

# Ribosomal Protein L5 and L11 Mutations Are Associated with Cleft Palate and Abnormal Thumbs in Diamond-Blackfan Anemia Patients

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Diamond-Blackfan anemia (DBA), a congenital bone-marrow-failure syndrome, is characterized by red blood cell aplasia, macrocytic anemia, clinical heterogeneity, and increased risk of malignancy. Although anemia is the most prominent feature of DBA, the disease is also characterized by growth retardation and congenital anomalies that are present in ~30%–50% of patients. The disease has been associated with mutations in four ribosomal protein (RP) genes, *RPS19*, *RPS24*, *RPS17*, and *RPL35A*, in about 30% of patients. However, the genetic basis of the remaining 70% of cases is still unknown. Here, we report the second known mutation in *RPS17* and probable pathogenic mutations in three more RP genes, *RPL5*, *RPL11*, and *RPS7*. In addition, we identified rare variants of unknown significance in three other genes, *RPL36*, *RPS15*, and *RPS27A*. Remarkably, careful review of the clinical data showed that mutations in *RPL5* are associated with multiple physical abnormalities, including craniofacial, thumb, and heart anomalies, whereas isolated thumb malformations are predominantly present in patients carrying mutations in *RPL11*. We also demonstrate that mutations of *RPL5*, *RPL11*, or *RPS7* in DBA cells is associated with diverse defects in the maturation of ribosomal RNAs in the large or the small ribosomal subunit production pathway, expanding the repertoire of ribosomal RNA processing defects associated with DBA.

Diamond-Blackfan anemia (DBA) (MIM 105650) is an inherited congenital bone-marrow-failure syndrome, characterized by normochromic macrocytic anemia and absence or insufficiency of erythroid precursors in otherwise normocellular bone marrow.<sup>1</sup> Although anemia is the most prominent feature of DBA, the disease is also characterized by growth retardation and congenital malformations, in particular craniofacial, upper limb, heart, and urinary-system defects, that are present in ~30%–50% of patients, reflecting the fact that DBA is a broad disorder of development.<sup>2–4</sup> Laboratory findings such as increased mean corpuscular volume (MCV), elevated erythrocyte adenosine deaminase activity (eADA), and hemoglobin F are observed in a majority of, but not in all, DBA patients.<sup>5,6</sup> The disease is clinically heterogeneous, and within affected families, some individuals can exhibit mild or absent anemia, with only subtle indications of erythroid abnormalities, such as macrocytosis or elevated eADA and/or HbF. Additionally, increased risk of malignancy—in particular, AML and solid tumors, including osteogenic sarcoma<sup>7,8</sup>—has been associated with DBA. Approximately 90% of affected individuals present during the first year of life or in early childhood, although patients with a “nonclassical” mild

phenotype are usually diagnosed later in life.<sup>3,4</sup> The incidence of DBA is 5–7 per million live births,<sup>2,3,9</sup> affecting both genders equally.<sup>4</sup> Although most cases are sporadic, about 10%–25%<sup>1,4</sup> and up to, according to recent data, even 45%<sup>10</sup> are familial, with disease inherited in an autosomal-dominant pattern.

Heterozygous mutations in ribosomal protein genes *RPS19* (MIM 603474), *RPS24* (MIM 602412), and *RPS17* (MIM 180472), encoding RPs of the small ribosomal subunit, and in *RPL35A* (MIM 180468), encoding an RP of the large subunit, have been reported in about 30% of DBA patients,<sup>6,10–15</sup> suggesting that DBA is a disorder of ribosomes. Haploinsufficiency of *RPS19* and *RPS24* has been shown to be the basis for DBA in patients with mutations of those genes. In a subset of patients, large deletions and frameshift mutations lead to degradation of the mutated transcripts.<sup>9,13,16,17</sup> Recent studies have also demonstrated that DBA missense mutations affect *RPS19* conformation and stability, triggering proteasome-mediated degradation or blocking *RPS19*'s incorporation into pre-ribosomes.<sup>18–20</sup> Despite mutations identified in four genes, no significant phenotype-genotype correlations have been reported to date, because *RPS24*, *RPS17*, and

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DOI 10.1016/j.ajhg.2008.11.004. ©2008 by The American Society of Human Genetics. All rights reserved.

*RPL35A* mutations are rare and have been reported in only eight, one, and six patients, respectively.

RPS19 protein has been shown to play an important role in 18S rRNA maturation in yeast and in human cells.<sup>21–24</sup> Similarly, alterations of pre-rRNA processing and small or large ribosomal-subunit synthesis were demonstrated in human cells with RPS24 and RPL35A deficiency, respectively, further indicating that DBA is a disorder of ribosomes.<sup>15,25</sup> Deficiency of RPS19 and RPL35A was shown to cause increased apoptosis in hematopoietic cell lines and in bone-marrow cells,<sup>15,26</sup> and it has been suggested that imbalance of the p53 family proteins is a mechanism of abnormal embryogenesis and anemia in zebrafish upon perturbation of RPS19 expression.<sup>27</sup>

Here, we report the results of a large-scale screen of 24 additional RP genes and of *RPS17* (in which one mutation among 24 tested patients was previously reported<sup>14</sup>) in a cohort of DBA probands. Remarkably, we identified probable pathogenic mutations in four of these genes, *RPL5* (MIM 603634), *RPL11* (MIM 604175), *RPS7* (MIM 603658), and *RPS17*, representing ~16% of our patient cohort. In addition, we report possible single mutations in three other RP genes, *RPL36*, *RPS15* (MIM 180535), and *RPS27A* (MIM 191343). In contrast to previously reported data on patients with *RPS19* mutations, mutations in *RPL5* are associated with multiple physical abnormalities, including craniofacial, thumb, and heart anomalies, whereas isolated thumb abnormalities are predominantly present in patients carrying mutations in *RPL11*. We also demonstrated defective rRNA maturation in *RPL5*-, *RPL11*-, and *RPS7*-mutated DBA cells.

To test the hypothesis that mutations in other RP genes can also cause DBA, we carried out direct sequencing of RP genes, starting with what we considered to be the most probable candidates. One hundred ninety-six DBA families participated in the study. Thirty-six of them were multiplex families, and 160 included only one clinically affected individual. All probands were negative for mutations in *RPS19* and *RPS24*, and 132 tested probands were negative for mutations in *RPL35A*. Informed consent was obtained from all patients and their family members participating in the study under a protocol at Children's Hospital Boston. The diagnosis of DBA in all probands was based on normochromic, often macrocytic, anemia; reticulocytopenia; a low number or lack of erythroid precursors in bone marrow; and, in some patients, congenital malformations and elevated eADA.

