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Molecular mechanisms of Diamond-Blackfan anemia

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I declare that this written submission represents my ideas in my own words and where others' ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission.

Prague, June 27, 2011

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Special thanks to Maťko, for being always here for me.

Abstract

Diamond-Blackfan anemia (DBA) is a rare congenital syndrome that presents with anemia and selective deficiency of erythroid precursors, while other blood lineages are usually unaffected. Approximately half of the patients display additional somatic anomalies and growth retardation. The therapy is mostly symptomatic and is dominated by corticosteroids, other modalities include regular blood transfusions or hematopoietic stem cell transplantation.

At the beginning of this work, only two DBA causal genes were known, *RPS19* and *RPS24*, being mutated in approximately 1/4 of all DBA patients. The goals of this work were to study the consequences of the known DBA causal mutations on cellular level, to select probable candidates for DBA, and to perform screening for mutations in confirmed DBA causal genes in the DBA registry of the Czech Republic.

To date, over a half of DBA patients have been reported to carry a mutation in one of nine known DBA causal genes, including RPS17, RPL11 and RPL5, that were identified by our team and are reported in this dissertation. All confirmed DBA causal genes encode for ribosomal proteins (RPs) that were essential for ribosome assembly.

We further hypothesized a non-ribosomal protein participating in this process might be involved in DBA pathogenesis, too. In one DBA patient, we identified a rare sequence variant in one such candidate, a protein arginine methyltransferase 3 (PRMT3). We reported that the patient PRMT3 variant was not fully active, however, we didn't confirm its causative role for DBA. In conclusion, our findings broadened the spectrum of known DBA causal genes and supported the hypothesis, that the mechanism of pathogenesis is a ribosomal defect.

Aims of the work

Specific aims of this work were:

- Identify probable candidates for novel causal genes for Diamond-Blackfan anemia (DBA) and search for their mutations in the patients from Czech DBA registry
- Study the role of such candidates in molecular pathogenesis of this syndrome, using cellular models
- Screen the patients from Czech DBA registry for mutations in known causal genes, search for genotype-phenotype correlations

Contents

1. Preface

Ribosomes are the only known enzymatic complex that can produce new proteins, and as such, they are essential for life of all living organisms. These ribonucleoprotein particles consist of two subunits, small and large, each being composed of 1–3 ribosomal RNA strands and a few dozens of proteins. The catalytic function of ribosomes (discrimination of correct codon-anticodon pair and peptide bond formation) is carried out by the rRNAs, while ribosomal proteins (RPs) stabilize the rRNA structure, interact with translation factors and chaperones, and some have regulatory functions.

Most of small subunit RPs and also some large subunit RPs are also essential for the biogenesis of eukaryotic ribosome, and therefore, homozygous knockouts of these essential RP genes are embryonic lethal at or before the blastocyst stage. Heterozygous RP gene mutations in higher eukaryotes give rise to characteristic phenotype first reported in *Drosophila* and termed *Minute*, from the latin expression meaning *(becoming) very small*. Since all RPs are closely functionally related, the phenotypes caused by mutations in different RP genes are exceptionally alike, and the parallels of *Drosophila Minutes* were later observed in other model organisms, although specific phenotypic features vary.

A disruption of a human RP gene was first reported in 1999: Draptchinskaia et al. reported a balanced chromosomal translocation in a patient diagnosed with a rare congenital syndrome, Diamond-Blackfan anemia (DBA). The mutation disrupted the *RPS19* gene that encoded an essential protein of the small ribosomal subunit, and interestingly, this gene was later found to be affected in approximately one quarter of all DBA patients.

Although DBA have some common characteristics to the various *Minute* phenotypes (such as various developmental anomalies and growth retardation), the hallmark of this syndrome is anemia caused by selective deficiency of erythroid cells and their precursors. Since *RPS19* was until 2006 the only known DBA causal gene, the original hypotheses supposed that RPS19 is not only a structural component of ribosomes but plays a specific role in erythroid differentiation. However, such extraribosomal function of RPS19 was not confirmed by any experimental data, and therefore, most researchers began to incline to the explanation that the common underlying defect in DBA is related to ribosome biogenesis and/or function. This hypothesis was soon after supported by the identification of DBA causal mutations in several other RP genes.

The aim of this work was to identify novel genes causal for DBA and to study the roles of promising candidate proteins on cellular models. Our flow of reasoning was based on the roles of known DBA causal genes and on the probable consequences of their mutations in human cells. Since all genes whose causality for DBA has been confirmed to date encode ribosomal proteins, my thesis begins with the description of ribosome structure and the roles of RPs (Section 2.1). Following sections are dedicated to the ribosome biogenesis (2.2) and mechanisms that participate in regulation and control of this complex process (2.3). Next sections outline the consequences of a defect in ribosome biogenesis and discuss its impact on the level of cells and tissues (2.4).

Understanding the link between ribosomal protein deficiency and aplastic anemia is indeed an essential step for finding novel genes causal for this syndrome. Therefore, the following section (2.5) briefly sketches the process of erythropoiesis, with a special attention to the role of stress in erythropoiesis and to the mechanisms underlying other examples of aplastic anemias in humans. The next section of the Review of literature describes the Diamond-Blackfan anemia (2.6), its clinical manifestation, known causal genes and proposed mechanisms of pathogenesis. The final section (2.7) presents the overview of promising candidates that might also be causal for DBA in a fraction of patients, or might modulate the phenotype.

The main focus of this work is on the the molecular pathogenesis of this syndrome, rather than on the medical aspects and patients' care. The reader is expected to be

familiar with molecular biology, genetics and biochemistry; knowledge of hematology and medical terminology is not assumed and therefore, the respective sections may seem too descriptive for a physician.

Extending the knowledge of the DBA pathogenesis and the identification of other genes causal for this syndrome is important for providing the patients with better care, namely for the development of more specific therapy with less adverse effects, and for assuring quality genetic counseling. Another motivation for studying this unique syndrome is to get better insight into such fundamental cellular processes such as ribosome biogenesis, cellular stress, and its impact on erythropoiesis.

In the past few years, significant progress has been made in understanding the consequences of RP haploinsuficiency and defects in ribosome biogenesis in general. Therefore, some of our original hypotheses became obsolete and from current perspective, the path we took was not always the straightest one. Our major limitations were the low number of DBA patients in the Czech DBA Registry, and in some cases also the unavailability of DBA patient-derived cells to test our hypotheses.

2. Review of literature

2.1 Ribosomes and their components

Ribosomes (from ***ribo****nucleic acid* and Greek ***soma*** meaning *body*) are complex ribonucleoprotein particles roughly spherical in shape dedicated to the synthesis of proteins. These particles are found in all living cells and also in semi-autonomous organelles of eukaryotic cells (mitochondria and chloroplasts), and they exist either free in cytoplasm or bound to endoplasmic reticulum.

Ribosomes are not only the most abundant macromolecular complexes but also one of the largest ones, with about 200 Å in diameter. They were first described in 1955 by a Romanian scientist George E. Palade as *"small particulate components of cytoplasm"* [5] for which he won a Nobel prize, but their role in translation was revealed by later studies in 1960s (reviewed in [6]).

2.1.1 Composition of ribosomes

Ribosomes consist of two subunits, small and large, that are designated according to their sedimentation coefficients[1] as 40S and 60S in eukaryotic ribosome; the completed eukaryotic ribosomes being sometimes referred to as 80S. Small and large ribosomal subunits are assembled independently, in eukaryotes, the major part of this process takes place in nucleolus and final steps occur in cytosol.

[1] where S or Svedberg equals exactly to 10^{-13} s

Eukaryotic[2] ribosomes contain 4 molecules of rRNA and 79–81 ribosomal proteins [7]; see Fig. 2.1. The molecular weight is approximately $2.5 \cdot 10^6$ daltons in mass. Ribosomes of prokaryotes or semi-autonomous organelles are smaller and much less structurally and functionally complex.

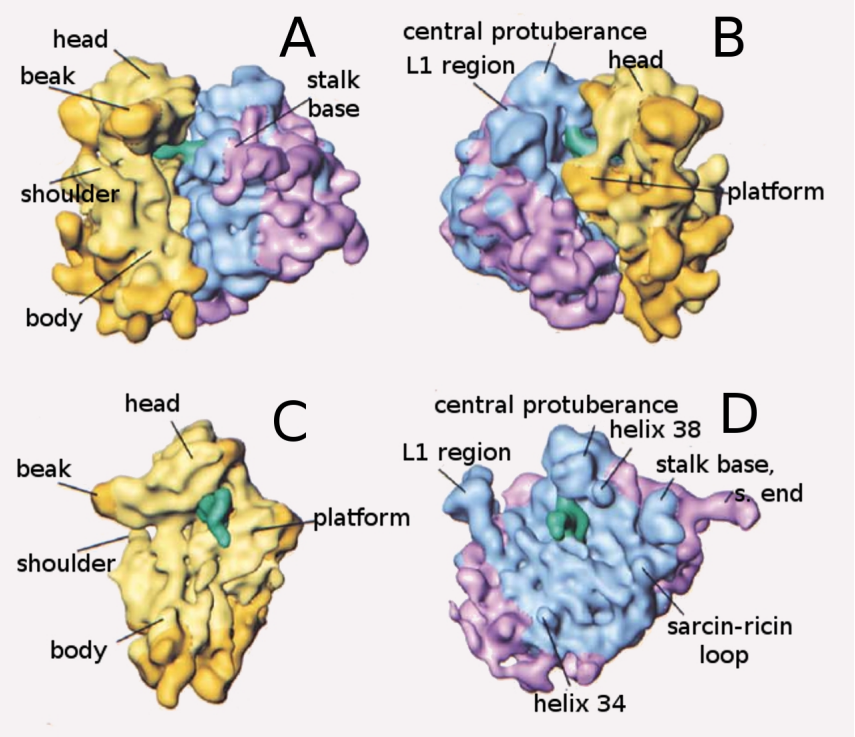

Figure 2.1: Schematic view of the eukaryotic ribosome and its separate subunits. Illustration of 80S ribosome based on the cryo-electron microscopic analysis of the yeast ribosome. Yellow: small subunit, blue and violet: large subunit components, green: catalytic centre. Dark yellow and violet denote proteins that doesn't have homologs in prokaryotes. From [8], altered

Despite the dissimilarities between ribosomes from different species, the primary sequences of the ribosomal components and especially the three dimensional structures of rRNAs show an extraordinary degree of conservation. The core principles of translation (the precise interaction of mRNA codon with tRNA anticodon, and the mechanism of the peptide bond formation catalysis) are conserved, too [9, 10]. The few exceptions from the otherwise universal genetic code include the usage of alternative start codons, an extra codon for selenocysteine in some bacteria, or different codon

[2] Although eukaryotic cells have *two* types of ribosomes – cytosolic and mitochondrial – the term "eukaryotic ribosome" will in this work always refer to the *cytosolic eukaryotic ribosome*, unless specified otherwise.

usage in ribosomes of semi-autonomous organelles. Because of the limited scope of this thesis, further description will be focused on eukaryotic, and especially mammalian ribosomes.

2.1.2 rRNAs

Eukaryotic ribosome contains four rRNAs, one (18S) in small subunit and three (5S, 5.8S, and 28S) in large subunit. They are subjects to many posttranscriptional modiffications, most frequent being 2'-O ribose methylations and pseudouridylations. Clusters of modified nucleotides are common especially in functionally important rRNA domains, such as regions associated with the peptidyltransferase center or the decoding center of the ribosome. They also facilitate the proper rRNA folding during ribosomal assembly and are required for a correct interaction with ribosomal proteins.

Ribosomal RNA belongs to a group of catalytically active RNAs (termed as *ribozymes*). The catalysis of the peptide bond formation is carried out by the longest rRNA of the large ribosomal subunit. The rRNA loops that form the active center contain sequences shared among ribosomes from all kingdoms, including mitochondrial ribosomes, while other regions of rRNA, including ribosomal protein binding sites, are much less conserved. The peptidyltransferase center of ribosome is composed exclusively of rRNA, with no protein in a close vicinity of the active site [11].

In comparison to proteins, the building blocks of RNAs have much lesser diversity and some functional groups that are frequently involved in protein catalysis are in ribozymes missing. The backbone of RNAs is highly negatively charged and the spectrum of secondary and tertially structure motifs is also very limited in contrast to proteins. Primitive rRNAs probably carried out only random peptide bond formation and the decoding capacity as we know it now evolved later [12] with the improvement of rRNA processing and the addition of ribosomal proteins.

During evolution, ribosomal proteins became indispensable for the catalysis of peptide bond formation: fragments of rRNAs from contemporary organisms containing the catalytic loops are not capable to catalyze the formation of peptide bond *in vitro*.

Moreover, while bacterial large ribosomal subunits retain some of their catalytic activity, the large ribosomal subunit is not active on its own.

2.1.3 Ribosomal proteins

Ribosomal proteins are vital for the following processes:

- ribosome biogenesis (rRNA processing and folding)
- translation (stabilization of the subunit structure, interaction with translation factors, determination of translational preferences, improvement of the rate and fidelity of translation)
- cotranslational processes (such as the interaction with protein chaperones at the exit tunnel of the ribosome or cotranslational translocation [13], and
- in many cases also for extraribosomal roles (i.e., unrelated to ribosome biogenesis or translation, such as regulation of cell cycle progression, stress response and apoptosis [6])

Human ribosomes contain approximately 80 ribosomal proteins [7], the size of human RPs ranges from 6.4 kDa for RPL39 to 47.7 kDa for RPL4 [14]. The *exact* number of RPs per ribosome (slightly) differ even between individual ribosomes of one species. These loosely associated RPs that can potentially dissociate from mature ribosomes under certain situations have been implicated in ribosome-independent regulatory functions [15].

Most RPs are very basic, with a pI>10, which is in concordance with their ability to bind the acidic backbone of ribonucleic acids. The few exceptions from this rule are the acidic phosphoproteins P0–P3 of the large ribosomal subunit that form the stalk recognizing translation factors. All human RPs are present in a single copy per ribosome, except for the large subunit proteins RPLP1 and RPLP2 that form homodimers [16].

Although ribosomal proteins belong to the most conserved proteins, regrettably, the RP names do not always denote the same protein ortholog accross different organisms.

Useful glossaries are accessible, for example, on the web sites of University of Miyzaki (`http://ribosome.med.miyazaki-u.ac.jp/`) [6].

