

# 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.

## Introduction

Diamond-Blackfan anemia (DBA) is a rare congenital hypoplastic anemia that manifests as moderate or severe macrocytic anemia associated with short stature, physical anomalies involving bone development, and a predisposition to malignancies.<sup>1-5</sup> More than 90% of patients are diagnosed during their first year of life (median age 12 weeks).<sup>6</sup> Elevated erythrocyte adenosine deaminase 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 exposure to these treatments often leads to intolerance.<sup>1,7,8</sup> Currently, hematopoietic stem cell transplantation is the only curative therapy.<sup>6,9</sup>

Erythroid failure in DBA patients is characterized by a significant reduction of erythroid precursors/progenitors in bone marrow, specifically, a blockade between the burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) stages or between the erythropoietin-independent and erythropoietin-dependent stages of erythroid development.<sup>10,11</sup> The mutant genes that encode ribosomal proteins (RP) are responsible for the

defect in ribosomal biogenesis 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.<sup>7</sup> In 2019, Ulirsch *et al.*<sup>12</sup> described the genetic landscape of DBA. Heterozygous mutations in one of the 16 RP genes or three non-RP genes are found in approximately 70-80% of DBA patients.<sup>1,7,12-14</sup> Mutations in *RPS19*, *RPL5*, *RPS26*, *RPL11*, *RPL35α*, *RSL10*, *RPS24*, and *RPS17* have been identified in 70% of DBA patients. Mutations in *GATA1* and *TSR2* (encoding a chaperone protein of eS26/*RPS26*) have also been detected in DBA patients.<sup>15-17</sup> *HEATR3* was reported to be associated with uL5 (*RPL11*) and uL18 (*RPL5*).<sup>18</sup> Recently, O'Donohue *et al.*<sup>13</sup> reported homozygous missense and splice site variants on *HEATR3* in six DBA patients from four distinct families, in which all parents are heterozygous for the variants found in their symptomatic children. A complete list of DBA-associated mutant genes that have been reported in patients is provided in *Online Supplementary Table S1*.

Although DBA patients share the unifying macrocytic anemia, they show a high degree of clinical heterogeneity, with disease severity and responsiveness to therapies

varying among patients. Notably, spontaneous remissions occur in 10–20% of transfusion-dependent DBA patients by the age of 25 years, even in those who have never been independent of transfusions.<sup>7,19–21</sup> In addition, asymptomatic family members of DBA patients with *RPS7* or *RPL15* mutations have been reported, and an identical mutant RP gene was detected in blood cells from a patient with DBA both prior to and after remission.<sup>22–24</sup> 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.<sup>25,26</sup> Major unresolved questions in DBA remain 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.<sup>15,16,27–30</sup> Dysregulation of cell cycle progression, apoptosis, and heme have also been linked to DBA.<sup>31–33</sup> Nemo-like kinase (NLK), a serine-threonine protein kinase, was reported to be hyperactive in erythroid progenitors from *RPS19* and *RPL11* knockdown human hematopoietic stem and progenitor cells.<sup>34</sup> Recently, Wilkes *et al.*<sup>35</sup> 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 the pathogenesis of DBA.

DBA provides a unique disease model for studying how RP deficiency has an impact on ribosome biogenesis and subsequent protein translation. However, because of the essential role of these proteins in 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 were published in 2011 by McGowan and Taylor,<sup>36,37</sup> research has advanced rapidly and is unraveling the pathogenesis of DBA. In this review, we focus on the murine models of DBA that recapitulate the hematologic features of DBA patients. We provide a comprehensive picture of recent progress and challenges in the development of animal models of 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 defects in the