Genomic DNA samples from 95 unrelated DBA probands enrolled in the study were amplified by polymerase chain reaction (PCR) and sequenced for mutations in 25 RP genes. Initially, we sequenced three genes, *RPS3A*, *RPS13*, and *RPS16*, which encode RPs and, like RPS19 and RPS24, are involved in binding of initiation factor eIF-2 to the 40S subunit.<sup>28</sup> Subsequently, we sequenced 11 RP genes located on chromosome 19 (*RPS5*, *RPS9*, *RPS11*, *RPL13A*, *RPL18*, *RPL18A*, *RPL28*, *RPL40*, *RPS28*, *RPL36*, *RPS15*), because previous studies had reported linkage of

DBA to this chromosome in families that did not segregate *RPS19* mutations.<sup>11</sup> Next, we screened five genes on chromosome 1 (*RPL5*, *RPL11*, *RPL22*, *RPS27*, *RPS8*), because Heyn et al. previously reported a pericentric inversion of chromosome 1 in a DBA patient with cleft lip and palate and a ventricular septal defect.<sup>29</sup> *RPS17* (chromosome 15) was screened because Cmejla et al. reported one mutation among 24 tested DBA patients,<sup>14</sup> and *RPL23* (chromosome 17) was screened because the RPS23 protein inhibits MDM2-mediated p53 ubiquitination and degradation in response to ribosomal stress in the same manner as RPL5 and RPL11.<sup>30</sup> Subsequently, we have started sequencing RP genes according to their chromosomal location, starting with four genes on chromosome 2 (*RPL31*, *RPL37A*, *RPS7*, *RPS27A*). Primers (sequences available on request) were designed with Primer3 software for amplification of the coding exons and intron-exon boundaries of the above genes. PCR products, between 200 and 600 bp, were robotically prepared with a Tecan Genesis Workstation (Tecan, Durham, NC), purified with Exo-SAP enzyme (USB, Cleveland, OH) and sequenced on both strands with an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The chromatograms were analyzed with Sequencher software, version 4.7 (Gene Codes, Ann Arbor, MI). When a sequence change was identified in a given gene, we sequenced an additional 101 samples from unrelated probands for mutations in this gene. Duplicate, independent PCR products were sequenced to confirm the observed nucleotide changes in the probands. We sequenced DNA samples from at least 150 control individuals, i.e., 300 chromosomes, to determine whether the observed sequence variations were nonpathogenic variants. Subsequently, we sequenced DNA samples from available family members to determine whether the mutation cosegregated with the DBA phenotype within the pedigree.

Interestingly, Heyn and colleagues reported a DBA patient with cleft lip and palate, VSD, and pericentric inversion of chromosome 1, inv (1p-; 1q+), resulting in the shortened p arm and lengthened q arm.<sup>29</sup> These findings suggest that the gene causing DBA might be located on chromosome 1. In accord with these findings, direct sequencing revealed multiple sequence changes in DBA patients in coding regions and intron-exon boundaries in two genes, *RPL5* and *RPL11*, located on chromosome 1p (1p22.1 and 1p35-p36.1, respectively). In addition, screening of 23 other RP genes revealed single sequence changes in five of them, *RPS7*, *RPS17*, *RPL36*, *RPS15*, and *RPS27A*, in DBA probands. In total, we found sequence changes in seven out of 25 screened RP genes (Table 1). DNA samples from available family members were also screened, demonstrating that the identified sequence changes cosegregated with the DBA phenotype within the pedigrees (Tables 1–4).

To summarize, we identified sequence changes in *RPL5* in 18 of 196 probands and in six additional family members, for a total of 24 individuals with *RPL5* sequence

**Table 1. Summary of Sequence Changes in Seven RP Genes Identified in 196 DBA Families**

Gene Symbol	No. of Tested DNA Samples		No. of Subjects with Mutations	Mutation Types
	from Unrelated Probands	No. of Probands with Mutations (%)		
RPL5	196	18 (9%)	24	nonsense, missense, splice site, small indel
RPL11	196	13 (6.5%)	18	nonsense, splice site, small indel
RPS7	159	1 < 1%	1	splice site
RPS17	193	1 < 1%	1	small del
RPL36	132	1 < 1%	1	small del
RPS15	151	1 < 1%	1	missense
RPS27A	159	1 < 1%	2	missense

"Indel" denotes insertion-deletion; "del" denotes deletion.

changes. Seventeen of the identified changes cause premature termination either by nonsense or splice-site mutations or deletions and/or insertions of 1–5 nucleotides causing frameshifts. The 18th is a missense change, 418G→A, resulting in G140S substitution (Tables 1 and 2). All of these changes are unique, (i.e., not previously described in the literature or databases), and 13 of them were found in only a single kindred, whereas one was seen in two and another in three apparently unrelated families. Thirteen sequence changes were found in *RPL11* among 196 DBA probands, and *RPL11* sequence changes were also identified in five other family members. These included acceptor or donor splice-site mutations, deletions or insertions of 1–4 nucleotides causing a frameshift, and one nonsense mutation. All of these changes are also unique, and 11 of them are distinct from each other, because two changes were seen in two unrelated probands (Tables 1 and 3). Parental DNA was available in seven probands with *RPL5* and in seven with *RPL11* mutations. De novo sequence changes were identified in four and six probands with sporadic disease, respectively, further supporting the assessment that these sequence changes are probably pathogenic mutations. The single sequence changes in the five other RP genes, *RPS7*, *RPS17*, *RPL36*, *RPS15*, and *RPS27A*, in DBA patients are summarized in Table 4. These are a donor splice-site mutation in intron 2 of *RPS7*, a deletion of 2 nt causing frameshifts in *RPS17* and *RPL36*, as well as two missense changes in *RPS15* and *RPS27A*. Interestingly, only one *RPS17* mutation was identified among 24 tested patients<sup>14</sup> and only one sequence change was found in our patient cohort, demonstrating that mutations in *RPS17* are rare events. None of the identified sequence changes was found on the NCBI SNP lists, and none was identified in at least 300 control chromosomes from a control population of similar, largely European origin. Since we consider it possible that the three missense changes in *RPL5*, *RPS15*, and *RPS27A* could be rare or private non-

pathogenic genetic variants, DNA samples carrying these sequence changes are currently being screened for mutations in the remaining 50 RP genes.