Heterogeneity of ribosomes

The protein composition of human ribosomes is rather heterogenous and this probably leads to their functional diversity, too. The concept of "alternative ribosomes" that differ in composition and function or translational preferences was recently reviewed by W. Gilbert in [17]. The heterogeneity of ribosomes stems from several reasons:

- male ribosomes contain one of two different isoforms of RPS4, which are encoded by homologous genes located on the sex chromosomes Y and X. These two isoforms share 93% identity, and ribosomes harboring RPS4X are in male cells about ten times as abundant as those containing RPS4Y [18, 19],
- the protein composition of ribosomes also varies depending on the type of tissue: some RPs have several tissue-specific isoforms that arise from alternative splicing of their mRNA [20],
- in addition, human genome contains more than 2000 RP pseudogenes and 16 duplicated RP genes, some of which may be translated under certain conditions [21, 22],
- some RPs aren't essential for translation of (some) mRNAs and under certain conditions may dissociate from ribosome and later assemble again (for example, ribosomal protein RACK1 or ribosomal protein L13a [17]), that are released from the large subunit upon phosphorylation induced by interferon-γ).
- and finally, many RPs are subjects to posttranslational modifications (PTMs) [23], many of them being reversible and serving for the modulation the functions of ribosomes.

Posttranslational modifications of RPs

Ribosomal proteins are subjects of a rich spectrum of posttranslational modifications, and the positions of PTMs are much less conserved compared with protein sequences.

Methylations, phosphorylations, acetylations, ubiquitinations and neddylations are the most frequent PTMs of ribosomal proteins (reviewed in [24, 25, 26, 27]).

The PTMs of ribosomal proteins can be divided into two categories: those introduced during or prior to the assembly into ribosomal precursors, and those modifying the properties of mature ribosomes. They may play both ribosomal and extraribosomal roles (i.e. affect events unrelated to translation).

PTMs introduced in the process of ribosomal subunit assembly are usually are vital for the process [28]. These PTMs also belong among the more conserved ones during evolution. The spectrum of these PTMs is dominated by methylations of arginine or lysine that serve for the modulation of protein-RNA interactions (reviewed in [29]).

So far, methylation of several RPs has been demonstrated to be important for ribosome biogenesis. For example, methylation of three arginines of mammalian RPS3 by protein arginine methyltransferase (PRMT) 1 is critical for the incorporation of RPS3 into the (mammalian) ribosome [30], and the methylation on two arginines of RPS10 by PRMT5 has similar function [31]. And finally, methylation of RPS2 by PRMT3 or perhaps the sole interaction of these two proteins is important for the assembly of the 80S ribosome [32, 33].

Modiffcations that occur post-assembly are highly dynamic and their roles are less understood. Methylation of some RPs may occur also in the assembled ribosome; the pattern of methylated ribosomal proteins fluctuates during the cell cycle [34] and differs also between IRES[3]-bound and native (free) 40S subunits [35]. Several RPs have been shown to be phosphorylated, most frequently on one or multiple serines.

Phosphorylation of the ribosomal protein S6 on five N-terminal serines was associated with preferential binding of ribosome to a group of mRNAs containing a characteristic oligopyrimidine stretch at their 5' terminus (5'TOP mRNAs, see page 23). This set of mRNAs includes messengers of all RPs, as well as of many regulatory proteins participating in the cell cycle control and translation. However, such a crucial role of RPS6 phosphorylation was not confirmed in animal models: the phenotype of mice

[3] **i**nternal **r**ibosome **e**ntry **s**ite, and alternative site for translation initiation harbored by some mRNAs

with the homozygous knockout of gene encoding the preferential RPS6 kinase, S6K1, is very mild, as well as that of mice expressing a variant of RPS6 with all 5 phosphorylable serines substituted to alanines [36]. The importance of this modification thus remains elusive.

Posttranslational modifications influencing the extraribosomal functions of RPs are often related with specific condition, such as cellular stress. For instance, DNA damage induces phosphorylation of RPS3 on threonine 42 via the Erk1/2 kinase. This modification is crucial for the translocation of RPS3 to nucleus and enhances the endonuclease activity of this protein, and thus promotes DNA repair [37]. Phosphorylation of other ribosomal proteins is even less understood.

Although ubiquitination is often a signal for proteasomal degradation of proteins, the attachment of ubiquitin to many RPs is important for the function of RPs, either free or built in ribosomes. Ubiquitination of the human RPL27a as a component of ribosome was shown to fluctuate during the cell cycle and was shown to be important for ribosomal function [38]. Ubiquitination of murine ribosomal protein S7 by Mdm2 interferes with the progress of cellular stress response: unmodified RPS7 inhibits Mdm2 and thus favors p53 accumulation and activation, and ubiquitinylation of RPS7 was shown to be important for the sustaining the p53 response and promotion of apoptosis [39]. Other RPs interacting with Mdm2 might play a similar role.

In addition, dozens of RPs were shown to be neddylated [27] (attachment of a small ubiquitin-like molecule NEDD8), and this modification is important for their stability. Neddylation of RPL11 might also be important for the mediation of ribosomal stress [40].

Extraribosomal roles of ribosomal proteins

The control of balanced production of ribosomal constituents is the most common extraribosomal function of RPs. This also includes the role of free RPs in the induction of cellular stress, which serves as a protective mechanism in case of a defect in ribosome biogenesis.

An inhibition of rDNA transcription or a block in ribosomal subunit assembly result in accumulation of unassembled ribosomal proteins in nucleolus. So far, several RPs have been shown to activate p53 by inhibiting its negative regulator, Mdm2. This response is also known as ribosomal stress, and will be discussed in more details in Section 2.4, page 27. Briefly, this response inhibits synthesis of all ribosomal components, blocks cell growth and eventually induces cell cycle arrest or leads to apoptosis.

Several other RPs have been reported to respond to different types of cellular stress, such as viral infection or DNA damage, including RPS3, RPL10, RPL13a or recently identified transient component of ribosome, RACK1 (**r**eceptor of **a**ctivated **C** **k**inase) (reviewed in [6]). However, their function in these processes is not fully understood yet. It can't be excluded that similar extraribosomal roles might be played by other ribosomal proteins, too.

Diverse phenotypes of distinct RP gene knockouts led to a hypothesis that some RPs play specific roles in organ development and differentiation of some cell types. However, no such tissue-specific role has been so far confirmed for human RPs. All cases of an RP gene mutation as a sole genetic defect are associated with a rare inherited syndrome, Diamond-Blackfan anemia. Although mutations in some of these RPs are more frequently associated with distinct somatic anomalies, the effect is likely due to their distinct potential to induce the p53 response. A similar manifestation is seen in 5q- myelodysplastic syndrome (heterozygous deletion of a region of 5th chromosome, which contains also the *RPS14* gene). And finally, although one RPS4X alelle is lost in Turner syndrome, the phenotype results from loss of heterozygosity of hundreds of other genes.

2.2 Ribosome biogenesis

The assembly of ribosomal subunits depends on coordinated synthesis, processing and packing of ribosomal RNAs with ribosomal proteins. In contrast to prokaryotic ribosomes that can self-assemble from its components, biogenesis of eukaryotic ribosomes requires hundreds of non-ribosomal proteins and tens of small nucleolar (sno)RNAs (in human [41, 42, 43] and in yeast [15]).

Eukaryotic ribosome biogenesis begins with the transcription of rDNA genes and the assembly of nascent pre-rRNAs with ribosomal and non-ribosomal proteins and snoRNAs into a large nucleoprotein particle (Fig. 2.2). In this large precursor particle, various posttranscriptional modifications of pre-rRNAs and also first pre-rRNA cleavage steps occur.

Subsequently, the particle divides into the precursor of the small (pre-40S) and large (pre-60S) subunit. At the late maturation stages are the subunit precursors exported to cytoplasm, where they form mature subunits (40S and 60S), which then join to form complete 80S ribosome capable of translation (reviewed in [15]). The following sections describe this complex process in more details.

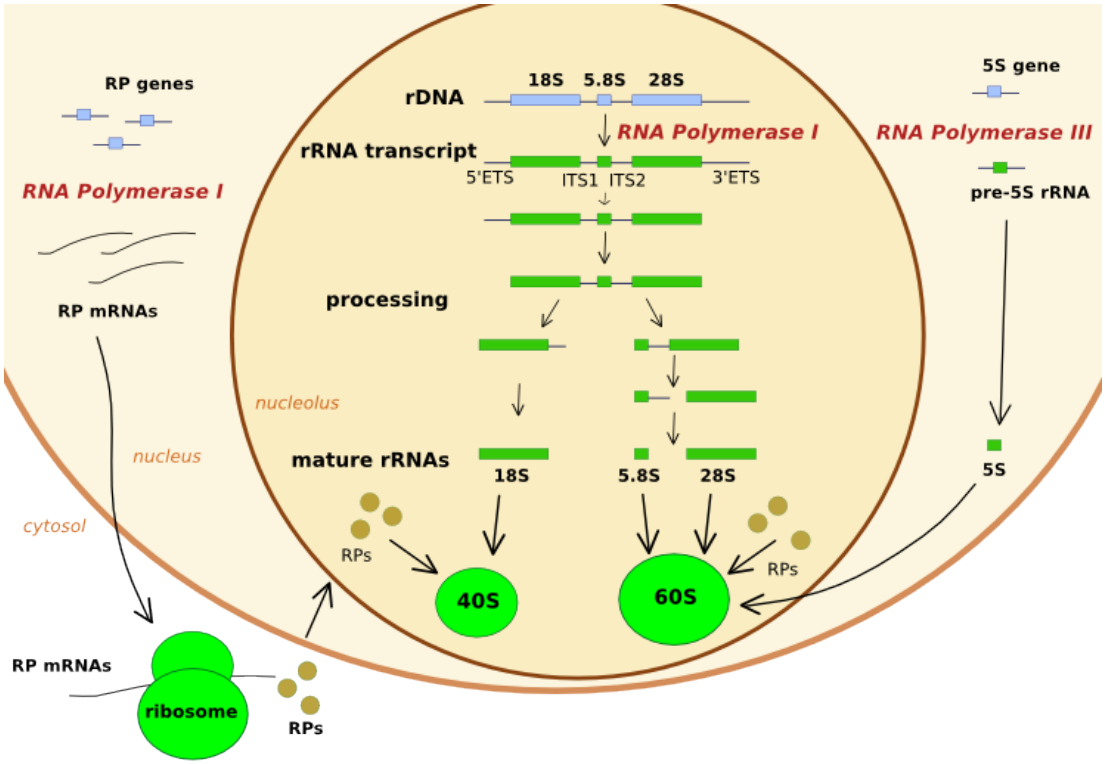

Figure 2.2: Ribosome biogenesis.
The primary rRNA transcripts associate with RPs and other proteins and small nucleolar RNAs into a 90S precursor. Inside this particle, pre-rRNAs are cleaved and undergo modifications of some residues. The 90S particle then splits into the precursors of the small and large subunit that then maturate separately. Each of these steps occurs in distinct nucleolar compartment and finally, the subunits are exported into cytosol.
Altered from `http://www.edk.fr`.

2.2.1 rDNA genes, transcription and initial processing steps

Eukaryotic rRNA genes (rDNAs) are arranged in multiple tandem clusters, arrayed in head-to-tail fashion. The small subunit 18S rRNA gene is localized upstream of genes encoding the large subunit rRNAs and is transcribed prior to them [25].

Three of the four eukaryotic rRNAs (18S, 5.8S, and 28S rRNAs) originate from the 35S–45S polycistronic pre-RNA that is transcribed by RNA polymerase I (RNA Pol-I) from the rDNA repeat, while the smallest 5S rRNA is transcribed separately by RNA Pol-III [44]. This organization of rDNA genes and the basic scheme of transcription and processing of rRNAs is conserved among all three kingdoms.

Because each copy of the rDNA gene is occupied by a large number of RNA polymerases, rDNA transcription gives rise to characteristic structures that are visible under elecron microscope and are known as *Christmas tree* structures (Fig. 2.3).

Figure 2.3: Christmas trees.
Left: electron micrograph of active rDNA genes (`http://www.nicerweb.com`). The similarity between structures formed by rDNA transcription and christmas spruce trees was first pointed out by Miller [45].

Transcription of rDNA, processing of pre-rRNAs and the predominant part of ribosomal subunit assembly and maturation take place in *nucleoli*, distinct regions inside nucleus that form around actively transcribed rDNA genes. Nucleoli aren't physically separated from the rest of the nucleus, nor are they supported by an inner scaffold, but because they contain an exceptional concentration of macromolecules, their density is

about two times higher than the density of the surrounding nucleoplasm [46]. They are thus easily observable even in the phase-contrast microscope as dark specks[4] in the nucleus. The inner structure of nucleoli is highly organized and compartmentalized with respect to both genome (the above mentioned organization of rDNA) and proteome (association of distinct sets of proteins with rDNA, rRNA or distinct or preribosomal intermediates) [49] and distinct steps of ribosome biogenesis take place in separate nucleolar compartments [50].

Simultaneously with the transcription, the primary 35–45S polycistronic pre-rRNA assembles with some of the large subunit RPs and most of the small subunit RPs, nucleolar proteins and snoRNAs to give rise to the early 90S ribonucleoprotein particle. The ribonucleoprotein complex containing the pre-5S rRNA, ribosomal proteins and several non-ribosomal components [51] joins the large ribosomal subunit precursor later (the exact point has not been resolved yet).

2.2.2 Maturation of ribosomal subunit precursors

The maturation of pre-rRNAs within the 90S particle proceeds with the introduction of posttranscriptional modifications (the predominant ones are 2'-O ribose methylations and pseudouridylations) that facilitate the proper rRNA folding and improve their stability and function. The transcript further undergoes series of endo- and exonucleolytic cleavages that remove the internal transcribed spacers and release the mature rRNAs. The order in which these events take place is not fixed and the pre-rRNA processing seem to follow several alternative pathways, giving rise to a broad spectrum of intermediates.

The resulting large ribonucleoprotein particles containing components of both ribosomal subunit (late 90S pre-ribosomal particles) are then divided into the precursors of the small and large ribosomal subunit (pre-40S and pre-60S particles), the former contains the precursor of 18S rRNA, while the latter contains common precursor of

[4] These specks were first described in 19th century by Rudolph Wagner who gave it a lyrical name *Keimfleck* (meaning *germ spot*, or *patch*) and *macula germinativa* in germinal vesicles. Now they are known under the term "nucleolus", which means *small nucleus*, or *nucleus of the nucleus* (reviewed in [47, 48]).

5.8S and 28S rRNAs, and is joined by the ribonucleoprotein particle harboring the smallest 5S pre-RNA. Both pre-ribosomal subunits are from this point processed independently first in the nucleus, then they translocate to the nucleoplasm, and finally to the cytoplasm.

As mentioned before, most RPs of the small subunit are incorporated in the 90S particle, and they are therefore present in the early pre-40 precursor. The remaining RPs of the small subunit join the pre-40S particle soon afterwards [43]. Majority of the small subunit RPs were shown to be required for the generation of mature 18S RNA (both in yeast [52] and in human cells [53]), and the timing of the assembly into the pre-ribosomal particle reflects their function in the rRNA processing. In contrast to the maturation of the large subunit precursor, only a small number of non-ribosomal proteins are found in the pre-40S particle, suggesting that most of the remodeling during the small subunit maturation is carried out by RPs themselves. Teminal maturation of the 40S particles occur in the cytoplasm.