regulation of erythropoiesis in DBA patients. Specifically, knockdown of *rps19* in zebrafish recapitulates the hematopoietic and developmental phenotypes of DBA, including failure of erythropoiesis with severe anemia, cell cycle arrest, and increased apoptosis, as well as upregulation of p53.<sup>28,38,39</sup> Bibikova *et al.*<sup>40</sup> reported that *gata1* expression was downregulated in *rps19*-deficient zebrafish, and that a tumor necrosis factor- $\alpha$  inhibitor, etanercept, could rescue the erythroid and developmental defects. Knockdown of *rpl11* in zebrafish leads to morphological defects in the brain, head, and eyes, and pericardial edema.<sup>29</sup> Danilova *et al.*<sup>41</sup> characterized the molecular and cellular impact of uL5 (*rpl11*) deficiency. The *rpl11* mutant zebrafish had anemia, decreased hematopoietic stem cells, 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.*<sup>42</sup> 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.<sup>38,43</sup> Although no *RPL18* mutations have been reported in DBA patients, mutant *rpl18* causes maturation arrest of red blood cells in zebrafish, due to increased p53 activation and JAK2-STAT3 activation.<sup>44</sup> Thus, in zebrafish models, researchers have successfully recapitulated maturation arrest of red blood cells, growth retardation, decreased globin synthesis, increased adenosine deaminase and apoptosis, and p53 activation, which are observed in DBA patients.<sup>28</sup> The advantage of zebrafish models is that they are relatively easy 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.*<sup>45</sup> published an excellent review covering these aspects in 2019.

## Mouse models harboring the mutations found in patients with Diamond-Blackfan anemia

### *Rps19* (eS19)

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

RNA interference (RNAi) exploits an endogenous mechanism of gene regulation that can be adapted to target and suppress specific genes *in vivo*.<sup>46</sup> Short hairpin (sh)RNA containing a specific sequence homologous to a target RNA transcript is embedded within a natural miRNA backbone; upon delivery to the cell, shRNA is processed to form short interfering (si)RNA which can bind to a target RNA and functionally inactivate it.<sup>47</sup> shRNA can silence transcripts without modifying the genomic locus, thus they can be reversible. Using transgenic RNAi, Jaako et al.<sup>48</sup> engineered a mouse model containing shRNA, which could be regulated by doxycycline, targeting the *Rps19* transcript, thereby 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 hematopoietic stem cells and impaired proliferation and apoptosis in hematopoietic progenitors, resulting in bone marrow failure.

Matsson et al.<sup>49</sup> described mice in which they engineered constitutive disruption of the *Rps19* gene by deletion of the 5' untranslated region and exons 1 to 4. Whereas zygotes homozygous for the knockout (*Rps19*<sup>-/-</sup>) could not develop to normal blastocysts leading to embryonic lethality, heterozygous mice with a single copy of wildtype *Rps19* (*Rps19*<sup>+/-</sup>) maintained normal ribosomal/extra-ribosomal functions. Interestingly, no erythrocyte abnormality was detected in the *Rps19*<sup>+/-</sup> mice. Kubik-Zahorodna et al.<sup>50</sup> reported that homozygous mice with an Arginine 67 deletion (Arg67del) in eS19 had growth retardation, macroscopic skeletal deformation, hydrocephalus, and behavioral defects without obvious hematologic abnormalities, whereas heterozygous mice had no phenotype. Devlin et al.<sup>51</sup> described 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 effect which manifested with many, but not all, of the pathological 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<sup>+</sup> cells from DBA patients with *RPS19* haploinsufficiency into sublethally irradiated immunocompromised non-obese diabetic/severe combined immunodeficient- $\beta_2$  microglobulin null mice provided conclusive evidence that *RPS19* mutation is the cause of the disease phenotype in DBA patients.<sup>43</sup> Additionally, Fly-

gare et al. and Zivny et al.<sup>52,53</sup> reported that overexpression of *RPS19* in DBA CD34<sup>+</sup> cells with *RPL19* mutation improved engraftment and erythropoiesis.

### **Rpl11 (uL5)**

Heterozygous loss-of-function mutations in the *RPL11* (uL5) gene are found in 5-20% of DBA patients.<sup>1</sup> Morgado-Palacin et al.<sup>54</sup> described transgenic mouse models with a Cre-mediated conditional knockout of exons 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*<sup>+/ $\Delta$</sup>  pups, suggesting that a single copy of *Rpl11* is not sufficient to support embryonic development. When they induced *Rpl11* deletion in adult mice with *Rpl11*<sup>loxP/loxP</sup> and a ubiquitous tamoxifen-inducible Cre recombinase (*Tx-Cre*), they found that complete deletion of *Rpl11* (*Rpl11* <sup>$\Delta$ / $\Delta$</sup> ) in adult mice (1.5 months old) was lethal (survival time <8 weeks after deletion). Furthermore, when compared to their wildtype littermates, adult mice with *Rpl11* haploinsufficiency (*Rpl11*<sup>+/ $\Delta$</sup> ) had lower red blood cell counts and macrocytosis because of a significant decrease in erythroblasts in bone marrow. It was shown that partial loss of *Rpl11* impairs erythroid maturation, reduces p53 responses, and increases cMYC levels. The data also recapitulated the *Cdkn1a* and *Bax* molecular defects observed in some DBA patients. The authors 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 increases susceptibility to cancer.<sup>54</sup>