Remarkably, the *RPL5* combined deletion-insertion (c.498\_502delTGTGG and c. 498, a 39 bp insertion) in exon 5 was identified in a family with anemia, triphalangeal thumbs, cleft lip, and a heart abnormality (P15; Table 2), a class of symptoms previously described as Aase syndrome (MIM 105650).<sup>31</sup> Strikingly, review of the available medical data on 20 patients with mutations in *RPL5* revealed that the majority of them (14/20) have physical malformations, including craniofacial, thumb, and heart anomalies (Tables 2 and 5). Similarly, a majority of patients with *RPL11* mutations (12/18) presented with physical malformations, whereas among 76 reported DBA patients with *RPS19* mutations, 35 (46%) presented with physical malformations, including short stature<sup>6,10,12,32</sup> (Tables 3 and 5). In general, regardless of genotype, congenital malformations were described in 30%–50% of DBA patients.<sup>4,6,9,10</sup> Remarkably, nine out of 14 patients with *RPL5* mutations and physical abnormalities have cleft lip and/or palate or cleft soft palate, isolated or in combination with other facial malformations—such as micrognathia, hypertelorism, or mandibular hypoplasia with retrognathia—and/or with other physical abnormalities, such as heart or thumb anomalies (Table 5). In contrast, none of the 12 patients with *RPL11* mutations and malformations have craniofacial abnormalities ( $p = 0.007$ , Fisher's exact test [FET]). Moreover, none of the 35 reported patients with *RPS19* mutations and malformations<sup>6,10,12,32</sup> presented with cleft lip and/or palate ( $p = 9.745 \times 10^{-7}$  for *RPL5* versus *RPS19*, FET). Furthermore, among a group of 21 DBA patients with craniofacial abnormalities, reported by the Diamond-Blackfan Anemia Registry (DBAR) of North America, no *RPS19* mutations were found, which strongly suggested that the DBA phenotype associated with cleft lip and/or palate is caused by gene(s) other than *RPS19*.<sup>33</sup> A report from DBAR revealed that 5.7% of DBA patients present with cleft lip and/or palate<sup>4</sup> and some cases were reported with additional microtia and/or ear abnormalities.<sup>34</sup> In contrast, in the general population, 0.1%–0.2% of children are born with cleft lip and/or palate.<sup>35</sup> Therefore, craniofacial clefting is clearly associated with DBA, particularly with mutations in *RPL5*. Because about half of DBA patients have unknown underlying genetic causes of DBA, it is likely that mutations in other DBA gene(s) yet to be discovered also cause craniofacial abnormalities. Importantly, it is possible that some DBA patients who present with craniofacial malformations and with only subtle (such as macrocytosis and/or elevated eADA) or no hematological abnormalities are underdiagnosed. *RPL5* screening for mutations in these patients could potentially help establish the diagnosis of DBA.

In addition to craniofacial malformations in patients with *RPL5* mutations, for patients whose medical records are available, eight out of 20 patients with mutated *RPL5* and eight out of 18 patients with mutated *RPL11* have

**Table 2. RPL5 Mutations in 18 Probands and Six Family Members from a Total of 196 Families Studied**

Mutation Type	Proband's ID (Gender) Inheritance	Family Members	DNA Mutation	Exon/ Intron	Predicted Amino Acid Change	Age at Diagnosis	Malformation Status	Response at First Steroid Therapy	Present Therapy
<b>Nonsense mutation</b>	P1 (F) sporadic	<i>m</i> : normal seq	c.48C→G	Ex2	Y16Stop	at birth	NA	responsive to high doses only	RBC trx
	P2 (F) de novo	<i>f, m</i> : normal seq	c.67C→T	Ex2	R23Stop	2mo	cleft lip, cleft palate	responsive SD	RBC trx
	P3 (F) sporadic		c.228C→A	Ex4	C76Stop	10 mo	cleft palate, ASD, hypoplastic thumb, micrognathia, tracheomalacia	initially responsive	stem cell transplant
<b>Missense mutation</b>	P4 (M) familial	<i>m, s, b</i> : normal seq	c.418G→A	Ex5	G140S	3 mo	none	unresponsive	RBC trx
<b>Deletion/insertion</b>	P5 (M) sporadic	<i>f</i>	c.418G→A c.46_47 insA	Ex5 Ex2	G140S Y16Stop	no anemia at birth	NA micrognathia, hypertelorism, soft cleft palate, triphalangeal right thumb, widened webbed spaced between first and second toes, hypospadias	no therapy no therapy	no therapy no therapy
	P6 (F) de novo	<i>f, m</i> : normal seq	c.156_159 delAGTT	Ex3	Frameshift at codon 52; stop at 54	10 mo	small jaw, cleft palate, triphalangeal thumb, hip dysplasia, rib anomalies	responsive SD	steroid therapy
	P7 (M) sporadic		c.169_172 delAACA	Ex3	Frameshift at codon 57; stop at 68	at birth	partial anomalous pulmonary venous return	unresponsive	RBC trx
	P8 (M) de novo	<i>f, m, b</i> : normal seq	c.173 delG	Ex3	Frameshift at codon 58; stop at 69	9 mo	multiple congenital heart defects <sup>a</sup>	responsive SD	RBC trx
	P9 (M) sporadic		c.173_4 delGA	Ex3	Frameshift at codon 58; stop at 111	NA	long proximal thumb phalanges bilateral, multiple congenital heart defects <sup>b</sup>	NA	NA
	P10 (M) sporadic		c.173_4 delGA	Ex3	Frameshift at codon 58; stop at 111	10 mo	small jaw, cleft palate, bronchiopharyngeal malacia, mild hydrocephalus	responsive SD	steroid therapy
	P11 (F) de novo	<i>f, m, b, s</i> : normal seq	c.173_4 delGA	Ex3	Frameshift at codon 58; stop at 111	NA	NA	NA	NA
	P12 (F) familial		c.235_236 insT	EX4	Frameshift at codon 79; stop at 112	11 y <sup>c</sup>	cleft soft palate	unresponsive	RBC trx
		<i>m</i> : familial	c.235_236 insT	EX4	Frameshift at codon 79; stop at 112	55 y	none	refused steroid trial	RBC trx
	P13 (F) familial		c.235_236 insT	EX4	Frameshift at codon 79; stop at 112	12 mo	cleft palate, bifid uvula, hypoplastic thumb,	NA	RBC trx
		<i>m</i> : familial	c.235_236 insT	EX4	Frameshift at codon 79; stop at 112	NA	none	NA	RBC trx
	P14 (F) familial		348_351 insTGGA	Ex5	Frameshift at codon 117; stop at 121	12 mo	mandibular hypoplasia with retrognathia, cleft palate with bifid uvula, dysplastic thumbs, ASD type II	unresponsive	RBC trx