Maturation of the 60S subunit is even more complicated: not because this subunit is larger, but mainly because (i) the maturation of 5.8S and 28S may follow at least two alternative splicing pathways, and (ii) a ribonucleoprotein complex with 5S rRNA seems to be incorporated to multiple possible intermediates [51].

The roles of large subunit RPs and the order in which they assemble into the pre-60S intermediates is still not fully understood, but only a fraction of the large subunit proteins is involved in the rRNA processing in eukaryotes. In human, numerous snoRNAs and at least 150 nonribosomal proteins (non-RPs) are involved in the process [41], and the number of participating factors is probably even much higher. The snoRNAs catalyze the posttranscriptional modifications of rRNA precursors and the majority of the non-RPs function as helicases, GTPases, AAA-ATPases and chaperones, and they assist in remodeling steps. Maturation of the large subunit is completed in nucleoplasm before its export to cytosol.

After their export to cytoplasm, mature small and large subunits join to form 80S ribosome capable of translation. This event depends on the methylation of a small subunit component, protein RPS2, by a dedicated protein arginine methyltransferase 3 (PRMT3) [28, 33, 32]. Further post-assembly changes in the structure and properties of

ribosomes (introduction of PTM, dissociation or re-association of several non-essential ribosomal proteins) were already mentioned in previous sections.

It should be noted that mutations affecting any component vital for ribosome biogenesis and function (RP or any of these vital extraribosomal components) manifest in model organisms with distinct defects. In human, defects in ribosome biogenesis are associated with rare inborn syndromes, which will be reviewed further in Section 2.4.3. To understand the consequences of ribosomal defects, it is necessary to mention first the principles of regulation of ribosome biogenesis, as well as the cellular response on the disruption of this process. These topics will be briefly reviewed in the next two sections.

2.3 Regulation of ribosome biogenesis

Ribosome biogenesis is one of the most energy consuming processes in the cell and presents the major limit to the cell growth, differentiation and proliferation. Therefore, it needs to be tightly coupled to the energy status and the availability of nutrients on one hand, and to the growth stimuli on the other hand. To prevent accumulation of some components over others, the process also demands accurate synchronization of production of all ribosomal components. The set of genes involved in ribosome biogenesis (sometimes referred to as the Ribi (*ri*bosome *bi*ogenesis) regulon) represents the largest set of coordinately expressed genes in eukaryotes [54].

The two major signaling pathways regulating cell growth and proliferation are the mTOR and the MAPK/Erk signaling [55, 56, 57]. Both these pathways are stimulated by various growth-promoting stimuli: the mTOR pathway plays a central role in the regulation of anabolic processes of the cell in response to various extra- and intracellular growth-promoting stimuli, MAPK/Erk signaling controls expression of genes involved in cell cycle progression and mitosis in response to growth factors (Fig. 2.4, page 22).

The downstream targets of mTOR and Erk1/2 cooperate to enhance the transcription of rRNA and RP genes via the regulation of availability/binding preferences of specific transcription factors, and via the regulation of activity of dedicated RNA polymerases. The mTOR pathway also enhances the translation of RP mRNAs by modulating both the initiation and elongation step. Because of their complexity, these pathways will be introduced separately and a focus will be given to the regulation of rRNA and RP synthesis.

2.3.1 The mTOR pathway

A serine/threonine kinase mTOR (**m**ammalian **t**arget of **r**apamycin[5]) regulates a wide range of cellular processes in response to various extra- and intracellular stimuli [59, 55]. These diverse functions of mTOR are accomplished by two distinct multiprotein

[5] An inhibitor of mTOR, rapamycin, is a macrolide lactone antibiotic first isolated in mid-1970s from soil bacterium Streptomyces hygroscopicus from Easter Island (locally known as Rapa Nui) [58].

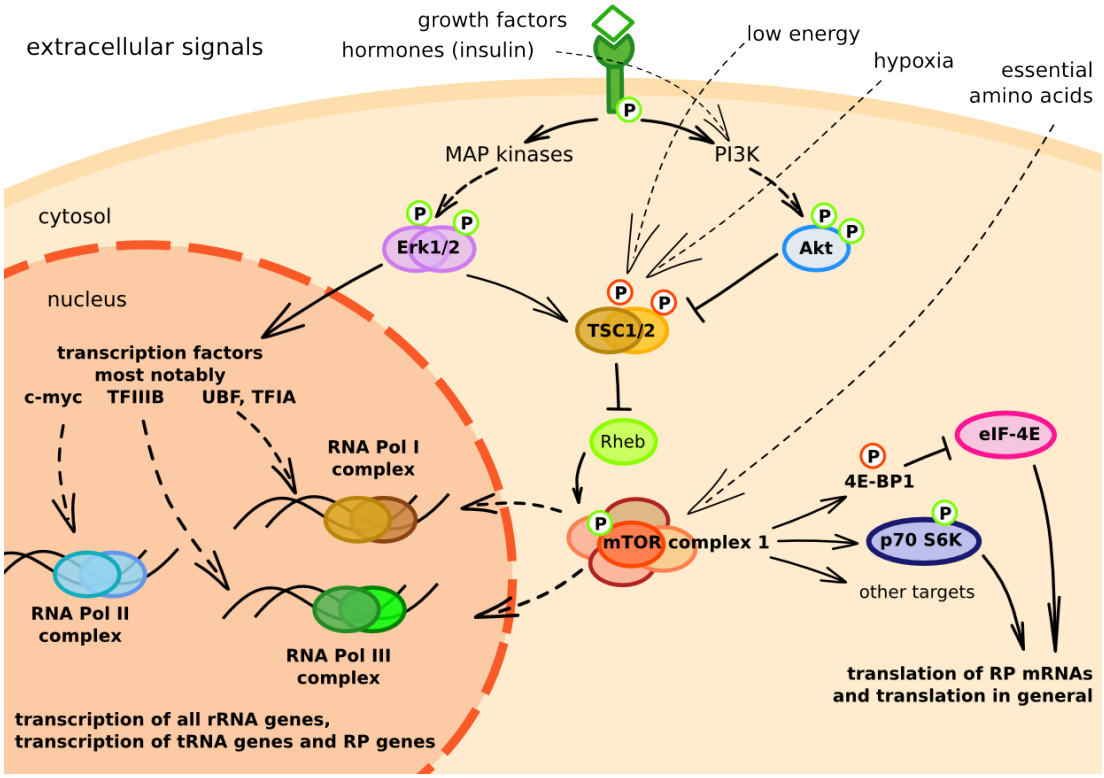

Figure 2.4: mTOR and MAPK/Erk pathways and control of ribosome biogenesis. Due to the complexity of the regulation, only the most important mechanisms are illustrated. Full arrow denotes direct connection (usually kinase and its substrate), dashed arrows indirect connection. Illustration is based on several schemes at `http://www.cellsignal.com`, visited 14.4.2011.

complexes, mTOR complex 1 and 2, only the first being involved in ribosome biogenesis control.

mTOR complex 1 (mTORC1) is composed of mTOR kinase and four associated proteins with regulatory and/or substrate determining roles[6]. The most important positive regulators of mTORC1 are insulin and growth factors that act via the canonical insulin and Ras/Raf/MAPK/Erk pathway; other positive signals are integrated from intracellular sensors of essential amino acids, and high ATP/AMP levels. Hypoxia, inflammatory or genotoxic stress pathways, as well as growth factor deprivation or low levels of nutrients negatively regulate this complex.

[6] Namely regulatory-associated protein of mTOR (Raptor), proline-rich AKT substrate 40 kDa (PRAS40), and DEP-domain-containing mTOR-interacting protein (Deptor), and mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL).

The main integrator of these signals is tuberous sclerosis complex protein TSC2. Under normal conditions, TSC2 is in its nonphosphorylated form and heterodimerizes with TSC1, and this complex (TSC1/2) inhibits an mTOR-specific kinase Rheb. Upon growth-promoting conditions or surface receptor activation (see above), TSC2 is phosphorylated by Akt or MAP kinase Erk, and looses its affinity to TSC1. The heterodimer thus disassembles, leading to Rheb activation, phosphorylation of mTOR and its activation. On the other hand, most of the signals that negatively regulate mTORC1 stabilize the TSC1/2 heterodimer. Other means of the regulation of mTORC1 include phosphorylation of other mTORC1 components, or upregulation of the expression of the mTORC1 proteins.

Activated mTORC1 phosphorylates various regulatory proteins, kinases or transcription factors, which in turn activate or inhibit various aspects of cell physiology. The best understood substrates of mTORC1 are ribosomal protein S6 kinase 1 (p70 S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1), other mTORC1 substrates include a component of mTORC1 protein PRAS40, transcription factors Stat3 and Hif-1, and repressor of RNA Pol-III Maf1. As a part of this complex, mTOR also phosphorylates itself.

Control of RP mRNAs translation

All RP mRNAs contain a 5'end sequence called 5'TOP (for 5'-terminal tract of oligopyrimidine), which allows a coordinated regulation of their translation. This 5' end motif is also shared by mRNA encoding a transcriptional factor c-myc, several mRNAs encoding other components of translational machinery, and also by mRNAs encoding other growth– and cell cycle–related proteins.

mTOR has long been established as a positive regulator of preferential translation of 5'TOP mRNAs, however, the mechanism is not as straightforward as was originally expected. mTORC1 associates with the 5'TOP end of mRNAs and with the associated proteins that are involved in translation initiation control. In the next step, mTORC1 phosphorylates two of those proteins, namely S6K1 and 4E-BP1.

S6K1 is a 70 kDa serine/threonine kinase that is activated upon phosphorylation by mTORC1. The first known cellular target of S6K1 was a protein of small ribosomal

subunit, RPS6, and the original hypothesis assumed that this modification was vital for preferential translation of mRNAs with the 5'TOP motif. This hypothesis provided an elegant explanation for the observed upregulation of translation of these mRNAs upon mTOR stimulation, however, it was in contradiction with the experimental evidence (reviewed in [60]). Nevertheless, S6K1 most likely stimulates translation via other proteins that control translation initiation and elongation (see [61] for more details), since RPS6 is not the sole substrate of this kinase.

The upregulation of 5'TOP mRNAs is now explained via a mechanism involving another mTORC1 substrate, 4E-BP1, and the initiation factor eIF4E. 4E-BP1 (for eIF4E-binding protein 1) is a negative regulator of eukaryotic initiation factor 4E (eIF4E), one of the components of translation initiation factor 4F (eIF4F)[7].

eIF4F binds to the 5' methylguanosine cap of mRNAs and is responsible for their recruitment to ribosomes, and this step is a rate-limiting for translation of most mRNAs. Under normal conditions, 4E-BP1 associates with eIF-4E and thus prevents eIF4E from forming the translation initiation complex. Phosphorylation of 4E-BP1 by mTORC1 weakens the affinity of this negative regulator towards eIF4E, and as a result, the pool of available eIF-4E increases, and the formation of translation initiation complexes and protein synthesis are promoted.

Although this mechanism was previously thought to be universal, recent findings suggest that the most marked increase in translation is observed for mRNAs encoding ribosomal proteins and other 5'TOP mRNAs, including c-myc.

Regulation of rDNA transcription

mTORC1 also interacts with a transcription initiation complex formed at the RNA Pol-I promoter and was reported to promote phosphorylation of several RNA Pol-I-dependent transcription factors. These direct or indirect targets of mTORC1 include

[7] Within this initiation factor complex, eIF4E a scaffold protein that is responsible for the recognition of the mRNA 5'-cap structure and supports the interaction of other eIF4F subunits. Namely, an RNA helicase eIF4A (that catalyzes unwinding the secondary structures of mRNA and therefore facilitate translation), and proteins eIF4B (responsible for the physical bridging of the mRNA with the ribosome) and eIF4G (required for circularization of the mRNA via interaction with poly(A)-binding protein).

TIF-IA and TIF-IB, upstream binding factor (UBF) and accessory protein SL1 (reviewed in [62, 63]). mTORC1 also phosphorylates and thus inactivates the protein phosphatase 2A (PP2A) that negatively regulates TIF-IA. This mechanism allows a several fold increase in rRNA synthesis without the detectable change in the number of active genes [64].

mTORC1 regulates also RNA polymerase III-dependent transcription via direct phosphorylation of the RNA Pol-III-specific repressor protein Maf1. Phosphorylation of Maf1 leads to its dissociation from the promotors of tRNA and 5S rRNA genes, and their transcription is thus promoted.

2.3.2 The MAPK/Erk pathway

The Ras/Raf/MAPK/Erk signaling cascade couples signals from cell surface receptors to transcription factors, which are involved in the regulation of cell cycle progression, apoptosis or differentiation (reviewed in [65, 66]). The pathway is activated by a wide variety of receptors for cytokines and factors regulating cell growth and differentiation, receptors for integrins and ion channels, and although the specific proteins involved vary depending on distinct stimuli, the architecture of the pathway is well conserved.

A signal from activated membrane receptor is transduced via a cascade of activating phosphorylations to the mitogen activated protein kinases (MAPK) and to kinase Erk, which dimerizes and becomes active. Erk1/2 dimer can enter nucleus and phosphorylate a variety of transcription factors, including c-myc, which in turn trigger the expression of target genes.

In addition, activated Erk1/2 phosphorylates a number of its cytosolic targets, including several components of other signaling pathways including an important negative regulator of the mTOR pathway, protein TSC2 (for more details, see the next section). Phosphorylation of TSC2 leads to dissociation of the TSC1/2 complex, and opens up the way to phosphorylation and activation of the mTOR pathway. This mechanism links together the regulation of metabolic pathways and translation by mTOR and the regulation of transcription by Erk1/2 targets.

Control of rRNA and RP gene transcription: c-myc

The protein c-myc is an essential transcription factor that is estimated to regulate the expression of up to 15% of all human genes, many of them being involved in ribosomal biogenesis (reviewed in [67]). c-myc acts in cooperation with other transcription factors, DNA modifying enzymes and chromating remodeling enzymes.

Transcription of rDNA genes is the limiting step in ribosome biogenesis. By regulating the transcription catalyzed by all three RNA polymerases, c-myc controls the expression of all components of ribosome:

- Promotes Pol-I–dependent transcription of 3 of 4 rRNAs via recruitment of general transcription factors (TBP and TBP-associated factors) and RNA polymerase I to the rDNA promoter.
- Enhances transcription of 5S rRNA and tRNAs by RNA Pol-III by binding to TFIIIB and promoting the assembly of Pol-III-dependent initiation complex.
- Stimulates Pol-II-catalyzed transcription of a many genes that encode proteins involved in ribosomal biogenesis and/or translation.

Interestingly, in this last case, transcription is regulated at the *elongation* and not *initiation* step [68]. Besides, the RNA Pol-I– and Pol-III–dependent[8] transcription is also enhanced via the mTOR pathway, as described before.