Using a mouse model with *Rpl11*<sup>+/ $\Delta$</sup>  *Tx-Cre*, Doty et al.<sup>55</sup> recently collected data from single-cell analysis of erythropoiesis in adult mice with *Rpl11*<sup>+/ $\Delta$</sup> . They found that the transcriptional pathways regulated by GATA1, GATA1-target genes, and genes involved in mitotic spindle formation were significantly downregulated in adult mice with *Rpl11*<sup>+/ $\Delta$</sup> , as observed in DBA patients.<sup>15</sup> 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.<sup>55</sup>

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

*RPL5* (uL18) is one of the most commonly mutated RP genes found in DBA patients (7%).<sup>1</sup> In 2016, Kazerounian et al.<sup>56</sup> reported two 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*. 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 quantitative polymerase chain reaction (qPCR) and immunoblot analysis demonstrated no significant reduction of *Rpl5* and



*Rps24* expression, suggesting that one copy of wildtype *Rpl5* and *Rps24* is sufficient to prevent the development of anemia. However, some older mice with heterozygous deletion of *Rpl5* or *Rps24* developed soft tissue sarcoma after the age of 17 months. Although the incidence was low, the authors concluded that prolonged RP deficiency may increase the risk of cancer, as observed in some DBA patients. Later, Kazerounian *et al.*<sup>57</sup> described a mouse model in which doxycycline could be used to regulate *Rpl5*-targeting shRNA 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 bone marrow erythroid blastopenia.

Recently, Yu *et al.*<sup>58</sup> reported a mouse model with an intronic ENU-induced mutation in the *Rpl5* gene (*Rpl5*<sup>Skax23-Jus/+</sup>), which alters the sixth nucleotide in intron 1 (c.3+6C>T). *Rpl5*<sup>Skax23-Jus/+</sup> 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 defects were observed in newborn mice with *Rpl5*<sup>+/-</sup>. Penetrance of a kinky tail phenotype was 100%. Most of the diseased mice died within 3 weeks after birth due to severe pancytopenia or ventricular septal defect. However, the anemia observed at birth in *Rpl5*<sup>+/-</sup> mice was, surprisingly, resolved completely in adult mice that survived to 7 weeks,<sup>58</sup> suggesting that epigenetic regulation may play an important role in spontaneous remission observed in DBA patients.

### **Rps7 (eS7)**

Watkins-Chow *et al.*<sup>59</sup> reported a mouse model with ENU-induced mutations in the *Rps7* (eS7) gene (*Rps7*<sup>Mtu</sup> and *Rps7*<sup>Zmo</sup>). Although the mutant mice did not show any defects in hematopoiesis, *Rps7* haploinsufficiency caused decreased mouse size, abnormal skeletal morphology, mid-ventral white spotting, and eye malformations, phenotypes that also occur in mice with haploinsufficiency for other RP subunits. In addition, significant apoptosis occurred in the central nervous system along with subtle behavioral phenotypes, suggesting that eS7 is particularly important for central nervous system development. The data reported by Sato *et al.*<sup>60</sup> also suggest that *RPS7* deficiency contributes to the pathogenesis of DBA indirectly through other mechanisms, such as protein-protein interactions in ribosome assembly.

### **Rps20 (uS10)**

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

### **Gata1**

GATA1 is an essential hematopoietic transcription factor in

early hematopoietic stem and progenitor cells for the specification of erythropoiesis, as well as in megakaryocytes and eosinophils.<sup>62</sup> Although *GATA1* mutations are found in <1% of DBA patients, it has been reported that a reduction of GATA1 expression is observed at early hematopoietic stem and progenitor cell stages in uncultured bone marrow cells from DBA patients with diverse RP gene mutations,<sup>15,16,40,63</sup> 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.<sup>15,16</sup> Fujiwara *et al.*<sup>64</sup> reported that mouse embryos lacking *Gata1* died at approximately E11.5 because of maturation arrest of erythroid progenitors. Female heterozygotes (*Gata1*<sup>+/-</sup>) were born pale due to random X chromosome inactivation. Interestingly, those mice recovered during the neonatal period, presumably as a result of *in vivo* selection for progenitors able to express *Gata1*. Later Gutiérrez *et al.*<sup>65</sup> described 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 not producing anemia, 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 bone marrow and spleen, resulting in severe anemia in adult mice. Furthermore, formation of both early and late erythroid progenitors was significantly reduced in adult bone marrow in the absence of *Gata1*.<sup>65</sup>