		<i>s</i> : familial	ND		ND	12 mo	mandibular hypoplasia with retrognathia, cleft palate, triphalangeal thumbs, persistent foramen ovale, ASD type II	no therapy	no therapy
		<i>m</i> : familial	348_351 insTGGA	Ex5	Frameshift at codon 117; stop at 121	20 yr	none	no therapy	no therapy
	P15 (M) familial	<i>f, m, s</i> : normal seq	c.498_502 delTGTTGG and 39bp ins	Ex5		NA	triphalangeal thumbs, VSD	responsive	no therapy
		<i>b</i> : familial	c.498_502 delTGTTGG and 39bp ins	Ex5		NA	cleft lip, triphalangeal thumbs	unresponsive	RBC trx
		<i>d</i> : familial	c.498_502 delTGTTGG and 39bp ins	Ex5		NA	NA	NA	NA
	P16 (M) sporadic		c.573_574 insG	Ex. 6	Frameshift at codon 192; stop at 216	NA	inability to flex right distal thumb phalanx	responsive SD	NA
<b>Splice-site mutation</b>	P17 (M) de novo	<i>f, m, b</i> : normal seq	Donor splice site IVS2 +2t→g	Intr2		NA	none	responsive SD	RBC trx
	P18 (M) sporadic		Donor splice site IVS3 +1g→t	Intr3		NA	none	responsive SD	RBC trx

Abbreviations are as follows: P, proband; *f*, father; *m*, mother; *s*, sister; *b*, brother; *d*, daughter; ins, insertion; del, deletion; Ex, exon; In, intron; seq, sequence; mo, month; y, years; ND, not done; NA, not available; SD, steroid dependent; RBC trx, red blood cell transfusions; ASD, atrial septal defect; VSD, ventricular septal defect.

<sup>a</sup> Small patent ductus arteriosus, mild mitral valve prolapse, mild mitral regurgitation.

<sup>b</sup> Double outlet right ventricle, pulmonary stenosis, left pulmonary artery stenosis, patent ductus arteriosus.

<sup>c</sup> macrocytic anemia diagnosed at age 11, DBA not diagnosed until years later.

**Table 3. RPL11 Mutations in 13 Proband and Five Family Members from a Total of 196 Families Studied**

Mutation Type	Proband's ID (Gender) Inheritance	Family Members	DNA Mutation	Exon/ Intron	Predicted Amino Acid Change	Age at Diagnosis	Malformation Status	Response at First Steroid Therapy	Present Therapy
<b>Nonsense mutation Deletion/ Insertion</b>	P19 (F) de novo	<i>f, m</i> : normal seq	c.223C→T	Ex 3	R75Stop	2 mo	triphalangeal thumbs	responsive SD	steroid therapy
	P20 (F) familial		c.60_61 delCT	Ex2	Frameshift at codon 20; stop at 53	9 mo	VSD, narrow pulmonary artery	responsive	RBC trx
		<i>m</i> : familial	c.60_61 delCT	Ex2	Frameshift at codon 20; stop at 53	NA	triphalangeal thumb	no steroid trial	RBC trx
		<i>gm</i> : familial	c.60_61 delCT	Ex2	Frameshift at codon 20; stop at 53	NA	triphalangeal thumb	NA	NA
	P21 (F) sporadic		c.94_97 delAGAC	Ex2	Frameshift at codon 32; stop at 32	1 mo	none	responsive SD	steroid therapy
	P22 (M) sporadic		c.160_161 insA	Ex3	Frameshift at codon 54; stop at 66	NA	abnormal thumbs	responsive SD	RBC trx
	P23 (F) de novo	<i>f, m</i> : normal seq	c.290 delA	Ex4	Frameshift at codon 97; stop 14 aa behind wt stop	1 mo	short neck	unresponsive	stem cell transplant
	P24 (M) de novo	<i>f, m</i> : normal seq	c.291_292 insA	Ex4	Frameshift at codon 97; stop at 120	NA	horseshoe kidney	responsive SD	steroid therapy
	P25 (M) familial		c.314_315 delTT	Ex4	Frameshift at codon 105; stop at 119	NA	none	responsive	no therapy
		son	c.314_315 delTT	Ex4	Frameshift at codon 105; stop at 119	NA	small extra thumbs	no therapy	no therapy
P26 (F) familial		c.482_484 delAGG	Ex5	161Edel	2 mo	none	responsive to high doses	RBC trx	
<b>Splice-site mutation</b>	P27 (F) de novo	<i>m</i> : familial	c.482_484 delAGG	Ex5	161Edel	NA	none	no therapy	no therapy
		<i>f, m</i> : normal seq	Donor splice site IVS1 +2t→c	In1		3 mo	none	unresponsive	RBC trx
	P28 (M) de novo	<i>f, m, b</i> : normal seq	Acceptor splice site IVS2 -1g→a	In2		3 mo	flat thenar muscle, small jaw	responsive SD	steroid therapy
	P29 (M) sporadic		Acceptor splice site IVS2 -1g→a	In2		2 y	Tetralogy of Fallot, bilateral grade 3 vesicoureteral reflex	responsive SD	steroid therapy
	P30 (F) de novo	<i>f, m, b</i> : normal seq	Donor splice site IVS4 +1g→t	In4		2.5 mo	hypoplastic thumb, flat thenar muscle	responsive/ stopped due to growth retardation	RBC trx
	P31 (M) familial		Donor splice site IVS4 +1g→a	In4		12 mo	small extra thumbs, Tetralogy of Fallot	responsive SD	steroid therapy
<i>m</i> : familial		Donor splice site IVS4 +1g→a	In4		21 y	left thumb: additional short phalanx	no steroid therapy	RBC trx (2x/yr)	

Abbreviations are as follows: P, proband; *f*, father; *m*, mother; *gm*, grandmother; *s*, sister; *b*, brother; ins, insertion; del, deletion; Ex, exon; In, intron; seq, sequence; mo, month; y, years; NA, not available; SD, steroid dependent; RBC trx, red blood cell transfusions; VSD, ventricular septal defect.