[8] It should be noted that in mammals, mTOR does not control the transcription of RP genes, in contrast to the role of its homolog in budding yeast, TOR.

2.4 Defects in ribosome biogenesis

Requirement of the production of all ribosomal components in stoichiometric amounts logically led to the development of mechanisms that ensure the coordinate production of both rRNAs and RPs, as well as mechanisms that monitor the progression of ribosome assembly.

Perturbations of virtually any step of ribosome biogenesis (insufficiency of any ribosomal component, defects in any processing or assembly step, or alteration of the inner compartmentalization of nucleoli) lead to the production of incorrectly assembled ribonucleoprotein particles and accumulation of free RPs [69, 70]. Ribonucleoprotein particles incapable of further maturation are recognized by specific mechanisms and subsequently degraded; first, proteins associated with aberrant rRNAs are targeted to proteasomal degradation, and next, rRNAs are digested by RNAses [71].

Ribosomal proteins that fail to assemble correctly into the pre-ribosomal particles accumulate in the nucleolus and some of them are responsible for the activation of an event called the *nucleolar* or *ribosomal stress response* [72, 73]. Although this response evolved as a protective mechanism, these stress-related events might have undesirable effects, too. Ribosomal stress response is very important for understanding the pathogenesis of diseases caused by ribosomal defects and therefore, it will be now described in more detail.

2.4.1 Ribosomal stress response

Distinct RPs (in addition to their canonical roles) act as guardians of different steps of ribosome biogenesis [72]. The mechanism how individual RPs trigger p53 stabilization and activation differs, some RPs are more potent than the others, but they act in a synergical manner.

The most common mechanism of p53 activation includes a specific binding of RPs to the ubiquitin ligase Mdm2 and thus blocking its enzymatic activity [74, 72]. Because Mdm2 is the major negative regulator of p53, inhibition of this negative feedback opens up the way to p53 accumulation and activation. So far, this extraribosomal function

was confirmed for large ribosomal subunit components RPL11, RPL23, RPL5, RPL26 [72]; and small ribosomal subunit proteins RPS7 [75] and RPS3 [76], but the list of RPs is probably not complete yet.

The efficiency of individual RPs in activating the p53 response varies since all these RPs recognize slightly different motifs in Mdm2 (although all in the central acidic domain of MDM2), and thus probably utilize *similar, but not identical* mechanism for the regulation of the E3 ubiquitin ligase activity of MDM2 [77, 78]. In addition to this scenario, some of these RPs employ also other mechanisms to activate the p53 response; refer to [75, 79, 80, 76, 81] for more information.

2.4.2 Physiological importance of ribosomal stress response

The outcomes of p53 pathway are diverse, including downregulation of anabolic pathways including rRNA and RP synthesis, delay or block in cell cycle progression, and/or increased susceptibility to apoptosis (Fig. 2.5).

Activated p53 not only dramatically affects the spectrum of expressed genes but also negatively regulates the mTOR complex 1, and therefore slow down many anabolic processes (translation, synthesis of lipids, biogenesis of organelles[9]) [82]. As a consequence, p53 slows cell growth by switching to the energy and resources-saving mode and activating mechanisms to overcome (repair or compensate) the original defect.

Other outcomes of p53 activation are cell- or tissue-specific: while some respond with transient or permanent cell cycle arrest, others are extremely prone to apoptosis almost regardless of the severity of the defect. The decidion depends on the expression of p53 and other proteins modulating its function in the respective cell type.

The apoptotic response is typical for cells capable of rapid clonal expansion, that can be easily replaced by proliferation of adult stem cells, and that are not essential for tissue structure or integrity [83], such as blood cells. This mechanism is to prevent

[9] Details: p53 activates the transcription of genes encoding proteins sestrin1 and sestrin2, both of which can bind to AMPK (AMP-activated kinase, sensor of intracellular ATP levels), promote its phosphorylation and activation. Since AMPK is a positive regulator of TSC2, therefore, a negative regulator of mTORC1, p53 activation leads to the inhibition of this central regulator of cellular metabolism.

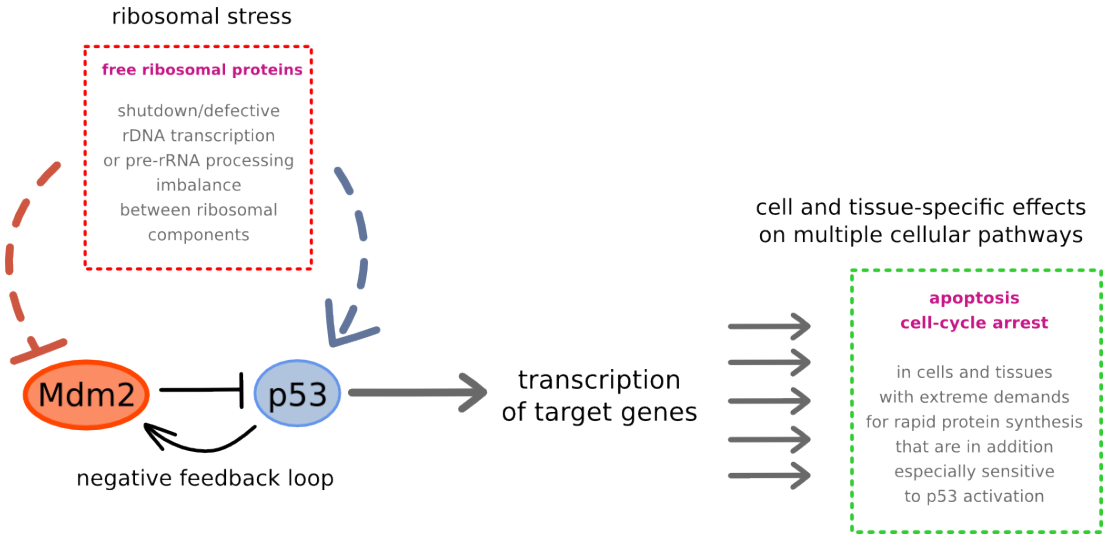

Figure 2.5: Ribosomal stress response.
Defects at any stage of ribosome biogenesis lead to activation of p53, which in turn triggers various stress responses. The outcome is tissue-specific and the most important responses are induction of cell cycle arrest and/or apoptosis. p53 also negatively regulates biogenesis of all ribosomal components and slows down anabolic pathways. Author's own illustration.

damaged cells (or in this context, cells incapable of meeting physiological standards for protein synthesis) from future divisions and from potentially "dangerous" spreading of the mutation(s) further.

On the contrary, cell cycle arrest is typical for cells of epithelia and connective tissue, as well as for pluripotent self-renewing hematopoietic progenitors. The transient entrance into the cell cycle arrest provide the cell with time for a *self-correction* of the imbalance between the balance of RPs, rRNAs and processing factors. Once the cell synthetizes a sufficient pool of functional ribosomes, it continues with cell growth and eventually proceeds to mitosis [71].

The link between free RPs and Mdm2 inhibition probably originally evolved as a control mechanism to prevent pointless production of some ribosomal components over others. However, the consequences of ribosomal stress (slow proliferation, apoptosis) are also very important for the phenotype associated with defect in ribosome biogenesis.

In higher eukaryotes, homozygous mutations affecting an essential RP genes or rDNA are early embryonic lethal (once the pool of maternal ribosomes is exhausted);

heterozygous mutations of *RP* genes manifest with distinct phenotype referred to as *Minute*[10].

The most apparent phenotype characteristics of *Minute* mutations common for all higher eukaryotes are smaller body size and developmental anomalies. Postnatally, these mutations result in distinct defects of rapidly growing tissues, including epithelia or bone marrow. And finally, mutations in ribosomal-related genes are also associated with an increased likelihood of cancer. Since RP gene mutations are associated with nucleolar stress and p53 activation, there is expected to be a strong selective advantage for those cells that override the p53-induced cell cycle arrest or apoptosis. Although it is not the sole factor, loss of control over p53 significantly contributes to tumorigenesis.

2.4.3 Ribosomes in human disease

In human, mutations in RP genes and in other components of ribosome biogenesis pathway are causal for a distinct group of syndromes termed *ribosomopathies*. Ribosomopathies [86] are a very heterogenous group of syndromes that include Diamond-Blackfan anemia, 5q- syndrome, Schwachman-Diamond syndrome, cartillage-hair hypoplasia, dyskeratosis congenita, and Treacher-Collins syndrome. Ribosomopathies are linked to mutations in genes encoding RPs, RNAs involved in posttranscriptional modifications of rRNAs, or non-RPs participating in ribosome assembly. In most cases, the mutations disrupt also non-ribosomal functions of the affected RNAs or proteins. Therefore, the underlying mechanisms of pathogenesis of ribosomopathies is thus often very complex.

All ribosomopathies affect mainly tissues extremely sensitive to the ribosomal stress, i.e, rapidly growing and proliferating cells. The most profound defects are seen in hematopoietic tissue, in epithelia and connective tissues, or tissues in developing embryo, though the severity of physical anomalies and growth retardation vary. The

[10] The term was first introduced in *Drosophila* but paralells of the phenotype can be found in mammals, too. Flies haploinsufficient for an RP gene displayed delay in larval development, short thin bristles and recessive lethality; other variable features include small body size, female sterility, large or rough eyes, and malformed wings. The only known non-RP gene deletion associated with *Minute* phenotype in *Drosophila* affected a locus harboring a gene encoding one subunit of translation initiation factor eIF-2α (reviewed in [84, 85])

severity of symptomes markedly differs even among individuals carrying a mutation in the same gene, likely depending on the genetic background and/or environmental factors.

Syndromes caused by defective ribosome biogenesis

Mutations in genes encoding protein components of cytosolic ribosomes are associated with **Diamond-Blackfan anemia (DBA)**. This syndrome manifests with a pure red cell aplasia of variable degree of severity, other cell lines are usually unaffected. Almost half of the patients also have somatic anomalies and show growth retardation.

In the begining of this work, only two DBA causal genes were known (namely *RPS19* and *RPS24*), having been mutated in approximately a quarter of DBA patients. To date, nine distinct RP genes have been confirmed to be causal in over a half of DBA cases (see section 2.6); in others, the causal gene(s) are still unknown.

The known DBA causal genes encode proteins of both ribosomal subunits, all of them participate in pre-rRNA maturation and are thus essential for ribosome biogenesis. The syndrome is explained as a defect in ribosome maturation, leading to the p53 activation and decreased translational capacity. More details regardings the molecular pathogenesis of DBA will be given in section 2.6.

5q- syndrome syndrome is also associated with an RP gene haploinsufficiency. 5q- is a myelodysplastic syndrome[11] with a deletion of a 1.5-megabase region on the long arm of the chromosome 5 (common deleted region (CDR)), del (5q), as the sole karyotypic abnormality. The main symptoms are refractory anemia (unresponsive to treatment), atypical megakaryocytes and a also slightly increased risk of transformation to acute myeloid leukemia. The CDR contains 40 genes, including the ribosomal protein *RPS14* gene.

The mechanism of 5q- pathogenesis is thought to be similar to DBA [2], which is also supported by a recent identification of *RPS14* mutation in one DBA patient [87].

[11] Myelodysplastic syndromes (MDS) are bone marrow disorders resulting in an inadequate production of one or more blood cell lineages. The blood cell progenitors show both impaired differentiation and also excessive apoptosis. Specific defects and mechanisms of pathogenesis of this heterogenous group of syndromes are poorly understood.

However, 5q- is an *acquired* condition while DBA is an *inborn* syndrome, and the loss of other genes or regulatory DNA might be important for the manifestation of 5q-, too.

Shwachman-Diamond syndrome (SDS) [1] is a rare congenital disorder characterized by pancreatic insufficiency, neutropenia and mild anemia, skeletal abnormalities and short stature. The *SBDS* gene which is causal for SDS encodes for a nucleolus-associated protein that has close homologs in both eukaryotes and prokaryotes, and several lines of evidence indicate that the SBDS protein is involved cellular RNA metabolism and/or ribosome assembly. However, the pathogenesis is not fully explained yet.

Cartilage-hair hypoplasia (CHH) is an unusual case of dwarfism accompanied with underdeveloped cartilage tissue, fine and scarce hair, paucity of lymphocytes, neutrophiles and erythrocytes, and increased risk of malignancies. CHH is caused by mutations in the RMRP (*RNA component of mitochondrial RNA processing endoribonuclease*) gene, which encodes a ribozyme with two distinct roles.

In mitochondria, RMRP participates in (i) priming mitochondrial DNA replication and (ii) in the function of telomerase reverse transcriptase domain [88]. In nucleus, it cleaves the rRNA precursors and is thus vital for the biogenesis of (cytosolic) ribosomes. The consequences of mutations in *RMRP* are thus very complex; the affected cells display ribosome biogenesis defects, premature senescence, and signs of aberrant mitochondrial DNA replication.

Dyskeratosis congenita (DC) is characterised by the triad of abnormal skin pigmentation, nail dystrophy and mucosal leucoplakia (sores or lesions that develop inside mouth); many patients also develop bone marrow failure/immunodefficiency and additional somatic anomalies may also be present. There are multiple causal genes that are directly or indirectly linked to the function of telomerase RNA component (TERC). As a result, affected cells suffer with telomere maintenance defects, namely with chromosomal instability, limited viability and slower proliferation [3].

The syndrome can be further classified into three subtypes, each being associated with a distinct defect in the telomerase function[12]. Dyskerin, whose gene is mutated in a fraction of DC patients with the X-linked form, catalyzes the pseudouridylation of specific motifs in RNA component of telomerase, but also in other non-coding RNAs, most notably also in ribosomal RNAs. Individuals carrying a mutation in dyskerin therefore display in addition to the telomerase insufficiency also defects in ribosome biogenesis, and the prognosis in this form of DC is usually worse than in the autosomal form.

Though not a bone marrow failure syndrome, **Treacher-Collins syndrome (TCS)** is also linked to defects in rRNA synthesis. *Nop56p*, the causal gene in TCS, encodes the protein treacle, a component of small ribonucleoprotein complexes that direct 2'-O-methylation of the precursor of 18S rRNA [89]. TCS manifests with craniofacial malformations and the mechanism of pathogenesis involves ribosomal stress and resulting p53-dependent apoptosis of developing neural crest (reviewed in [90]. Interestingly, the application of p53 inhibitors were shown to prevent the defect [91] in mouse model of TCS.

A point mutation of a gene encoding Nep1, a methyltransferase necessary for the processing of the 18S-rRNA precursor is associated with **Bowen-Conradi syndrome (BCS)** [92]. The mutation leads to D86G substitution in Nep1 and causes partial loss of function, which manifests with abberant methylation of pre-18S rRNA in the affected cells. However, since the later pre-rRNA modifications are dependend on the earlier ones, improper rRNA methylation causes other pre-18S rRNA processing defect and thus a production of aberrant ribonucleoprotein particles.