## **Mouse models with molecular defects not found in patients with Diamond-Blackfan anemia but relevant to its pathogenesis**

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

### **Rps14 (uS11)**

The *Rps14* (uS11) mutation, like the 5q deletion, has never been reported in DBA patients. In an attempt to mimic 5q deletion in mice, Barlow *et al.*<sup>66</sup> investigated mice engin-

eered with LIM domain only 2 (*Lmo2*)-*Cre* and *Cd74<sup>+/lox</sup>Nid67<sup>+/lox</sup>* (containing the *Rps14* gene for uS11, *Cd74<sup>+/Δ</sup>Nid67<sup>+/Δ</sup>*) and reported that haploinsufficiency of the *CD74-Nid67* interval caused a DBA-like phenotype, including macrocytic anemia, thrombocytopenia, and hypocellularity in bone marrow, which was associated with increased p53 expression and apoptosis, as observed in DBA samples.<sup>27</sup> They also demonstrated that deletion of *Trp53* completely restored the populations of hematopoietic stem cells, common myeloid progenitors, megakaryocyte-erythrocyte progenitors and granulocyte/macrophage progenitors, and reversed the dysplasia in bone marrow of mice carrying *Cd74<sup>+/Δ</sup>Nid67<sup>+/Δ</sup>*.

### **Flvcr**

Feline leukemia virus subgroup C receptor (FLVCR) is a heme export protein, and delayed globin synthesis leads to excess heme in DBA patients.<sup>67</sup> Although a DBA-causative mutation in the *FLVCR1* gene has never been identified in DBA families,<sup>68</sup> Keel *et al.*<sup>69</sup> reported mouse models with constitutive *Flvcr<sup>+/-</sup>* and inducible *Flvcr* mutations (*Flvcr<sup>+/flox</sup>;Mx-cre*). They demonstrated that *Flvcr*-null mice lack definitive erythropoiesis and have craniofacial and limb deformities, while deletion of *Flvcr* in the neonatal period causes severe macrocytic anemia with proerythroblast maturation arrest, resembling the clinical features of DBA patients. Later, the same group reported the data from single-cell analyses of mice with *Flvcr* deletion.<sup>70</sup> They demonstrated that the heme-GATA1 feedback loop regulates red cell differentiation and that 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<sup>+</sup>Ter119<sup>lo-hi</sup>*). Their data suggest that excessive heme may be responsible for the progression of RP imbalance and prematurely lower *GATA1*, and may impede mitosis in patients in the late stages of DBA.

### **Rps6 (eS6)**

Mouse embryos with haploinsufficiency of *Rps6* (*Rps6<sup>+/Δ</sup>*) are small and die at gastrulation; genetic inactivation of p53 bypasses this checkpoint, prolonging the embryonic development until E12.5, at which point the embryos likely die from anemia.<sup>71</sup> Two groups reported their observations in mice with inducible *Rps6* deletion using conditional *Rps6* knockout mediated by *Mx-Cre*.<sup>72,73</sup> Regardless of the timing of deletion, induced in either the neonatal period (5–7 days after birth) or in adulthood (7–9 weeks), mice with *Rps6<sup>+/Δ</sup>* exhibited robust macrocytic anemia, granulocytopenia, lymphopenia, and progressive thrombocytosis. Elevated levels of erythroid ADA (eADA) were also observed in these mice, a finding present in >80% of DBA patients. McGowan *et al.*<sup>72</sup> demonstrated that deletion of *Trp53* rescued the red blood cell counts and burst-forming

unit-erythroid or mature erythroid precursors in *Rps6*-deficient mice, a finding that was also reported by Tiu *et al.*,<sup>30</sup> collectively confirming that p53 plays an important role in the pathogenesis of DBA. In particular, Tiu *et al.*<sup>30</sup> characterized limb development defects upon *Rps6* deletion during the development of the limb bud mesenchyme; these defects 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 essential eukaryotic initiation factor eIF4E. 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.<sup>30</sup>

## **Other mutations**

Mouse models with mutations in *Rpl24* (eL24)<sup>74</sup> and *Rpl27a* (eL27) have been described.<sup>75</sup> These animals had non-hematologic phenotypes (Table 1).

