapitulating the phenotype observed in DBA patients, such as the presence of combination of anemia and physical anomalies, those models suggest that the complexity of haploinsufficiency of various RPs depend on the tissue-specificity, resulting in varying disease severity in children with DBA. In the past decades, we learned much about the genetic changes in DBA,[12] but we know very little about the role of epigenetics in the pathogenesis of DBA. It is striking that most DBA patients are diagnosed during their first year of life, when epigenetic regulation is required for developmental hematopoiesis, as the newborn adapts to normoxia and rapid growth. However, this age-specificity and the dynamics of developmental hematopoiesis have been underappreciated in most of the reported DBA mouse models.

Spontaneous remissions occur in 10-20% of transfusion-dependent DBA patients, and some DBA patients experience periods of anemia remission and relapse due to unknown mechanisms.[24] Additionally, asymptomatic family members of DBA patients with *RPS7* or *RPL15* mutation have been reported, and the mutation persistently presented in the blood cells from a DBA patient post remission.[22-24] Furthermore, female mice with *Gata1*$^{+/-}$ were born pale due to the random inactivation of X chromosome bearing the normal allele, but those mice recovered during the neonatal period, presumably a result of *in vivo* selection for progenitors able to express *Gata1*.[64] Recently, it was reported that the severe anemia and cardiac defect could

be resolved spontaneously in surviving adult mice with *Rpl5*$^{+/-}$.[58] Collectively, these data suggest that epigenetic regulation may contribute to the pathogenesis of DBA.

Fetal HSCs may follow various tracks to migrate into adult hematopoiesis based upon the status of the hematopoiesis switch, at which the RP mutations occur. In addition, the effects of mutant RP on the assembled ribosome and subsequent translation likely dictate the spectrum and severity of the disease. Previous reports suggest that ribosome levels are rate-limiting and selectively regulate the translation and lineage commitment in human hematopoiesis.[63] Therefore, insufficient ribosomes most likely alter the dynamics of hematopoiesis during development in early childhood. Elevated fetal hemoglobin is often detected in DBA patients.[6] It has been reported that Pten deficiency in mice sustains fetal-like hematopoiesis at an age when the fetal-to-adult-hematopoiesis switch should be completed,[79] and the timing of Pten deficiency determines the disease severity in juvenile leukemia mice.[80] Recently, Strahm *et al*[81] reported favorable outcomes of hematopoietic stem cell transplantation in children and adolescents with DBA, suggesting the benefit of correcting the molecular defects at an early stage of development of DBA patients. Emerging data support the idea that developmental hematopoiesis plays critical roles in pediatric hematopoietic disorders. Therefore, understanding the molecular mechanisms of fetal-to-adult hematopoiesis will be key in the future efforts of mimicking DBA in mouse models. In the future, investigation of the mutation timing in different developmental stages may help to uncover how the developmental hematopoiesis contributes to the pathogenesis of DBA, particularly in pediatric patients.

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Table 1. Mouse models with DBA associated mutations