**Table 4. Sequence Changes in RPS7, RPS17, RPL36, RPS15, and RPS27A in DBA Patients**

Mutated Gene	Proband's ID (Gender) Inheritance	Family Members	DNA Mutation	Exon/Intron	Predicted Amino Acid Change	Age at Diagnosis	Malformation Status	Response at First Steroid Therapy	Present Therapy
RPS7	P32 (M) sporadic	s – normal sequence	Donor splice site IVS3+1g>a	In3		NA	none	responsive SD	steroid therapy
RPS17	P33 (M) de novo	f, m, s – normal sequence	c. 200_201 delGA	Ex3	Frameshift at codon 67; stop at 86	4 mo	none	responsive SD	steroid therapy
RPL36	P34 (M) sporadic		c. 250_251 delGA	Ex3	Frameshift at codon 84; stop 27 nt behind wt stop codon	2 mo	left thumb abnormality	responsive SD	steroid therapy
RPS15	P35 (F) sporadic		c.208A→G	Ex3	Met70Val	1 d	truncus arteriosus type 1, VSD, colobomata, absent right radius, ulna and thumb, absent left ulna, small left thumb and 5th finger, unilateral absent fibulae and club foot	unresponsive	bone marrow transplant
RPS27A	P36 (F) familial	m, b – normal sequence	c.169T→C	Ex4	Ser57Pro	15 mo	none	no therapy	no therapy
		f familial	c.169T→C	Ex4	Ser57Pro	no DBA diagnosis	none	no therapy	no therapy

Abbreviations are as follows: P, proband; f, father; m, mother; s, sister; b, brother; del, deletion; Ex, exon; In, intron; nt, nucleotide; wt, wild-type; mo, month; d, day; NA, not available; SD, steroid-dependent; VSD, ventricular septal defect.

thumb abnormalities, including triphalangeal or dysplastic thumbs, additional bilateral small thumbs, and bilateral long proximal thumb phalanges. In addition, two patients with *RPL11* mutation have flat thenar muscles, one isolated and the other associated with thumb abnormality. In contrast, review of the literature reveals that various thumb abnormalities were reported in only seven out of 76 (9%) patients with *RPS19* mutations<sup>6,10,12,32</sup> ( $p = 0.0024$  for *RPL5* versus *RPS19* and  $p = 0.0012$  for *RPL11* versus *RPS19*, FET). Moreover, congenital heart defects were found more often among patients with *RPL5* mutations (5/20) compared with *RPL11* (3/18) and *RPS19* (4/76) (Table 5) ( $p = 0.017$  for *RPL5* versus *RPS19*, FET). Strikingly, the majority (11/20) of patients with *RPL5* mutations presented with multiple, severe abnormalities, including craniofacial, heart, and/or thumb malforma-

tions. In contrast, patients with *RPL11* and *RPS19* mutations who presented with multiple physical abnormalities were uncommon: three patients out of 18 and 16 out of 76, respectively<sup>6,10,12,32</sup> (Table 5) ( $p = 0.02$  for *RPL5* versus *RPL11* and  $p = 0.0047$  for *RPL5* versus *RPS19*, FET). Elevated eADA was found in all eight patients with *RPL5* mutations and in eight patients with *RPL11* mutations for whom the results are available. Willig and colleagues<sup>6</sup> described 11 families with *RPS19* mutations and eADA measurements. They have found cosegregation of the *RPS19* mutations and high eADA in four families, although partial cosegregation of the *RPS19* mutations and elevated eADA were found in seven other families.

Our finding of an *RPL5* mutation in the family first diagnosed with Aase syndrome,<sup>31</sup> as well as other mutations of *RPL5*, *RPL11*, and, as described by Willigs and colleagues,<sup>6</sup>

**Table 5. Congenital Physical Abnormality in DBA Patients with RPL5, RPL11, and RPS19 Mutations**

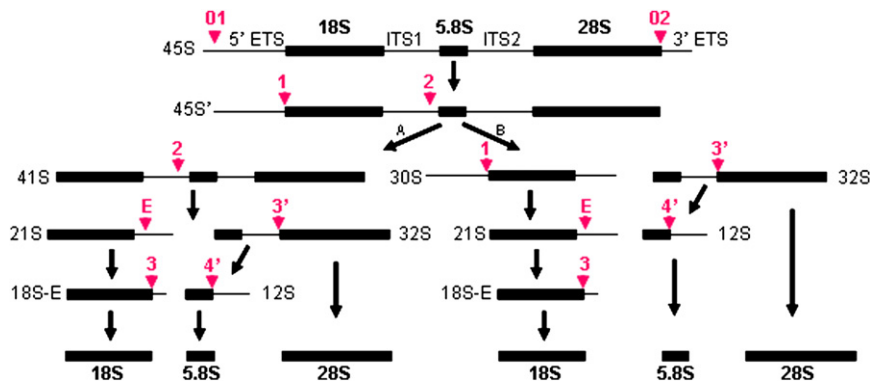
	Patients with Mutations <sup>a</sup>	Patients with Malformations	Cleft Lip and/or Palate <sup>b</sup>	Thumb Abnormality <sup>b</sup>	Heart Abnormality <sup>b</sup>	Other Abnormalities, Including Short Stature <sup>b</sup>	Multiple Malformations
RPL5	20	14	9 <sup>c</sup>	8	5	13	11
RPL11	18	12	0	8	3	4	3
RPS19 <sup>d</sup>	76	35	0	7	4	47	16

<sup>a</sup> Total number of patients whose medical data are available.

<sup>b</sup> Isolated or in association with other abnormalities.

<sup>c</sup> Including two patients with soft cleft palate.

<sup>d</sup> Based on <sup>6,10,12,32</sup>.