## **Conclusions and future directions**

Most molecular defects in DBA patients, are caused by haploinsufficiency in one of the RP genes.<sup>1,12</sup> In 2005, DBA was recognized as the first ribosomopathy in humans.<sup>76</sup> Ribosomopathies broadly comprise two categories: disorders caused by single-copy mutations in specific RP, 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 bone marrow-derived cell lineages and skeletal tissues. It is unknown how the phenotypic diversity among the ribosomopathies is regulated. The common tissue specificities of the different ribosomopathies are very challenging to reconcile with the ubiquitous requirement for ribosomes in all cells.<sup>77</sup> Due to the limited numbers of bone marrow cells in DBA patients, studies on the critical roles of ribosomes in protein biosynthesis have 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

**Table 1.** Mouse models with Diamond-Blackfan anemia-associated mutations.

Gene (RP)	Defect in patients	Alteration	Mechanism	Age (mut. induced)	Characteristics		Reference	
					Anemia	Other systems	Author	Year
<i>Rps19</i> (eS19)	Yes	Del. exons 1-4	+/-	Constitutive	No	Growth	Matsson et al. <sup>49</sup>	2004
		Rps19R62W	+/- (DN)	Fetal ( <i>Prion-Cre</i> )	Yes	Growth	Devlin et al. <sup>51</sup>	2010
		shRNA	<i>M2-rtTA</i>	>4 weeks (DOX)	Yes	Growth	Jaako et al. <sup>48</sup>	2011
		<i>Dsk3</i>	+/ <i>Dsk3</i>	Constitutive	Yes	Dark skin	McGowan et al. <sup>72</sup>	2011
		Arg67del	-/-	Constitutive	No	Growth, behavior	Kubik-Zahorodna et al. <sup>50</sup>	2016
		na	Xenograft	na	Yes	na	Flygare et al. <sup>52</sup>	2008
<i>Rpl11</i> (uL5)	Yes	Del. exons 3-4	+/ <i>loxP</i>	5-8 weeks ( <i>Tg.UbC-CreERT2</i> )	Yes	GI	Morgado-Palacin et al. <sup>54</sup>	2015
<i>Rpl5</i> (uL18)	Yes	shRNA	<i>ColA1/rtTA</i>	5-6 weeks (TRE)	Yes	na	Kazerounian et al. <sup>57</sup>	2019
		Intron 1	<i>Skax23m1 Jus/+</i>	Constitutive	Yes	Bone, heart	Yu et al. <sup>58</sup>	2021
		Del. exons 1-8	+/ <i>loxP</i>	Embryonic ( <i>pGK-gb2 Loxp/FRT</i> )	No	Soft tissue sarcoma	Kazerounian et al. <sup>56</sup>	2016
<i>Rps24</i> (eS24)	Yes	Del. exons 2-3	+/ <i>loxP</i>	Embryonic ( <i>pGK-gb2 Loxp/FRT</i> )	No	Soft tissue sarcoma	Kazerounian et al. <sup>56</sup>	2016
<i>Rps14</i> (uS11)	Yes	Del. <i>Cd74</i> to <i>Nid67</i>	+/ <i>loxP</i>	Embryonic ( <i>Lmo2-Cre</i> )	Yes	na	Barlow et al. <sup>66</sup>	2010
<i>Rps7</i> (eS7)	Yes	<i>Rps7<sup>Mtu</sup>, Rps7<sup>Zma</sup></i>	+/-	Constitutive	No	Growth, eye, CNS	Watkins-Chow et al. <sup>59</sup>	2013
<i>Rps20</i> (uS10)	Yes	<i>Dsk4</i>	+/ <i>Dsk4</i>	Constitutive	No	Dark skin	McGowan et al. <sup>61</sup>	2008
<i>GATA1</i>	Yes	Del. exons 2-6	<i>loxP</i>	8-10 weeks ( <i>Mx1-Cre</i> )	No	na	Gutiérrez et al. <sup>65</sup>	2008
				8-10 weeks ( <i>Tx-Cre</i> )	Yes	na		
<i>Flvcr</i>	na	Del. exon 3	<i>loxP/loxP</i>	PND 7,9,11 ( <i>Mx1-Cre</i> )	Yes	Bone (limb), heart	Keel et al. <sup>69</sup>	2008
<i>Rps6</i> (eS6)	na	Del. exons 3-5	+/ <i>loxP</i>	PND 5-7 ( <i>Mx1-Cre</i> )	Yes	eADA	Keel et al. <sup>73</sup>	2012
				7-9 weeks ( <i>Mx1-Cre</i> )	Yes	eADA, dark skin	McGowan et al. <sup>72</sup>	2011
				Fetal ( <i>Prix/Msx2-Cre</i> )	Yes	Bone (limb)	Tiu et al. <sup>30</sup>	2021
<i>Rpl24</i> (eL24)	na	Intron 1	+/ <i>Bst</i>	Constitutive	No	Belly spot and tail kinks	Oliver et al. <sup>74</sup>	2004
<i>Rpl27a</i> (eL27)	na	<i>IVS4-15A &gt; G</i>	+/-	Constitutive	No	Sooty foot ataxia	Terzian et al. <sup>75</sup>	2011