Gene (RP)	Defect in patients	Alternation	Mechanism	Age (mut. induced)	Characteristics		Reference	
					Anemia	Other system	Author	Year
Rps19 (eS19)	Yes	*Del. Exon 1-4*	*+/-*	Constitutive	No	Growth	Matsson *et al*[49]	2004
		Rps19R62W	*+/-(DN)*	Fetal (*Prion-Cre*)	Yes	Growth	Devlin *et al*[51]	2010
		shRNA	*M2-rtTA*	>4 wks (DOX)	Yes	Growth	Jaako *et al*[48]	2011
		Dsk3	*+/Dsk3*	Constitutive	Yes	Dark skin	McGowan *et al*[72]	2011
		Arg67del	*-/-*	Constitutive	No	Growth, behavior	Kubik-Zahorodna *et al*[50]	2016
		N/A	Xenograft	N/A	Yes	N/A	Flygare *et al*[52]	2008
Rpl11 (uL5)	Yes	*Del.Exon 3-4*	*+/loxP*	5-8 wks (*Tg.UbC-CreERT2*)	Yes	G/I	Morgado-Palacin *et al*[54]	2015
Rpl5 (uL18)	Yes	*shRNA*	*ColA1/rtTA*	5-6 wks (TRE)	Yes	N/A	Kazerounian *et al*[57]	2019
		Intron 1	*Skax23m1Jus/+*	Constitutive	Yes	Bone, heart	Yu *et al*[58]	2021
		Del. Exon 1-8	*+/loxP*	Embryonic (*pGK-gb2 Loxp/FRT*)	No	Soft tissue sarcoma	Kazerounian *et al*[56]	2016
Rps24 (eS24)	Yes	*Del. Exon 2-3*	*+/loxP*	Embryonic (*pGK-gb2 Loxp/FRT*)	No	Soft tissue sarcoma		
Rps14 (uS11)	Yes	*Del. Cd74 to Nid67*	*+/loxP*	Embryonic (*Lmo2-Cre*)	Yes	N/A	Barlow *et al*[66]	2010
Rps7 (eS7)	Yes	$Rps7^{Mtu}$, $Rps7^{Zma}$	*+/-*	Constitutive	No	Growth, eye, CNS	Watkins-Chow *et al*[59]	2013
Rps20 (uS10)	Yes	*Dsk4*	*+/Dsk4*	Constitutive	No	Dark skin	McGowan *et al*[61]	2008
GATA1	Yes	Del. Exon 2-6	*loxP*	8-10 weeks (*Mx1-Cre*)	No	N/A	Gutiérrez *et al*[65]	2008
				8-10 weeks (*Tx-Cre*)	Yes	N/A		
Flvcr	N/A	*Del. Exon 3*	*loxP/loxP*	PND7,9,11 (*Mx1-Cre*)	Yes	Bone (limb), heart	Keel *et al*[69]	2008
Rps6 (eS6)	N/A	*Del. Exon 3-5*	*+/loxP*	PND5-7 (*Mx1-Cre*)	Yes	eADA	Keel *et al*[73]	2012
				7-9 wks (*Mx1-Cre*)	Yes	eADA, dark skin	McGowan *et al*[72]	2011
				Fetal (*Prix/Msx2-Cre*)	Yes	Bone (limb)	Tiu *et al*[30]	2021
Rpl24 (eL24)	N/A	*Intron 1*	*+/Bst*	Constitutive	No	Belly spot and tail kinks	Oliver *et al*[74]	2004
Rpl27a (eL27)	N/A	*IVS4-15A > G*	*+/-*	Constitutive	No	Sooty foot ataxia	Terzian. *et al*[75]	2011