**Figure 1. Pre-rRNA Processing in Human Cells, According to Hadjiolova<sup>49</sup> and Choessel<sup>22</sup>**

The mature 18S, 5.8S, and 28S rRNAs are separated by ITS1 and ITS2 and flanked by external transcribed spacers, 5'-ETS and 3'-ETS. The red arrows and the numbers in red indicate the cleavage sites. "A" and "B" represent two pathways of the rRNA processing.

*RPS19* in DBA patients presenting with anemia and triphalangeal thumbs, confirms the notion that the Aase syndrome is a subtype of DBA and not a separate syndrome.

Interestingly, 16 out of 18 patients with *RPL5* mutations whose treatment data were available required steroid treatment, and nine of them were responsive to the first steroid treatment, five were unresponsive, and one patient refused steroid treatment, choosing red blood cell transfusions. Similarly, 13 out of 17 patients with *RPL11* mutations and available data of their medical treatment also underwent steroid therapy. Eleven of them responded well to the treatment, and two were unresponsive. According to data from the DBA Registry of North America, 79% of DBA patients have been initially responsive to steroid, 17% have been unresponsive, and 4% were never treated with steroids.<sup>4</sup> In light of these data, steroid treatment of DBA patients with *RPL5* and *RPL11* mutations seems to have a similar outcome as that in the general DBA population. At present, 11 *RPL5*-mutation patients are dependent on red blood cell transfusions, two are steroid-dependent, one patient underwent stem cell transplantation, and three do not need any treatment. Seven *RPL11*-mutation patients are red blood cell transfusion-dependent, six are dependent on steroid treatment, one patient underwent stem cell transplantation, and three do not need any treatment.

Review of available medical data of 14 patients with *RPL5* mutations revealed one patient with melanoma. Similarly, one patient out of 11 with *RPL11* mutations and available medical data had uterine cancer. None of the patients with *RPS7*, *RPS17*, *RPS15*, *RPL36*, and *RPS27A* sequence changes suffered from cancer. Data from the DBA Registry of North America revealed eight DBA patients among 420 registered as having hematopoietic, nonhematopoietic, or myelodysplastic syndrome.<sup>4</sup>

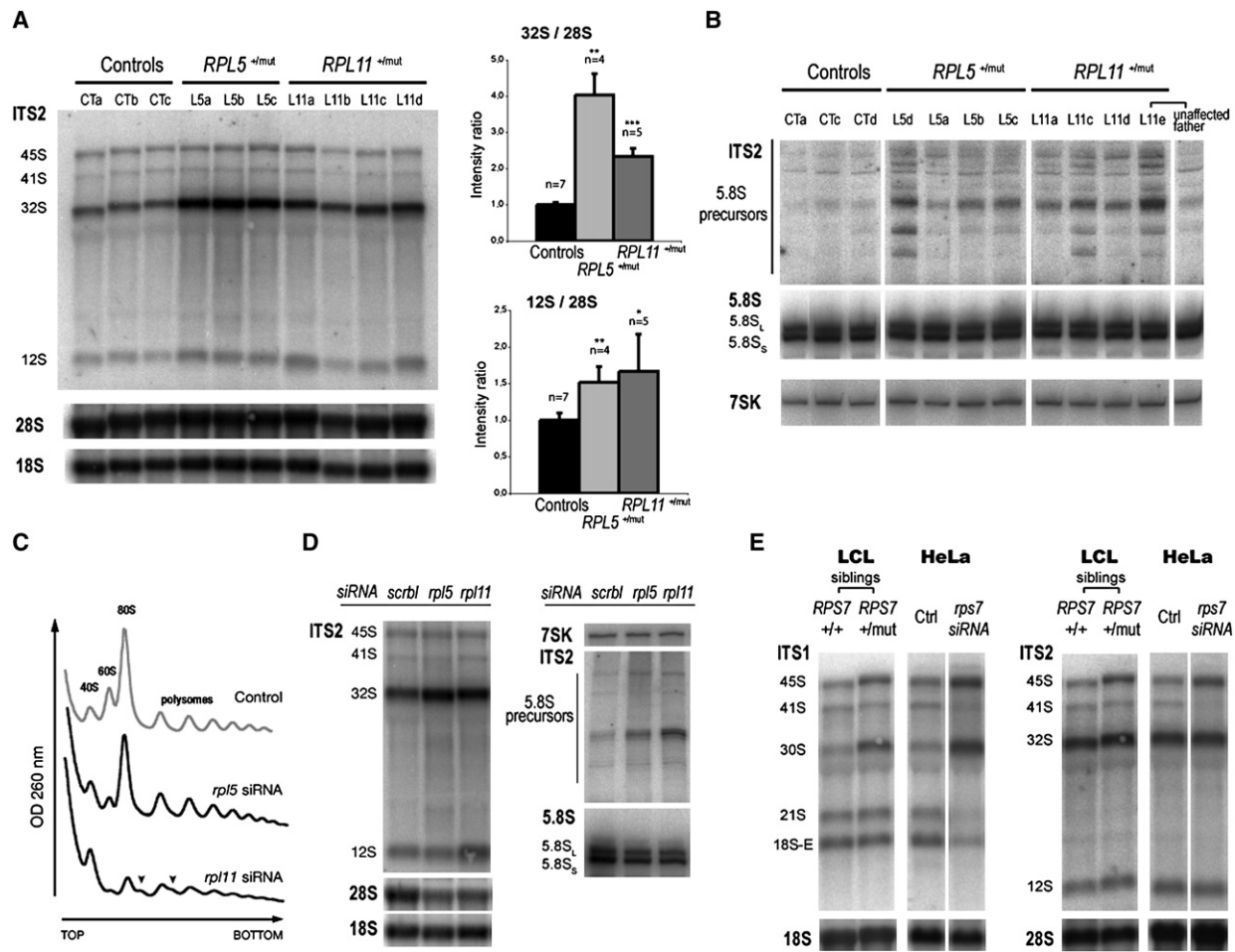
DBA-associated ribosomal proteins *RPS19*, *RPS24*, and *RPL35A* were shown to be required for pre-rRNA maturation, a complex process that leads to the production of three rRNAs from a common precursor (Figure 1). To determine whether DBA mutations in *RPL5* and *RPL11* affected pre-rRNA maturation, we analyzed pre-rRNAs from lymphoblastoid cells established from DBA patients by northern blotting, with probes complementary to the internal transcribed spacer 2 (ITS2), and the signals were quantified by phosphoimager. Cells mutated in either of these genes

displayed a conspicuous accumulation of 32S pre-rRNA when compared to cells derived from control individuals, this phenotype being more pronounced for mutations in *RPL5* (Figure 2A). In parallel, we also observed a higher amount of 12S pre-rRNA (Figure 2A), as well as accumulation of smaller precursors of 5.8S rRNA, which were not detected in cells derived from the unaffected father of one of the patients with *RPL11* mutation (Figure 2B). These results indicate defective maturation of ITS2, both at the initial endonucleolytic cleavage in the 32S pre-rRNA and during subsequent processing steps. The ITS1 probe also revealed a moderate increase in the levels of 30S and 18S-E pre-rRNAs (data not shown), consistent with several reports in yeast showing that perturbation of the pre-60S particle maturation affects maturation of the 18S rRNA.<sup>36</sup>