RP: ribosomal protein; DOX: doxycycline; na: not available; CNS: central nervous system; PND: post natal day; eADA: erythroid adenosine deaminase; GI: gastrointestinal.

of how ribosome dysfunction affects erythropoiesis and contributes to the pathogenesis of DBA. Haploinsufficiency of *Rpl11* and *Rpl5* in mice also produces manifestations resembling the clinical features of DBA. While in some models the RP composition of ribosomes varies,<sup>78</sup> Khajuria et al.<sup>63</sup> 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 reduced cellular levels of ribosomes, suggest-

ing that ribosome levels are rate-limiting and selectively regulate 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 survive to birth, such as homozygous *Rps19* fetuses and heterozygous *Rpl11* fetuses.<sup>49,54</sup> This suggests that gene dosage and the timing



of mutation hits play critical roles in recapitulating the clinical manifestations of DBA patients. Although each of the reported mouse models has various limitations in recapitulating the phenotype observed in DBA patients, such as the presence of a combination of anemia and physical anomalies, the models suggest that the complexity of haploinsufficiency of various RP depends on tissue specificity, resulting in varying disease severity in children with DBA. In the past decades, we have learnt much about the genetic changes in DBA,<sup>12</sup> 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 neonate 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 as a result of unknown mechanisms.<sup>24</sup> Additionally, asymptomatic family members of DBA patients with *RPS7* or *RPL15* mutation have been reported, and the mutation persisted in the blood cells from a DBA patient after remission.<sup>22–24</sup> Furthermore, female mice with *Gata1*<sup>+/-</sup> were born pale due to the random inactivation of the X chromosome bearing the normal allele, but recovered during the neonatal period, presumably as a result of *in vivo* selection for progenitors able to express *Gata1*.<sup>64</sup> Recently, it was reported that the severe anemia and cardiac defects in young mice with *Rpl5*<sup>+/-</sup> could resolve spontaneously in surviving adults.<sup>58</sup> Collectively, these data suggest that epigenetic regulation may contribute to the pathogenesis of DBA.

Fetal hematopoietic stem cells may follow various tracks to migrate into adult hematopoiesis based on 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 translation and lineage commitment in human hematopoiesis.<sup>63</sup> Therefore, insufficient ribosomes most likely alter the dynamics of hematopoiesis during development in early childhood. Elevated fetal hemoglobin levels are often detected in DBA patients.<sup>6</sup> It has been reported that *Pten* deficiency in mice sustains fetal-like hematopoiesis at an age at which the fetal-to-adult-hematopoiesis switch should be completed,<sup>79</sup> and the timing of *Pten* deficiency determines the disease severity

in mice with juvenile leukemia.<sup>80</sup> Recently, Strahm et al.<sup>81</sup> 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. 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 for future efforts to mimic DBA in mouse models. In the future, investigation of the timing of mutations in different developmental stages may help to uncover how developmental hematopoiesis contributes to the pathogenesis of DBA, particularly in pediatric patients.

### Disclosures

*No conflicts of interest to disclose.*

### Contributions

*YLL designed the original layout, conducted the literature search, created the tables, and wrote the manuscript. AS conducted the literature search for the Online Supplementary Table, and edited the manuscript. BG, MCW, and MB edited the manuscript. KMS designed the original layout and edited the manuscript. All authors approved the final version of the manuscript.*

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### Data-sharing statement

*There are no data to share.*

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