[12] Vital component of TERC is the mammalian H/ACA ribonucleoprotein complex, that consists of four protein subunits: dyskerin, Gar1, Nop10, and Nhp2, and mutations in any of them lead to the development of the DC-like phenotype. Autosomal dominant form of DC has been associated with mutations in TERC proteins Nop10 and Nhp2, autosomal recessive form is due to a defect in the H/ACA domain of RNA component of telomerase, and X-linked form is caused by mutations in dyskerin.

Syndromes associated with inefficient translation

Decreased translational rate of (all or some) mRNAs can be also a result of mutations affecting one of the following groups of proteins or RNAs:

- non-ribosomal components involved in the translation process
    1. translation initiation/elongation/termination factors
    2. tRNAs or amino acyl-tRNA transferases
- proteins involved in the regulation of translation
    1. members of mTOR pathway
    2. members of the MAPK/Erk pathway

Genes encoding these proteins are causal for some rare congenital syndromes but unlike ribosomopathies, these diseases are associated only with inefficient or limited translation, but not with ribosomal stress response. Therefore, the comparison of these two groups of syndromes might be useful for the interpretation of the molecular mechanism of their pathogenesis.

Defective function of several essential translation initiation factors and enzymes modifying their function have been reported in various neurological disorders; for review, see [93] and references within. For example, mutations in in genes encoding subunits of eIF2α, of eIF2B complex are associated with **leukoencephalopathy with vanishing white matter** and **childhood ataxia with central hypomyelination**. The molecular basis of these syndromes is not fully understood. The probable mechanism include hypersensitivity of unfolded protein response and altered expression of stress-activated translation factors, while the translational rates ale almost normal. Genes encoding elongation factors has not been associated yet with human disease, but mouse models develop similar, yet more serious phenotype.

Mutations in genes encoding aminoacyl-tRNA synthetases (Gly-tRNA and Tyr-tRNA synthetases) have been associated with various neuropathies known as **Charcot-Marie-Tooth syndromes**. The clinical manifestation includes spinal muscular dystrophy, signs of vanishing white matter, hypo- or demyelination, childhood ataxia and other defects.

Most reported cases of sequence variants in proteins regulating ribosome biogenesis and cell growth (kinases in the mTOR or MAPK/Erk pathways) are associated with deregulation of these processes, leading to uncontrolled cell growth and eventually, to cancer. However, there are also a few examples leading to reduced synthesis of ribosomal components, slower growth, and a *Minute*-like phenotype. However, since the mTOR and MAPK signaling pathways play a central role in cellular metabolism, mutations affecting the members of this pathway are usually also associatated with complex metabolic disorders (including cardiovascular diseases, obesity and type 2 diabetes [94, 95]), or with developmental syndromes (manifesting with anomalies affecting heart, bone and skin development [96].

Ribosomal stress versus limited translation: summary

Disorders affecting the mRNA translation might be divided into two categories: defects of ribosome assembly, and defects of translation itself. Disorders caused by defects in ribosome biogenesis (ribosomopathies) manifest with bone marrow aplasias of variable severity, skin defects and developmental anomalies. On the contrary, syndromes caused by defects in translation present with dissimilar features, such as neurological and metabolic disorders.

In general, defects in ribosome biogenesis have two major consequences: (i) decreased translational capacity due to lower pool of mature ribosomes, and (ii) cellular stress due to activation of p53. The dissimilarity between ribosomopathies and translational syndromes might indicate that the second outcome of ribosome maturation defect, namely the induction of cellular stress, might have a dominant role in the pathogenesis of ribosomopathies. In most cases, the proteins or RNAs that are causal for ribosomopathies play other roles in addition to participation in ribosome biogenesis. However, Diamond-Blackfan anemia seems to be a purely ribosomal defect since it is associated with mutations in ribosomal protein genes as the sole causal genes.

In order to understand the possible link between a ribosomal protein haploinsufficiency, cellular stress and anemia, it is necessary to briefly recapitulate the process of normal erythropoiesis.

2.5 Erythropoiesis and its defects

An adult human body contain approximately 20–25 trillion erythrocytes in circulation, and the number is slightly higher in males. This is not only due to the larger average volume of blood in circulation, but also due to the stimulatory effect of androgens (male sex hormones) on erythropoiesis, in contrast to the female sex hormones. Androgens were even tested as therapeutical modality in some types of anemia, such as Fanconi anemia. Erythrocytes live approximately 110–120 days, which means that about 1% of erythrocytes (approximately $2.2 \cdot 10^{11}$ cells) must be produced every day from erythroid progenitors in red bone marrow, and the same number of senescent erythrocytes must be removed from the circulation (mostly by spleen) [97].

Hematopoiesis at a glance

The process of maturation of erythrocytes (erythropoiesis) starts by the differentiation of hematopoietic stem cells (HSC) (Fig. 2.6, page 37). Hematopoietic stem cells have the capacity to divide (without senescence) or differentiate into progenitor cells of any hematopoietic lineage. Adult hematopoiesis in humans takes place almost exclusively in the bone marrow, while embryonic/fetal hematopoiesis occurs in the yolk sac and is also transiently found in the fetal liver [98].

HSC differentiate first into common lymphoid or common myeloid progenitors. These progenitors further divide and differentiate either into B or T lymphocytes or natural killer (NK) cells following the lymphoid lineage, or to granulocyte/monocyte, megakaryocyte or erythrocyte precursors (CFU-GEMM)[13] within the myeloid lineage. The pluripotent CFU-GEMM progenitor then differentiates to progenitors of the respective cell lineages, erythroid and myeloid.

[13] for ***c****olony-****f****orming* ***u****nits–****g****ranulocyte–****e****rythrocyte–****m****acrophage–****m****egakaryocyte*); this name originated from their ability to generate various hematopoietic colonies in the spleen after transplantation into lethally irradiated mice

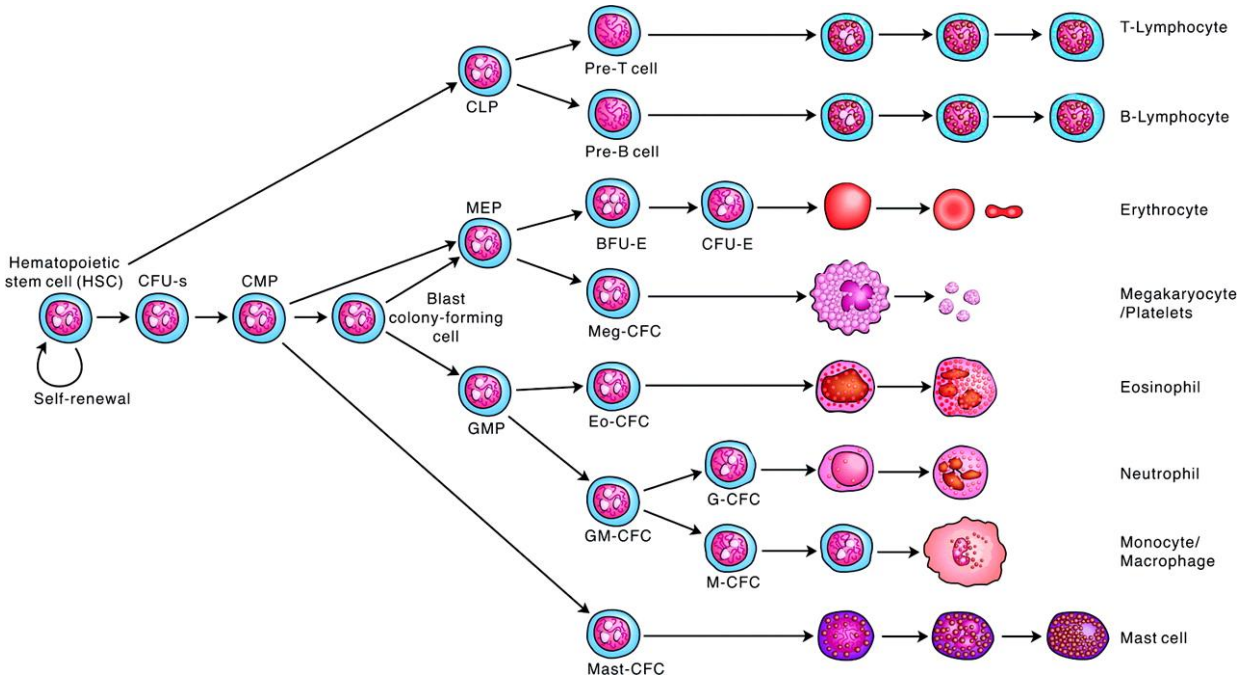

Figure 2.6: Hematopoiesis.
Differentiation of hematopoietic stem cells into pluripotent progenitors and finally to diverse blood cell types. A hematopoietic stem cell (HSC) gives rise to common lymphoid progenitor (CLP), or to the common progenitor of all other blood lineages (colony forming unit – spleen, CFU-S). CFU-S further differentiate into common myeloid progenitor (CMP). Further differentiation separates common granulocyte-monocyte progenitors (GMP), mast cell precursors, and common megakaryocyte-erythrocyte progenitors (MEP). MEPs give rise to the populations of megakaryocytes and erythrocytes.
Source: `http://www.lifeethics.org/`

Erythropoiesis at a glance

Erythroid differentiation takes place in *erythroblastic islands*, specialized niches that are composed of erythroid precursors at different stages of development that are in an intimate contact with a central macrophage. This microenvironment ensures that developing erythroid cells get a regulatory feedback via cell-cell and cell-extracellular matrix interactions. The central macrophage is regarded as a *nurse cell* that provides developing erythroblasts with iron and growth factors [99].

The earliest progenitors committed exclusively to the erythroid differentiation are termed as ***b**urst-**f**orming **u**nits (**e**rythroid)* (BFU-E), that form multi-clustered colonies of hemoglobin-producing cells. They give rise to more differentiated progenitors, ***c**olony-**f**orming **u**nits (**e**rythroid)* (CFU-E), that further differentiate to proerythroblasts and then erythroblasts. During these steps, cells accumulate hemoglobin and at late erythroblast stages, chromatin condenses, nucleoli disappear, and nuclei are then extruded.

Reticulocytes, the enucleated erythroid precursors, then undergo the final maturation in bone marrow and are released into circulation, giving rise to erythrocytes [97]. The whole process (from commited progenitors to mature erythrocytes, Fig. 2.7) takes about 10 days.

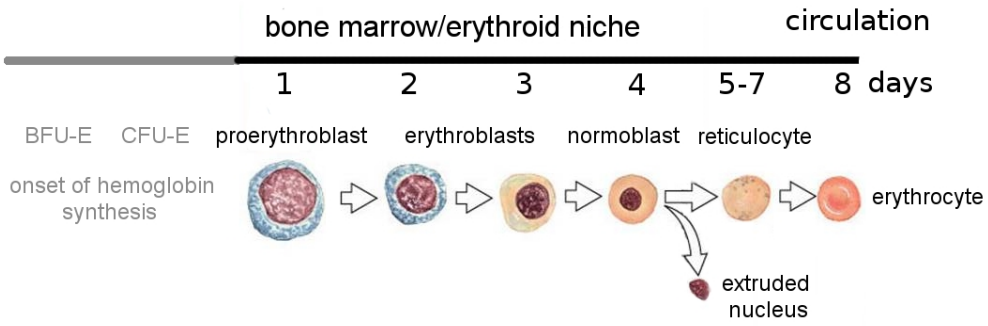

Figure 2.7: Erythropoiesis.
The first commited erythroid progenitor is BFU-E (burst forming unit – erythroid) that develops from the common progenitor of erythroid and megakaryocyte lineages, MEP (megakaryocyte-erythrocyte progenitor). BFU-E further differentiates into CFU-E (colony-forming unit – erythroid), proerythroblast, erythroblast, and then reticulocyte. After enucleation, the maturation of erythrocyte is completed.
Altered from `http://www.as.miami.edu`

Erythroid differentiation is a complex and tightly regulated process. Progenitor cells must synthesize large amounts of proteins, most notably globin chains, in a relatively short period of time. This is accomplished by a massive upregulation of mTOR pathway (described earlier in 2.3.1 on page 21) by stem cell factor (SCF) and possibly other growth factors [100, 101].

The synthesis of hemoglobin can be first traced in proerythroblast and further increases until late erythroblast stages. Because all building blocks of hemoglobin (free porphyrins, heme and monomeric globin molecules) are toxic to the cell, the synthesis of globin and heme must be accurately matched. Synthesis of heme requires an uptake and processing of large quantities of iron ionts, and cells must be able to protect themselves against reactive oxygen species that are generated as undesirable byproducts of iron metabolism. Iron uptake and metabolism are also tightly coupled to the synthesis of porfyrin.

The high demands for the production of globin chains require a steep increase in the production of ribosomes. As the synthesis of hemoglobin declines in late stages

of erythroid differentiation, the cellular pool of ribosomes and the overall translation ceases, too, but seem to persist to some extend even after the enucleation and in mature erythrocytes [102].

The cytokine receptors and membrane transporters that integrate growth signals exchange gradually as cell maturate. Finally, the membrane remodels to achieve the characteristic biconcave shape. During the terminal stages of erythroid differentiation, the morphology of erythroblasts changes dramatically: the nucleus and chromatin condensates, the organelles disappear, and subsequently, the enucleation occurs.

The loss of mitochondria leave mature erythrocytes dependent on anaerobic metabolism (glycolysis), but many metabolic processes (pentose phosphate pathway, methemoglobin reduction, function of membrane iont channels) persist to some extend even in mature erythrocytes [102].

2.5.1 The role of apoptosis in normal erythropoiesis

At advanced stages of erythroid differentiation (late erythroblasts to normoblasts), the levels of protein p53 gradually rise [103]. This is probably the result of a nuclear condensation and preparation to the repulsion of nucleus: activation of p53 as a result of DNA breaks is a part of normal protective mechanisms of cells [104].

The DNA degradation starts in the perinuclear region, diffusing all over the nucleus and similarly to the programmed cell death, nuclear apoptotic bodies are eventually formed. p53 activates (or contributes to the activation) proapoptotic caspases 3/7, which is necessary for the profound morphological rearrangements (cleavage of proteins maintaining nucleolar integrity, degradation of organelles, etc.) [105, 104]. However, instead of classic p53-dependent apoptosis, an alternative pathway is followed and cells survive.

Several factors contribute to the escape from classic apoptosis. Erythropoietin (Epo) supresses (via the Jak/STAT signaling pathway) transcription of several proapoptotic proteins, and induces transcription of antiapoptotic proteins and of erythroid-specific genes (reviewed in [104]). The action of erythropoietin is further supported by the effect of several other cytokines.

GATA-1 is another differentiation and survival promoting transcription factor in play. This crucial erythroid-specific transcription factor was shown to be activated by erythropoietin [106]. GATA-1 not only promotes the expression of erythroid-specific genes, but also negatively regulates the p53 activity (and perhaps also cellular levels) [90]. A low activity of p53 throughout the differentiation process probably also keeps low the levels and activities of the major proapoptotic caspases 3 and 7. As a consequence, the exhaustion of the active caspases 3 and 7 prevents the completion of apoptosis after the enucleation [104].