HeLa cells transfected with siRNAs complementary to mRNAs encoding *RPL5* and *RPL11* had reduced levels of 28S and 5.8S when compared to controls. Consistently, analysis of cytoplasmic ribosomes on sucrose gradient showed low levels of free 60S subunit and formation of half-mers in the polysomes upon knockdown of *RPL5* or *RPL11* expression (Figure 2C), indicating that these proteins are essential for synthesis of the 60S subunit. Indeed, inhibition of *RPL5* or *RPL11* synthesis led to accumulation of 32S and 12S pre-rRNAs, together with shorter precursors to 5.8S (Figure 2D). Given the similarity of these pre-rRNA processing defects with those observed above in lymphoblastoid cells, we conclude that heterozygous mutation of *RPL5* and *RPL11* in DBA has a direct impact on pre-rRNA processing.

We applied a similar approach to evaluate the consequence of the mutation in *RPS7* found in a single DBA patient. Lymphoblastoid cells established from this patient's cells displayed higher levels of 45S and 30S pre-rRNAs when compared to cells derived from an unaffected sibling (Figure 2E). Accordingly, knockdown of *RPS7* synthesis in HeLa cells with siRNAs resulted in a strong defect in 5'-ETS processing. We observed accumulation of 45S and 30S pre-rRNAs and a marked decrease of the levels of the 41S, 21S, and 18S-E intermediates, whereas the amount of precursors to the large ribosomal subunit RNAs was unchanged (Figure 2E). This defective processing pattern is very reminiscent of that observed upon depletion of





**Figure 2. Impact of DBA Mutations in *RPL5*, *RPL11*, and *RPS7* on Pre-rRNA Processing**

(A) Northern blot analysis of total RNAs extracted from lymphoblastoid cells with mutations in *RPL5* and *RPL11*. Precursor and mature rRNAs were detected with probes complementary to the ITS2, 18S rRNA, and 28S rRNA sequences. Bar graphs show signal quantification with a phosphoimager and include experiments from additional samples processed separately. Bars indicate variation of 32S/28S and 12S/28S intensity ratio relative to controls (means  $\pm$  SD). Significance of the difference compared to controls was assessed with a Student's *t* test assuming nonequal variance (single asterisk indicates  $p \leq 0.05$ ; double asterisk indicates  $p \leq 0.01$ ; triple asterisk indicates  $p \leq 0.001$ ).

(B) Northern blot analysis of total RNAs fractionated on denaturing 6% polyacrylamide gel. After transfer, the blot was successively hybridized with probes complementary either to the junction between 5.8S rRNA and ITS2 (probe 5'-ITS2) or to 5.8S rRNA. 7SK RNA, a stable and abundant noncoding RNA involved in the regulation of RNA polymerase II, was chosen as a loading control.

(C) Analysis on sucrose gradient of cytoplasmic ribosomes isolated from HeLa cells 48 hr after transfection with siRNAs directed against the mRNAs encoding *RPL5* or *RPL11*. Arrowheads indicate polysomes containing half-mers. Control: transfection without siRNA. Expression of both siRNAs results in significant reduction of 60S particles. Differences in the extent of reduction are related to differing efficiencies of the siRNAs used.

(D) Northern blot analysis of total RNAs from cells treated as in (C) with probes complementary to the ITS2, the 18S rRNA and the 28S rRNA (1% agarose gel), or with probes complementary to the junction between the 5.8S rRNA and the ITS2 (probe 5'-ITS2) and to the 5.8S (6% polyacrylamide gel). The 7SK RNA is shown as a loading control for the polyacrylamide gel. Scrbl: scramble siRNA.

(E) Northern blot analysis of pre-rRNA processing in lymphoblastoid cell lines (LCL) with mutation in *RPS7* and in HeLa cells transfected with siRNAs targeting *RPS7* mRNAs. The control *RPS7*<sup>+/+</sup> lymphoblastoid cells are derived from an unaffected sibling of the *RPS7*<sup>+mut</sup> patient. Ctrl: HeLa cells transfected without siRNAs. Methods: The 21 nt siRNA duplexes with a 3' dTdT overhang, corresponding to L5 mRNA (5'-AAGGGAGCTGTGGATGGAGGC-3')<sup>47</sup> and to L11 mRNA (5'-AAGGTGCGGGAGTATGAGTA-3')<sup>48</sup> were purchased from Eurogentec (Seraing, Belgium) and transfected via electrotransformation, as described previously.<sup>42</sup> Detection of pre-rRNA on northern blots was performed as described previously<sup>50</sup> with oligonucleotide probes 18S, 28S, ITS1, ITS2b, and ITS2-d/e.<sup>42</sup> For detection of ITS2, ITS2-b and ITS2-d/e probes were mixed in equal amounts. The remaining probes had the following sequences: 5'-ITS1, 5'-CCTCGCCCTCCGGGCTCCGTTAATGATC-3', 5.8S, 5'-CAATGTGTCTGCAATTAC-3'; 5'-ITS2, 5'-GGGCGGATTGATCGCAAGCGAGCCTC-3', 7SK (mix of two probes), 5'-CATGGAGCGGTGAGGGAGGA-3', and 5'-GTGTCTGGAGTCTTGAAGC-3'. For ribosome analysis on sucrose gradient, HeLa cells transfected with siRNAs for 48 hr were treated with 100 mg/ml cycloheximide (Sigma Aldrich, St. Louis, MO) for 10 min and fractionated, and the cytoplasmic fraction was analyzed on a 10%–50% sucrose gradient as described previously.<sup>42</sup>

RPS24.<sup>25</sup> As for mutations in *RPL5* and *RPL11*, these results strongly suggest that mutation of *RPS7* in this DBA patient directly affects maturation of the pre-rRNA.