Suppl. Table-1: Genetic alternations and clinical manifestations in DBA patients

Gene (Protein)	% in DBA*	Alternation	Age at diagnosis	Hematopoiesis			Malformation**	Reference	
				Anemia	Elevated HBF	Elevated eADA		Author	Year
RPS19 **(eS19)**	25-30%	*G120A*	2 months	Yes	N/A***	N/A	No	Matsson et al	1999
		329delG	1 year						
		C302T	10 months						
		160insCT	15 months						
		C302T	1 month	Yes	Yes	Yes	Yes	Draptchinskaia *et a*l	1999
		c.172+1G>A	8 months	Yes	Yes	N/A	N/A	Jahan *et al*	2020
		c.185G >A	Birth -23 yeas	Yes	Yes	Yes	Yes	Cole *et al*	2022
RPL11 **(uL5)**	5-20%	*c.59T>A, c.357T>G*	Birth	Yes	Yes	N/A	Yes	Cmejla *et al*	2009
		Small indel	Birth-2 months	Yes	N/A	Yes	Yes	Quarello *et al*	2020
		Nonsense, splice site, small indel	1 month-21 yrs	Yes	N/A	N/A	Yes	*Gazda et al*	2008
RPL5 **(uL18)**	7-12%	Nonsense, missense, splice site, small indel	Birth-10 months	Yes	N/A	N/A	Yes	*Gazda et al*	2008
		Small indel	Birth-24 months	Yes	N/A	Yes	Yes	Quarello *et al*	2020
		Point mutations, splice site	Birth	Yes	N/A	N/A	Yes	Cmejla *et al*	2009
RPL36 (eL36)	<1%	Small indel	2 months	Yes	N/A	N/A	Yes	*Gazda et al*	2008
RPS26 (eS26)	6.6-9%	9 distinct mutations	6 weeks-8 months	Yes	N/A	N/A	No	Doherty *et al*	2010
RPS10 (eS10)	1-3%	3 distinct mutations	Birth-12 years	Yes	N/A	N/A	No	Doherty *et al*	2010
RPS7 **(eS7)**	~1%	Misssense mutation	Adulthood	Yes	N/A	Yes	No	Volejnikova *et al*	2020
		Splice site variant	1st year	Yes	N/A	N/A	Yes	Akram *et al*	2020
		c.76-1G>T	Early infancy	Yes	N/A	Yes	Yes	Ichimura *et al*	2017
		Splice site in intron 1	1st year	Yes	N/A	N/A	Yes	Smetanina *et al*	2015
RPL35A **(eL33)**	2-3%	Gene deletion	4 months	Yes	N/A	Yes	Yes	Quarello *et al*	2020
		Allelic del, c.304 C>T, c.82_84delCTT	2-14 months	Yes	N/A	Yes	Yes	Farrar *et al*	2008
		Large deletion	Birth	Yes	Yes	Yes	Yes	Gianferante *et al*	2021
RPS24 **(eS24)**	2-3%	Nonsense mutation in Exon 4	N/A	Yes	No	Yes	N/A	Gazda *et al*	2006
RPS17 **(eS17)**	1-3%	Nonsense mutation	Birth, 11 months	Yes	N/A	Yes	Yes	Gerrard *et al*	2013
		c.2T>G	6 months	Yes	N/A	Yes	Yes	Cmejla *et al*	2007
		c.1A>G	5 months	Yes	N/A	N/A	Yes	Song *et al*	2010
RPL9 **(uL6)**	<1%	*c.-2+1G>C, c.59T>C; c.59T>C*	6 months	Yes	Yes	No	Yes	Lezzerini *et al*	2020
RPL15 **(eL15)**	~1%	Deletion of intron 3 and extron 4	Birth	Yes	N/A	N/A	Yes	Landowski *et al*	2013
		c.242dupA, c.85C>T, c.29T>C, c.458A>C	Prenatal to 6 months	Yes	Yes	Yes	Yes	Wlodarski *et al*	2018
RPS29 **(uS14)**	~1%	*p.I31F, p.I50T*	3 months-9 years	Yes	No	Yes	No	Mirabello *et al*	2014
RPS20 **(uS10)**	rare	*c.251T>A, c.251T>G*	1st month	Yes	N/A	Yes	No	Bhar *et al*	2020
RPL26 **(uL24)**	Rare	C.120_121delGA (nonsense mutation)	Birth	Yes	Yes	N/A	Yes	Gazda *et al*	2012
RPS27A **(eS31)**	Rare	*c.169T>C*	15 months	Yes	N/A	N/A	Yes	Gazda *et al*	2008
RPS28 **(eS28)**	Rare	*c.1A>G*	Infancy	Yes	N/A	N/A	Yes	Gripp *et al*	2014
GATA1	<1%	chX: 48,649,736 G>C; chX: 48,649,736–48,649,737Gdel	Birth, 1.6 months	Yes	Yes	Yes	N/A	Sankaran *et al*	2012
		c.220G>C	3 months	Yes	Yes	Yes	No	Klar *et al*	2014
		c.2T>C	27 and 28 yrs	Yes	N/A	N/A	N/A	Ludwig *et al*	2014
		c.2T>C	9 months	Yes	N/A	Yes	No	Parrella *et al*	2014
		c.2T>C, c.220+2T>C	7 months and 5 yrs	Yes	Yes	Yes	No	van Dooijeweert *et al*	2022
TSR2	Rare	*c.191A>G*	10 months	Yes	Yes	Yes	Yes	Gripp *et al*	2014
HEATR3	Rare	*c.1751G>A, c.1337G>A, c.399+1G>T, c.719C>T, c.400T>C*	5-20yrs	Yes	N/A	N/A	Yes	O'Donohue *et al*	2022
EPO	One case	chr7:100320704 G>A	1st year	Yes	N/A	N/A	N/A	Kim *et al*	2017

* Resource from https://www.ncbi.nlm.nih.gov/books/NBK7047/ and https://www.ncbi.nlm.nih.gov/pubmed/34889440

**Malformation

*** N/A: Not available