Nevertheless, erythropoietin levels are under normal conditions kept relatively low, and therefore, a fraction of maturating erythrocytes complete the apoptotic pathway. This balance between the maturation and apoptosis is important for maintaining homeostasis and keeping stable levels of erythrocytes in the circulation.

Hypoxia (reduced oxygen supply) stimulates erythropoiesis by inducing erythropoietin, which stimulates differentiation and proliferation of erythroid precursors by preventing the entrance to apoptosis. Once the red cell mass becomes sufficient, the decrease in erythropoietin levels leads to the shift of the balance towards apoptosis, and as a result, a steady level of erythrocytes is achieved [107]. Various stress conditions have also been shown to activate glucocorticoids, which cooperate with erythropoietin in shifting the balance towards erythroid maturation. The mechanism of action of glucocorticoids is very complex, and their role in erythropoiesis is not fully understood yet. However, it was demonstrated that glucocorticoid receptors are essential for stress erythropoiesis [108].

2.5.2 The role of apoptosis in anemias

As mentioned before, the elimination of a fraction of maturating erythroid precursors by apoptosis is a physiological mechanism that serves for maintaining the stable red blood cell count [109]. On the other hand, an increased apoptosis of the erythroid precursors resulting from the p53 activation have been reported to cause anemias. p53 blocks pro-proliferative programme and stimulates pro-apoptotic pathways, and p53 also weakens or blocks physiological mechanism that stimulates erythropoiesis under stress conditions by antagonizing a glucocorticoid receptor [110].

The activation of p53 pathway by various types of cellular stress (such as DNA damage, blocks in cell cycle progression, etc.) plays an important role in the pathogenesis of several congenital anemias and also some acquired hematologic diseases. There are several likely explanations why are hematopoietic and namely erythroid progenitors exceptionally sensitive to p53 activation and subsequent apoptosis. A higher sensitivity of stem cells and pluripotent progenitors probably originated as an effective mechanism to prevent the clonal expansion of malignant cells that might arise by DNA mutations [83]. The reason why the erythroid progenitors appear to be especially prone to apoptosis might be that the balance between differentiation and apoptosis is very delicate, as discussed above.

A typical example of inherited anemia due to a genotoxic stress is Fanconi anemia (FA). This disorder has several causal genes, all encoding components of a complex vital for the crosslinked DNA strand repair [111]. FA manifests with a profound bone marrow failure, developmental anomalies, abnormal skin pigmentaion and a higher incidency of malignancies.

Several other congenital dyserythropoietic anemias (CDAs) have been linked to insufficiency in DNA repair machinery or in the control of cell cycle progression. Interestingly, in addition to anemia, their symptomes also include also growth retardation, heart defects and distal limb anomalies in a fraction of patients. These examples include CDA type I, which is caused by mutations codanin [112, 113], which plays an indispensable role in cell cycle progression. Other rare congenital anemias due to mutations in checkpoint kinase 1 [114] (that also controls the cell cycle progression), or in geminin [115] (that is involved in DNA repair), have also been reported.

Activation of p53 might also result from various types of biological, chemical or physical stress, such as radioactive radiation or chemotherapy. Cell cycle arrest and apoptosis have also been implied in the infection of erythroid progenitors by human parvovirus B19. Several parvovirus B19 proteins interfere with cell cycle progression and induce cellular stress, and the infected cells are highly prone to apoptosis. Hematopoietic tissue is also extremely sensitive to cytostatics or to irradiation [116].

Anemia and other cytopenias are also present in ribosomopathies, syndromes associated with defective ribosome biogenesis and another type of cellular stress termed

the ribosomal stress (see Section 2.4.3, page 30). Genes mutated in these syndromes affect various aspects of RNA metabolism including rRNA modifications and ribosome biogenesis. The mechanism of pathogenesis of these syndromes is very complex and in most of the syndromes, is still not fully explained. One such disease, Diamond-Blackfan anemia, was the main subject of my work and will be described in more detail.

2.6 Diamond-Blackfan anemia

Diamond-Blackfan anemia (DBA, OMIM[14]:105650) is a rare congenital syndrome that manifests usually during the first year of life with normochromic macrocytic anemia (erythrocytes are larger but with normal concentration of hemoglobin). Almost half of DBA patients display additional congenital malformations and/or slower growth. The majority of DBA cases are sporadic, familiar cases show autosomal dominant inheritence. Incidence is 5–7 cases in one milion of live births, and both sexes are affected equally [117, 118]. This disorder is named after Louis K. Diamond M and Kenneth D. Blackfan [119]; synonyms include Aase syndrome and Aase-Smith syndrome.

2.6.1 Clinical symptoms and diagnosis

The clinical manifestation of DBA is very diverse and therefore the diagnostic criteria for this syndrome have been reviewed several times (most recently in [118, 120]). The main diagnostic criteria include:

- early onset of anemia (in 90% of cases in the first year of life and the median age of the manifestation of DBA is two to three months)
- erythrocytes are normochromic, (in most cases) with markedly increased mean corpuscular volume (MCV)
- profound reticulocytopenia (erythroblasts represent less than 5% of nucleated cells in the bone marrow)
- apart from a selective defficiency of erythroid precursors, bone marrow is normocellular (other blood cell precursors are present in normal counts[15])

The diagnosis is further supported by the presence of the following additional characteristics and laboratory findings, which are to various extent present in most patients:

[14] Mendelian inheritance in man, `http://www.ncbi.nlm.nih.gov/omim/`

[15] Additional cytopenias (deficiencies of hematopoietic progenitors of other lineages) may be rarely present, sometimes severe enough to require independent therapy [121]

- slower growth
- various congenital malformations (mainly affecting head, upper limbs, genitourinary tract and heart)
- elevated erythrocyte adenosine deaminase activity (eADA)[16]
- persistent expression of fetal hemoglobin (HbF) and a presence of the fetal i antigen
- high levels of folic acid, vitamin B12 and erythropoietin in serum

Approximately 1/4 of DBA patients are small for gestational age and scarcely, *hydrops fetalis* (accumulation of fluids in tissues, causing swollen arms and legs and breathing difficulties) may be present in newborns with DBA ([125] and [126]). Symptomes of inadequate oxygenation of tissues are similar to those in other anemias: pale skin and difficulties in breathing or shortness of breath, especially during feeding or while sucking.

Growth retardation is reported in about one third of DBA patients, in some cases as a primary defect while in others, it might be a secondary complication from side effects of therapy (iron overload, steroids) [127, 128]. Slower growth is often accompanied with somatic anomalies, which are reported in 35–50% of DBA patients (statistical data from DBA registries of different countries are summarized in [118]). Most common craniofacial anomalies include micro- and macrocephaly (unusually small or large head), cleft lip or palate, glaucoma, low ear set, small lower jaw, flat nasal bridge and hypertelorism (wide face and wide-set eyes[17]). Thumb defects also range from mild, such as thenar hypoplasia (underdeveloped thumb joint and muscle), to more severe bifid (bifurcated last phalanx of thumb), triphalangeal (thumb having three instead of two phalanges) or duplicated thumb [130], or even the absence of the radius or forearm.

[16] eADA actvity is significantly increased in 80–85% of all DBA patients ([122, 123]). High levels of eADA suggesting the stimulation of adenine catabolism may be explained by the fact that improperly processed rRNA precursors accumulate in the nucleoli and must be degraded [124].

[17] As in some other inherited syndromes (Down syndrome, for example), some DBA patients look more alike other unrelated DBA patients, than alike their other relatives. The craniofacial anomalies observed in DBA are also referred to as *Cathie* or *classical DBA faces* [129].

Less frequent additional anomalies include heart septal defects, spine defects, kidney aplasia, and anogenital fistula (abnormal passage from anus and urogenital system). In many cases more than one anomaly is present.

In some cases, some first degree relatives of a DBA patient carry an identical mutation in a DBA causal gene as the proband, yet display normal or nearly normal hematological findings. Such individuals should be assigned as having non-classic DBA (*silent*) DBA regardless of the phenotype [118]. Identification of such individuals is important in order to exclude them as stem cell transplant donors for their affected family members and to provide them genetic counseling and medical care, if necessary.

Patients suspected for DBA diagnosis are screened for mutations in genes known to be associated with DBA. In cases where the causative gene is not known, diagnosis is made indirectly by rejection of other diseases (please refer to [118] for more details).

2.6.2 Etiology and causal genes

The incidence of DBA is estimated to be 5–7 in one million of live births, men and women are affected equally and no ethnic predominance was observed so far [132, 133, 118]. Approximately 2/3 of DBA cases are familiar with autosomal dominant inheritance [117, 134, 120].

Causal genes

At the beginning of this work, in autumn 2006, RPS19 was the only gene with causal association with DBA, being affected in 25% of DBA patients. Since then, significant progress has been made and eight other RP genes encoding RPs of small and large subunits of cytosolic ribosomes were identified (*RPS* genes and *RPL* genes). Together, these other RP genes have been reported to be affected in another 25–35% of DBA patients, and the causal gene thus remains to be identified in slightly less than half of the patients. Genes listed in listed below in **bold face** are subjects of articles that are part of this work.

Currently known DBA causal genes include *RPS19* [4], *RPS24* [134], ***RPS17*** [135] (see Appendix 1), *RPS10* and *RPS26* [136], ***RPL5*** and ***RPL11*** [137, 133] (see Ap-

pendix 2), *RPS7* [133], *RPL35a* [138]. In addition, single cases of DBA patients carrying rare sequence variants in several other RP genes (RPL36, RPS27a, RPS8, RPS14[18] and RPS15) were reported, but the causality of these variants hasn't been confirmed yet [139, 140].

All types of mutations (missense, nonsense, deletions, insertions, splice site alterations) have been reported in DBA patients. So far, only one case has been reported with more than one RP affected, namely an RPL5 mutation and an RPS24 variant of unknown significance [141]. In all cases reported to date, mutations were heterozygous. Considering ribosomal proteins are highly conservative and indispensable for the proper function of ribosome, homozygosity would be most likely (according to animal models) lethal.

The causality of mutations in these RP genes for DBA has been confirmed several times both in animal models and in human $CD34^+$ progenitor cells. The reduction in the amount of RP mRNA by 50–60% (by siRNA or antisense morpholino analogs that acts as DNA mimicks) was confirmed to be sufficient to recapitulate the defect in rRNA processing and ribosome biogenensis observed in cells of DBA patients [53, 142], along with subsequent defect in erythroid maturation.

The frequency of mutations in causal DBA genes, as well as their chromosomal loci, are summarized in table 2.1. The data were extracted and pooled from the above cited sources and from an online database set by the University of Torino, collecting the identified mutations in RP genes in DBA patients from several national registries (`http://www.dbagenes.unito.it` [143]). However, the data are far from being complete and therefore, the number of DBA patients refer to the *minimal* numbers, and therefore the percentages should also be taken merely as *approximate* values.

[18] Interestingly, RPS14 gene resides in a chromosomal region that is deleted in the 5q- syndrome and was previously confirmed to be the major, if not the sole causal gene in this bone marrow syndrome (see page 31 for more details).

Table 2.1: Genes mutated in DBA and their incidence

DBA locus (OMIM)	gene	chromosome	number of variants identified to date	number of affected patients	percentage of patients worldwide	fraction of affected families in the Czech DBA Registry[a]
DBA1 (603474)	*RPS19*	19q13.2	129	219	25 %	24%
DBA3 (610629)	*RPS24*	10q22-q23	6	12	>2 %	0
DBA4 (612527)	*RPS17*	15q	3	3	>1 %	3%
DBA5 (612528)	*RPL35a*	3q29-qter	5	7	>3 %	0
DBA6 (612561)	*RPL5*	1p22.1	39	59	>7 %	18%
DBA7 (612562)	*RPL11*	1p36.1-p35	26	37	>5 %	9%
DBA8 (612563)	*RPS7*	2p25	1	1	<1 %	0
DBA9 (613308)	*RPS10*	6p21.31	3	5	>6 %	0
DBA10 (613309)	*RPS26*	12q13	8	13	>2 %	15%
	In total:		**over 230**	**over 350**	**over 50**	**70% (22/33 families)**

[a] Dr. Cmejla, personal communication (19. 5. 2011)

General and specific roles of DBA causal genes

To date, mutations in 9 RP genes have been confirmed to be causal for DBA, though some of these genes were mutated only in one or a few patients. *None* of the proteins encoded by these genes seem to play a specific role in erythroid differentiation. Although there were some attempts to prove such extraribosomal function for RPS19, no such role has been confirmed yet.

Almost all small subunit RP genes causal for DBA (RPS19, RPS24, RPS17, RPS7, RPS10, RPS26), and also two possibly causal DBA genes (RPS8 and RPS14) participate in different steps of pre-18S rRNA maturation and the small subunit assembly. Another small subunit RP possibly causal for DBA, RPS15, is required in the export of the pre-40S subunit to cytoplasm [53]. Although the participation of large subunit RPs in the maturation of pre-rRNAs is definitely much less frequent, *all* three large subunit RPs associated with DBA (RPL35a, RPL5 and RPL11) were confirmed to be important this process [41].

Several of the RPs associated with DBA (RPL5, RPL11, RPL26 and RPS7) were reported to serve as mediators activating the p53 response in case of ribosome maturation defects (as overviewed before in Section 2.4). Nevertheless, since the p53-inducing RPs act in a cooperative manner, haploinsufficiency of one of them doesn't disrupt this signaling, because it is functionally compensated by others [72].

It is also worth mentioning that introns of some *RP* genes contain small nucleolar RNAs that participate in posttranscriptional modification of rRNA precursors. Longer RP gene deletions thus may lead to a more severe defect in pre-ribosomal particle processing and possibly also contribute to the DBA phenotype, although this hypothesis remains to be confirmed.

Probable consequences of *RP* mutations

A common outcome of the haploinsufficiencies of all DBA causal genes is a block in the maturation of the respective subunit. To recapitulate, this has two major consequences:

- decrease in the pool of mature subunits and of assembled 80S ribosomes, resulting in reduced translational capacity,

- generation of incorrectly processed subunit precursors and accumulation of unassembled ribosomal proteins in nucleus, causing the induction of p53 and leading to cell cycle arrest and/or apoptosis.

The impact of these two effects on cells varies substantially among different cell and tissue types, depending on growth rates and specific protein requirements. The reason why are hematopoietic and especially erythropoietic cells one of the most affected ones might be their very high demands for translation.

Irrespective of a causal gene, cells derived from DBA patients have previously been reported by our team to have decreased translational efficiency [144]. The addition of essential amino acid leucine[19] to the cell culture medium was shown to significantly increase the translational rate in model cells K562 [144], leucine having the most pronounced effect. This indicated that limited translation is an important factor in DBA pathogenesis.