RPL5, a 297 amino acid, 34 kDa protein component of the 60S large ribosomal subunit, localizes to the cytoplasm and the nucleolus. RPL5 is a nucleocytoplasmic shuttle protein that plays an important role in 5S rRNA intracellular transport during assembly of the large ribosomal subunit.<sup>37,38</sup> Likewise, RPL11, a 178 amino acid, 21 kDa protein, is also a component of the large ribosomal subunit. RPL5 and RPL11 are closely connected during ribosome biogenesis; it has been shown, by the recent demonstration in yeast *Saccharomyces cerevisiae*, that they form a subcomplex with the 5S rRNA prior to incorporation into preribosomes.<sup>39</sup> RPS19 deficiency was shown to affect 18S rRNA maturation and small-ribosomal-subunit formation in yeast and in human cells.<sup>22–24,40</sup> We showed that mutations of RPS24 in human cells alter maturation of the 18S rRNA at an earlier stage than do mutations in RPS19.<sup>25</sup> Similarly, alterations of pre-rRNA processing and large-ribosomal-subunit synthesis were demonstrated in cell lines and in DBA cells with RPL35A deficiency.<sup>15</sup>

Here, we show that mutations in RPL5 and RPL11 in DBA cells lead to accumulation of the 32S and 12S intermediates of 28S rRNA and smaller precursors on the 5.8S rRNA maturation pathways. As shown by the analysis of cytoplasmic ribosomes on sucrose gradient, both RPL5 and RPL11 proteins are essential for rRNA maturation and 60S ribosomal subunit formation. *RPL5* and *RPL11* knock-down induces accumulation of precursors to 5.8S rRNA, whereas siRNA-mediated knockdown of *RPL35A* transcripts results in a decrease of these rRNA species,<sup>15</sup> indicating that these RPs fulfill different functions in pre-rRNA processing, in agreement with recently published data.<sup>41</sup> RPS7 mutation, in turn, affects the 5'-ETS cleavage, similar to *RPS24* mutation. The role of RPS15 in nuclear export of the 40S subunit has been documented in mammalian cells<sup>42</sup> and in *Saccharomyces cerevisiae*,<sup>21,43</sup> whereas depletion of RPS31, the ortholog of RPS27A in yeast, is also necessary for production of the 40S ribosomal subunit.<sup>43</sup> The findings of several ribosomal protein genes mutated in DBA, which subsequently cause aberrant rRNA maturation and small or large subunit formations, supports a hypothesis of a unifying model of DBA as a disorder of ribosome biogenesis that might stem from diverse defects in pre-rRNA processing.

Interestingly, RPL11 has been recently implicated in the negative regulation of c-Myc function. The c-Myc oncoprotein enhances ribosome biogenesis and promotes cell growth. Overexpression of c-Myc was shown to alter ribosome production and tumorigenesis, whereas overexpression of RPL11 inhibits these actions of c-Myc. In contrast, reduction of RPL11 expression increases c-Myc-induced transcription and cell proliferation.<sup>44</sup> Both RPL5 and RPL11, as well as RPL23, were shown to inhibit MDM2-mediated p53 ubiquitination and degradation in response

to ribosomal stress by restoring p53-mediated transactivation, accumulating p21 protein levels, and inducing a p53-dependent cell-cycle arrest.<sup>30,45–48</sup> It will be interesting to evaluate the effects of RPL5 and RPL11 mutations of these pathways in future studies for assessment of their potential role(s) in apoptosis and in promoting tumorigenesis in patients with DBA.

In summary, we found that mutations in *RPL5* and *RPL11* are present in about 9% and 6.5% of our DBA proband cohort, respectively. After correcting for the fact that our study population was prescreened for *RPS19*, *RPS24*, and *RPL35A* mutations, we estimate that *RPL5* and *RPL11* mutations are present in about 6.6% and in 4.8% of the overall DBA population, respectively. Remarkably, *RPL5* and *RPL11* mutations are associated with physical malformations in about 70% and 67% of mutated patients, respectively, as compared to only 46% of patients with *RPS19* mutations, none of whom have been reported to have cleft lip and/or cleft palate.<sup>6,10,12,32</sup> Thus, to our knowledge, *RPL5* is the first ribosomal protein gene to be associated with cleft lip and/or cleft palate abnormalities in DBA patients. Moreover, mutations in *RPL5* appear to cause a more severe phenotype, compared to mutations in *RPL11* and *RPS19*, whereas mutations in *RPL11* are predominantly associated with thumb abnormalities. The data do not permit definitive determination of pathogenicity for the mutations that we found in *RPL36*, *RPS15*, and *RPS27A*, which will require further study by groups with access to additional DBA patient cohorts.

## Acknowledgments

This work was supported by the Diamond-Blackfan Anemia Foundation (to H.T.G.), the Manton Foundation (to H.T.G. and A.H.B.) and the French National Research Agency (Agence Nationale de la Recherche—RIBODBA project). DNA sequencing was performed by the Children's Hospital Boston Program in Genomics, Molecular Genetics Core, supported by the Developmental Disabilities Research Center (NIH P30 HD18655) and the Harvard Neuromuscular Disease Project (NIH P50 NS040828). The authors thank Kyriacos Markianos for his help with statistical analysis. Special thanks to Marie Arturi for stimulating DBA research collaboration and to physicians and DBA patients for participating in the study.

Received: September 26, 2008

Revised: October 31, 2008

Accepted: November 6, 2008

Published online: December 4, 2008

## Web Resources

The URLs for data presented herein are as follows:

Entrez Gene, <http://www.ncbi.nlm.nih.gov/sites/entrez> and <http://www.ncbi.nlm.nih.gov/SNP/>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim/>

Primer3 Input, [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)

UCSC Genome Browser, <http://genome.ucsc.edu/cgi-bin/hgGateway>

## Accession Numbers

The GenBank accession numbers for human *RPL5*, *RPL11*, *RPS7*, *RPS17*, *RPS15*, *RPL36*, and *RPS27A* are [NM\\_000969](#), [NM\\_000975](#), [NM\\_001011](#), [NM\\_001021](#), [NM\\_001018](#), [NM\\_015414](#), and [NM\\_002954](#), respectively.

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