Activation of p53 is also crucial in the pathogenesis of several other inborn aplastic anemias, such as Fanconi anemia or type I congenital dyserythropoietic anemia. Recently, Dutt et al. demonstrated that activation of p53 led to a lineage-specific cell cycle arrest in erythroid progenitors in DBA, and pifithrin as a specific p53 inhibitor resued the defect [145]. These authors proposed that the mechanism of the ribosomal stress might cause p53 activation predominantly in erythroid progenitors, since these cells have lower treshold for the induction of p53. This is in agreement with previous studies: delayed cell cycle progression and/or increased apoptosis of bone marrow precursors have been observed in several animal models of DBA, and also in human progenitors with induced silencing of *RPS19* or other *RP* genes [146, 147, 148]. This mechanism would explain not only the pathogenesis of DBA but might be applicable to other ribosomopathies, however, more studies are needed to clarify this issue.

The activation of p53 is also connected with the observed lower translational rate. p53 negatively regulates the mTOR pathway and the activation of mTOR thus at least partially compensate the defect. Since mTOR controls various metabolic pathways,

[19] These branched chain amino acids (leucine, valine, and isoleucine) are known to stimulate the main controller of cellular translation, protein kinase mTOR.

the suppression of mTOR via the elevated p53 activity was also expected to affect the cellular metabolism, and this assumption was already confirmed experimentally[20].

2.6.3 Correlations between the causal gene and phenotype

Despite the above discussed common mechanisms of pathogenesis, the DBA phenotype is extremely heterogeneous, ranging from silent to severe, and may markedly differ even among patients bearing an identical mutation. There is no unambiguous correlation between the affected gene, the type of mutation and severity of anemia and other phenotypic characteristics of DBA patients. However, some links have been established.

Mutations in *RPL5* cause in most cases more severe anemia and often also multiple somatic anomalies, in comparison with the phenotype associated with mutations in *RPL11* and *RPS19* [137, 133]. Characteristic facial dysmorfisms, namely cleft lip and/or cleft palate (CL/P) were reported in about 40% of patients with *RPL5* mutations [133, 141]. *RPL5* mutations are also often accompanied with triphalangeal thumb and thenar anomalies [137].

In contrast, isolated thumb defects are predominantly present in patients carrying mutations in *RPL11* [133]. Other somatic anomalies, such as the tetralogy of Fallot (a congenital heart anomaly), are less frequent [137] in this subset of patients.

Mutations in *RPS10* and *RPS26* have also in a few cases been accompanied by physical anomalies (CL/P and webbed neck (*pterygium colli*, short neck with skin fold that runs along the sides of the neck down to the shoulders)) [136], but the number of patients reported with those causal genes is too low to prove any correlation. One patient with RPS26 mutation also displayed Klippel-Feil syndrome [150], which presents by short, immobile neck and is caused by defect in cervical vertebrae segmentation.

Mutations in *RPS19* gene haven't been associated with any distinct physical anomalies [117, 151, 152]. Patients carrying *RPS19* mutations have in general poorer response to steroids [120], missense mutations in the *hot spot region* [153] (codons 52 to 62 in

[20] RPL11-deficient zebrafish also displayed alterations in energy metabolism and changes in biochemical composition of affected cells [149].

humans) and loss-of-function mutations are associated with more severe phenotype [154].

It was proposed that the expression of the affected *RP* might be influenced by genetic variations in the non-coding regions and thus might affect the phenotype. However, to date, no correlations have been observed [155], [156], and this possibility will be discussed later in section 2.6.6 (page 59).

2.6.4 Therapy

There are several treatment options for DBA, but none of them specific or causal. The choice of the therapy depends on the severity of anemia, age and physical state of the patient and on the response to previous therapies, if administered. Current options of treatment are dominated by corticosteroid therapy, red blood cell transfusions and hematopoietic stem cell transplantation (HSCT) [157]. Most of the patients are infants or small children, which must be also taken into account in the selection of the therapeutic approach.

Corticosteroids

Glucocorticoids are steroid hormones that are produced by the cortex of the adrenal gland, but the term also applies to their synthetic analogs. They bind to specific glucocorticoid receptors (GR) and although the name refers to their role in controlling the metabolism of glucose, their functions aren't limited to regulating anabolic pathways. Most notably, glucocorticoids also have anti-imflammatory effects: synthetic analogs of glucocorticoids (such as dexamethasone) were originally developed for the therapy of immune system diseases, allergic reactions, inflammatory diseases, arthritis, and even multiple myeloma.

Glucocorticoid receptor signaling was also demonstrated to cooperate with the erythropoietin receptor and stem cell factor receptor (c-kit) signaling, and is essential for balancing proliferation and maturation of erythroid precursors. In response to hypoxia, GR signaling (together with erythropoietin) promotes erythroid maturation by

upregulation of genes required for erythroblast proliferation, such as c-myb, and repression of genes specific to other lineages [110] (and references therein). GR signaling is negatively regulated by p53 [158].

Glucocorticoids are effective also in the treatment of DBA [159]. The possible mode of action of glucocorticoid-based drugs in DBA is that they override the inhibitory effect of p53 on the proliferation of erythroid progenitors, and specific inhibitors of p53-GR interaction would be potentially promising therapeutic agents in DBA. From unknown reasons, some patients, especially those carrying mutations in RPS19, have usually poorer response to steroids [157].

Corticosteroids are still the therapy of choice, but since they have profound adverse effects on physical and neurocognitive development in infants, the onset of the therapy should be delayed to at least until 1 year of age [157]. Up to 80% of patients initially respond to corticosteroids. An increase in hemoglobin is usually seen within several weeks and the dose is then gradually decreased to establish the minimal effective dose. Approximately 25% of these patients later enter remission[21] or require only very small doses of corticosteroids, while remaining three quarters require much higher doses of these drugs to maintain transfusion independence [118]. Some patients have to discontinue the terapy because of severe side effects, such as developmental retardation, diabetes, glaucoma, high blood pressure, and bone weakening (reviewed in [160] and [118]).

Transfusion

Long term red blood cell transfusions are necessary in individuals that can't be given steroids or don't respond to them. The goal of transfusion therapy is to sustain hemoglobin concentration of 80–100 g/l, which is usually sufficient for maintaining growth and development (reviewed in [160] and [118]). Transfusion therapy has to be combined with iron chelation therapy to minimize the risk of iron overload.

[21] *Remission* of a DBA patient is defined as a state when he/she reaches a hemoglobin level adequate for their age lasting six months without any treatment, but NOT as a result of the transplantation of HSC.

Alternative therapies

A number of other agents were (with limited success) reported as alternative therapeutic modalities, including intravenous antithymocyte globulin (ATG), high-dose erythropoietin, androgens, cyclosporine, interleukin-3 and metoclopramide. However, little is known about the mechanism of action of these drugs in DBA. It is highly probable that the effect of many of these agents is indirect and mediated by the microenvironmental cells in bone marrow ([157] and references therein).

As mentioned before, essential branched-chain amino acids (**leucine**, isoleucine, valine) stimulate translation via the protein kinase mTOR. This anabolic effect is specific and can't be attributed only to the increased availability of one of the limiting protein components. Of all essential (branched-chain) amino acids, leucine seems to be the most important in stimulating protein synthesis [161].

The effect of leucine on translation was previously tested by our team using lymphocytes of several DBA patients. Leucine markedly improved translational rate in most samples, however, only a little effect was observed in patients bearing an *RPS19* mutation [144]. Leucine treatment evoked remission in one patient with DBA [162] and is currently being tested in a larger cohort of patients. The highest advantage of leucine in the therapy is that it is not accompanied with any risks and adverse side-effects associated with current therapies.

Valproic acid, a structural analog of butyric acid that is routinely prescribed to control tonic seizures, was reported to induce stabilization of hemoglobin levels in one DBA patient, who soon after valproic acid therapy achieved remission [163]. The same treatment was also successful in some patients with myelodysplastic syndromes [164].

Valproic acid acts as a histone deacetylase and alters the gene expression programme but the exact mechanism and target genes are not known. However, it was reported to stimulate both proliferation and self-renewal of HSC, and increase the expression of HbF[22] and the percentage of HbF-containing red blood cells [165].

[22] fetal hemoglobin

Another drug that is now being clinically tested in a cohort of DBA patients is **lenalidomide**. This medication was previously successful in the treatment of 5q- syndrome. The possible action of lenalidomide involves inhibition of two G_2/M checkpoint regulators, Cdc25C and PP2Aα. Interestingly, the cell cycle arrest is restricted only to clones carrying the 5q- deletion; the drug doesn't affect normal cells [166].

Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is currently the only curative treatment of DBA but because of risks associated with this option, it is recommended usually mainly for transfusion-dependent or steroid-dependent patients (approximately 10% of total patients), preferably between 3 and 9 years because younger patients accept grafts better (reviewed in [157]).

Whenever possible, the prefered donor is a HLA[23]-matched sibling, however, significant progress has been made in recent years also in HSC transplantations from HLA-matched unrelated donors. Grafts are either bone marrow or (less frequently) cord blood. Most of the HLA-matched donor transplants required myeloablative chemotherapy alone as a conditioning regimen, while in the majority of the alternative donor (mismatched family or unrelated donor) transplants, a combination of chemotherapy and radiation therapy was used for pre-transplant conditioning [139].

Summary: Effectivity of current DBA therapy

Glucocorticosteroid analogs as a therapy of choice improves the red blood count and other laboratory findings in approximately 80% of patients after the initial course. Subsequent treatment is highly variable, some patients continue with initial doses of steroids, some individuals eventuallly require very small doses or even achieve remission. Some patients become refractory to steroids (i.e. had been on steroids but lost the response to steroids over time) or have to discontinue the therapy due to unacceptable side effects [118, 157]. Eventually, 40% of DBA patients are long-term steroid depen-

[23] human leukocyte antigen

dent, 40% receive regular blood transfusion, and 20% enter remission. The reasons for this variable response are unknown, except for the above mentioned correlation with some types of *RPS19* mutations.

The probability of achieving remission is about 1:4 by age 25 years. From all individuals diagnosed with DBA that eventually entered remission, the majority of them (4/5) were children younger than 10 years [157]. Some patients in remission (without any history of treatment for anemia for more than 10 years) display evidence of persistent functional abnormalities of erythropoiesis (macrocytosis, low/borderline hemoglobin concentration, and elevated eADA) with normal leukocyte and platelet counts. Some patients may regain the symptoms and eventually experience another remission later in their lifetime. Relapses often follow viral infections or pregnancy.

The overall survival, as reported by the DBA Registry [139, 157] of North America, is 75% at 40 years of age; being higher for the steroid-responsive patients. The main causes of premature death (in 2/3 of cases) are the adverse effects of treatment (complications from HSCT, iron overload from transfusions, higher susceptibility to infections, etc.), less than 1/4 of patients die from DBA-related complications (severe anemia, malignancies).

2.6.5 Other risks and restrictions associated with DBA

Current options of therapy allow DBA patients to have a long and productive life, although regular medical examinations are advised, and there are also a few risks and limitations.

Depending on their actual condition, most DBA patients are allowed and encouraged to get some daily physical excercise. Unlike in many other anemias, DBA patients aren't recommended to eat iron-rich diet; in male patients requiring regular blood transfusions it's quite the contrary because there is a risk of iron overload [167]. Patients who take regular doses of steroids are recommended to take preventive measures to avoid infections, because they are more prone to complications due to immune system suppression [160].

DBA had long been considered a disease that affects only erythroid differentiation. However, other cell lineages might also display slowed proliferation, reduced translational rates, impaired pre-rRNA processing and abnormal nucleoli [144, 168, 169]. There were several reports of reduced leukocyte and altered thrombocyte counts in a fraction of DBA patients, and some patients even developed trilineage aplasia [121, 159]. Resulting complications (immunodeficiency or blood clotting defects) are sometimes severe enough to deserve special precausions or medical care.

In a survey of 64 pregnancies in women with DBA [170], an increased incidence of complications was found in both mothers and foeti. The main risks include intrauterine growth retardation, miscarriages or preterm delivery [160].

Ribosomopathies are also associated with increased predisposition to cancer (from reasons discussed before, on page 30), and the prognosis is generally worse. However, the reported increased incidence of cancer in DBA patients (4 % compared to < 1 % in healthy controls [157, 118, 132]) is not significant due to the small size of the cohort.

2.6.6 Searching for new DBA causative genes

Although Diamond-Blackfan anemia was recognized as a distinct hematological syndrome as early as in 1936, the underlying somatic cause remained a mystery for a long time. The first known DBA causal gene, *RPS19*, was found in 1999 by a lucky chance[24], and with later identified mutations in other *RP*s, the underlying defect is now known in over a half of DBA patients.

DBA is now classified as a ribosomopathy and its pathogenesis is explained by a combination of reduced translational capacity and events triggered by the p53 activation. The spectrum of mutated genes in other ribosomopathies is fairly heterogenous, and many of them affect also cellular processes unrelated to ribosomes. Therefore, a question still open is whether DBA is caused by *RP* haploinsufficiency only, or whether a non-ribosomal proteins related to ribosome biogenesis might be involved as well.

Searching for the underlying defects in DBA patients is complicated by the fact that the syndrome is very rare and therefore, the cohorts of patients are relatively small. Patients' data are collected in national registries (which again exist only in a few countries); the largest set of patients (currently over 600) is in the Mexican–USA–Canadian Registry of North America (`http://diamondblackfananemia.org/resources/dbar/registry`). The collection of statistical data is further complicated by the fact that the cohorts of patients in each registry have been screened (except for *RPS19*) only for some of the causal and/or candidate genes for DBA.

Assuming that the pathogenesis of DBA is very similar in all patients, the most likely candidates with causal connection to DBA include:

- RP genes that haven't been sequenced yet
- regulatory segments of RP genes or intronic snoRNAs participating in ribosome biogenesis
- non-RP genes involved in ribosome biogenesis

[24] A 7-year-old Swedish girl diagnosed with DBA carried a balanced translocation t(X;19)(p21;q13) [171], and the gene disrupted by this event was later cloned and identified as a gene encoding a protein component of ribosome, RPS19 [4].

In addition, the following possibilities also should not be excluded:

- genes affecting proteins regulating ribosome biogenesis (such as downstream targets of the mTOR pathway, transcription factors, etc.)
- genes involved in translation *per se*
- genes involved in regulation of cell cycle progression, controlling differentiation or division
- genes whose deletion trigger erytroid-specific p53 response

Or, alternatively, DBA might also be a polygenic disease caused by a combination of the above. All these possibilities will now be discussed in more details.

Other RP genes as candidates in DBA

So far, RP genes are currently the only genes whose causal role in DBA has been confirmed; additional RP genes thus seem as the most likely candidates. Recently, Gazda et al. completed the screening of all 80 RP genes for moderate to large RP deletions in a large cohort of patients from the North American DBA registry [87] with yet unknown causal gene, and they identified only three additional *RP* genes to contain exon deletions or duplication.

Currently, the number of known DBA causal genes rised to nine[25], but this number is probably not final yet because mutations in some of these genes are very rare (mutations in some of the 9 RPs associated with DBA have been so far identified only in a single patient), and because of the costs of sequencing of all RP genes, only a handful of RP genes have been so far sequenced in patients from other (smaller) DBA registries [26]. In addition to these RPs whose causative role in DBA pathogenesis has

[25] Namely, RPS19, RPS24, RPS17, RPL35a, RPL5, RPL11, RPS7, RPS10, RPS26 [157, 136]; see also Section 2.6.2, page 45

[26] The list of RP genes sequenced by our group include *RPS2, RPS3, RPS3a, RPS10, RPS12, RPS13, RPS14, RPS16,* **RPS17**, **RPS19**, *RPS24, RPS25,* **RPS26**, *RPS30,* **RPL5**, **RPL11**, *RPL13, RPL23, RPL26, RPL27, RPL35a* and *RPL36* (Dr. Čmejla, unpublished data). Genes mutated in at least 1 of total 37 patients from 33 families are in bold face.

been confirmed, rare sequence variants were identified in additional 8 RP genes[27] in several unrelated DBA patients. In some cases, these sequence variants encoded substitutions for amino acids of very similar properties, and there was no reason to expect an alteration of their physiological role. In other cases, the protein encoded by the patient RP gene variant was shown to be fully functional, i.e. was incorporated into ribosome and there were no defects in the rRNA maturation process and no changes in the ribosomal profile [136, 172].

Non-coding parts of RP genes: 5'UTR or introns

The promoters of all RP genes share some common features, and all contain binding sites for a common set of transcription factors (TFs) [156], ensuring that all RP mRNAs are produced at roughly equal levels. Some of the TFs are tissue-specific, and a disruption of their binding sites would make the respective RP limiting only in some tissues [173].

Several groups [174, 156, 155] performed the analysis of upstream sequences or other non-coding regions of the most frequently mutated DBA causal gene, *RPS19*, but none of the reported sequence variants segregated with the disease. Upstream sequences of other RP genes in DBA patients haven't been adressed yet, and so far, no data support the hypothesis of the importance of promotor variants.

Introns of some ribosomal protein genes [175] encode small nucleolar RNAs (snoRNAs) that guide post-transcriptional modifications of rRNA molecules necessary for the maturation and incorporation of rRNAs into ribosomes. The expression of intronic RNAs depends upon the transcription and processing of mRNAs from the host gene. Mutations that reduce expression or splicing of RP transcripts harboring snoRNAs would thus probably result in combinated insufficiency of both the RP mRNA and small helper snoRNAs, thereby impairing ribosome biogenesis in two different ways. However, this hypothesis has yet to be tested.

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[27] *RPL36*, *RPS15*, and *RPS27a* [133], *RPL19*, *RPS8*, *RPS14*, *RPS15* [87], *RPL9*, *RPL14*, *RPL23a*, *RPL7*, *RPL35* and *RPL3* [140]

Proteins regulating ribosome biogenesis or translation

Production of all components of ribosomes is controlled by several signaling pathways, most importantly the mTOR pathway (see section 2.3.1, page 21 for the details), and this pathway was demonstrated to be vital for the induction of ribosome biogenesis during erythroid maturation [176].

The improvement of the phenotype or symptoms upon stimulation of the mTOR pathway by leucine has already been confirmed [144, 177], both in *RP*-deficient $CD34^+$ progenitors and notably, also in DBA patients. Therefore, additional explanation for the inadequate synthesis of ribosomes could be a mutation disrupting a component of the mTOR pathway.

Since the many roles of mTOR complexes and their upstream effector(s), animal models haploinsufficient for some of the mTORC1 pathway components develop very heterogenous spectrum of anomalies. The phenotypes are more alike to phenotypes associated with mutations in genes encoding the members of Ras/MAPK/Erk pathways. Both signaling pathways control cell growth, proliferation and differentiation, and cooperate with one another in the regulation of ribosome biogenesis.

Proteins directly responsible for the positive downregulation of ribosome biogenesis are more likely candidates but so far, only a couple of genes encoding kinases of the mTOR pathway have been sequenced by our lab (namely, the mTOR and Rheb), and only in a small number of patients [144], and the results were negative so far.

Non-RPs that are involved in ribosome biogenesis

Eukaryotic ribosomes require for their maturation the assistance of snoRNAs and a large number of non-ribosomal proteins that function either as chaperones or catalyze the processing of pre-rRNAs. According to animal models, mutations affecting these non-ribosomal components lead to similar phenotypic manifestation as RP gene mutations and therefore, these genes may be promising candidates for DBA causal genes as well.

To date, mutations disrupting some of these extraribosomal components have been reported to some other ribosomal syndromes (see section 2.4.3). Despite some sim-

ilarities, these syndromes are classified as distinct clinical entities requiring different therapeutic approaches.

Other candidates unrelated to ribosome biogenesis and function

It is commonly accepted that DBA is a ribosomal syndrome. However, some DBA patients have been reported to carry mutations or at least unusual sequence variants in genes that didn't fall into any of the categories above. None of these variants has been proven to be causal, but since their role is only now beginning to be understood, it can't be excluded that they contribute to the phenotype.

The possibility of a non-RP gene involvement in DBA pathogenesis was first raised by the the identification of the DBA2 locus (OMIM: 606129) that has been mapped to the telomeric regions of chromosome 8p23-p22 [178]. This region doesn't contain any gene functionally related to ribosome biogenesis or erythropoiesis. Some authors emphasize the importance of the transcription factor GATA-4, residing in this region, but this TF is rather than in hematopoietic tissue involved in the development of mesoderm-derived tissues, such as heart. GATA-4 is able to induce the expresion of several globin chains and possibly other erythroid-specific genes, but the link between this locus and DBA remains elusive.

FLVCR1[28]is another candidate for a DBA causal gene. FLVCR1 is a heme exporter that is thought to play a role in the control of intracellular heme level, and this protein is expressed ubiquitously, being especially abundant in early stages of erythroid precursors and cancer cell lines.

The phenotype of *FLVCR1*-deficient mice is in many aspects similar to DBA phenotype: severe aplastic anemia, smaller body size and limb or craniofacial anomalies resembling those found in DBA [179]. The lower expression of FLVCR1 and/or abnormal presence of alternatively spliced FLVCR1 isoforms were detected in immature erythroid precursors of some DBA patients negative for RPS19 gene mutations [179]. These isoforms encode truncated and probably not fully functional proteins with al-

[28] **f**eline **l**eukemia **v**irus subgroup **C** **r**eceptor; this virus causes severe erythroid aplasia in cats

tered cellular localisation [180]. The gene encoding FLVCR1 resides in chromosomal region (1q31) that has been linked to DBA in several families with yet unknown causal gene. Nevertheless, no mutations in FLVCR1 have been found in DBA patients to date. Interestingly, a mutation affecting FLVCR1 gene was recently reported in a clinically unrelated inborn neurodegenerative syndrome manifesting with sensory ataxia and *retinitis pigmentosa* [181]. Therefore, more studies are necessary to explain the roles of FLVCR1 in human.

Another candidate protein which might be important in DBA pathogenesis is a serine/threonine kinase PIM-1[29], that was originally identified as an interacting partner of RPS19. The expression of PIM-1 is strongly induced upon the stimulation by erythropoietin and PIM-1 phosphorylates various cell cycle regulators, transcription factors and other proteins involved in signal transduction [182, 183]. Among other effects, phosphorylation and activation of the transcription factor c-myc (see page 26 for details) by PIM-1 leads to the enhancement of ribosome biogenesis.

PIM-1 has been associated with a protection of hematopoietic cells from apoptosis induced by genotoxic stress or growth factor withdrawal. Mice defective for *pim-1* have mild microcytosis, whereas those hyperexpressing the gene display macrocytosis [184]. Loss of Pim-1 seems to be compensated by the induction of other two Pim kinases that are highly homologous to Pim-1. Knockout of all three Pim kinaseses (Pim-1 to 3) result in smaller phenotype and display impaired colony formation of all hematopoietic lineages, as a result of weaker responses to hematopoietic growth factors [185].

Two missense mutations (P311T and C17Y) have been identified in DBA patients [184], but neither of them affect important PIM-1 domains and can't therefore be considered as major causal mutations. Another case of PIM-1 mutation was reported in acute myeloid leukemia [183].

Another mutation in a non-RP gene that was found by our group affected *E2F4* gene (Dr. Cmejla, unpublished data). E2F4 is an essential transcription factor that is expressed at early stages of cell cycle and is indispensable for correct proliferation and differentiation [186], and seems to be important especially in cells of intestinal epithe-

[29] Proviral Integration Moloney virus

lium [187]. However, neither this mutation is causative for DBA, since the proband carrying this mutation was later found to be positive for a mutation in the (causal) *RPL5* gene [137].

Candidates for DBA causal genes: summary

The causal gene is still to be identified in 30–50% of all DBA patients (the data from different registries vary). Given the current insight into DBA pathogenesis, *RP* genes that haven't been sequenced yet are still the most probable candidates. Other possibilities (mutations affecting noncoding regions of RP genes, non-RPs participating in ribosome biogenesis,or translation, as well as mutations seemingly unrelated to this process) are purely speculative at the moment. Although alterations in these genes were reported in several DBA cases, none of them had a causative relation to the syndrome, although they may modulate the resulting phenotype.

The finding of novel genes that are causative for this rare disease was the main aim of this work. The identification of such genes will allow us to refine our hypotheses on the molecular mechanism of DBA and related syndromes. Equally important will also be improvement of the DBA diagnosis and genetic counseling.

3. Results

3.1 Searching for novel mutations and causal genes: our approach

In autumn 2006, when I joined the team for a DBA research, *RPS19* was the only known causal gene for DBA. Our original approach to select promising candidates for DBA causal genes was based on the hypothesis that proteins rarely act on their own, but rather in multiprotein complexes. Mutations affecting any component of the complex usually have a similar phenotypic manifestation, and this principle can be illustrated by several human syndromes (including above mentioned dyskeratosis congenita and Fanconi anemia).

Following this assumtion, we reasoned that a suitable candidate protein would interact with RPS19, and that mutations in *RPS19* would abolish the assembly and therefore the biological role of the multiprotein complex. In our pilot studies, we carried out co-immunoprecipitations of RPS19 complexes to compare the interactomes of wild-type RPS19 and its frequent *hot-spot* variants causal for DBA. We identified small subunit components RPS2 and RPS3 as proteins missing in complexes of mutated RPS19 protein, and we anticipated that this loss of interaction might be important for the pathogenesis in some DBA patients. However, later sequencing of RPS2 and RPS3 genes in patients from Czech DBA registry didn't reveal any mutation. Nevertheless, taking into account the low incidency of mutations of some DBA causal genes and the small size of the Czech DBA cohort (currently 37 patients), it can't be excluded that RPS2 or RPS3 might be affected in some patients from other DBA registries.

Since our original hypothesis pointed to other small subunit ribosomal proteins and previous results from our lab demonstrated that cells derived from DBA patients showed decreased rate of translation irrespective of the mutation in the RPS19 gene [144], we focused on ribosomal proteins functionally related to RPS19.

RPS19 interacts with 18S rRNA but is partially displayed on the surface of the 40S subunit, namely in a so-called "head" region, in a close vicinity to the binding site for the eukaryotic initiation factor eIF-2 [188]. Interestingly, the second DBA causal gene to be reported, RPS24 [134] was also localized in this ribosomal region[1]. No mutation in this gene has been found in the Czech cohort of DBA patients, but we reasoned that some of the other proteins residing in this area (RPS3a, RPS13, RPS16 and RPS17) might be involved in DBA pathogenesis as well.

Sequencing of genes encoding these four RPs in 24 patients from the Czech DBA registry by our team revealed a mutation in the RPS17 gene (see Cmejla et al., 2007, **Appendix I.**).

In 2008, a mutation in the first gene encoding a large ribosomal subunit protein, namely RPL35a[2] [138], was reported, which brought a strong support to the hypothesis that DBA is a ribosomal defect. A few months later, mutations in two other genes, *RPL5* and *RPL11*, were reported [189]. Interestingly, RPL5 and RPL11 were known to stimulate the p53-stress response by inhibiting its important negative regulator, Hdm2. Since a similar role has been proven also for RPL23, we decided to screen our DBA patients for mutations in genes encoding all these three large ribosomal subunit proteins and found mutations in the first two of them, RPL5 and RPL11 (published in Cmejla et al., 2009, **Appendix II.**).

Returning back to the now widely accepted hypothesis that DBA is caused by a defect in ribosome biogenesis, we speculated that DBA might not be limited to mutations in RP genes only, instead, non-ribosomal components vital for this process might be involved, too. In our selection of promising candidates, we took an advantage of the

[1] Gazda et al. selected *RPS24* as a candidate gene by a genomewide linkage screen, not according to its functional role within ribosome

[2] They decide to sequence RPL35a because of reports of deletions on chromosome 3 in a region harboring this gene.

initial experiment in which we identified RPS2 and RPS3 as proteins that were present in a complex with wild-type RPS19, but not the mutated variant. Both of them are methylated on arginines [28, 30], which is a common posttranslational modification of RNA-binding proteins. Methylation of both these proteins was shown to be important in the small ribosomal subunit assembly in eukaryotes: while RPS2 methylation is important for the cytosolic assembly of 80S ribosome (association of the small and large ribosomal subunits), methylation of RPS3 is necessary for the import of RPS3 to nucleolus and its building into the pre-ribosomal particle.

We identified a mutation in the gene encoding PRMT3, an enzyme dedicated to RPS2 methylation, in one patient from the Czech DBA registry with no known mutation in RP genes. Although we didn't confirm the causality of the mutation for DBA, we confirmed that the mutation decreased the PRMT3-RPS2 affinity and also the enzymatic activity of PRMT3 (published in Handrkova et al., 2010, **Appendix III.**). In the following sections, these three discoveries representing the core of my thesis will be discussed in detail.

3.2 Part 1.: Identification of novel DBA causal gene, RPS17

3.2.1 Background

RPS19 was the first and for several years the only known causal gene for Diamond-Blackfan anemia. The mutation of RPS19 was discovered by a chance, as it was the gene disrupted by a chromosomal translocation and mutations in this gene were later found in 1/4 of DBA cases. Although mutations in RPS19 segregated with the disease and were confirmed to be causal for the defect in erythroid maturation, there were no hints what might be the underlying defect in the other 3/4 of the patients. Identification of another gene encoding the ribosomal small subunit protein, RPS24 in DBA patients suggested that DBA might have a common underlying defect in translation.

There were several hypotheses (not mutually exclusive):

1. paucity of an indispensable component of ribosome, which would result in decreased translational capacity due to a lower count of ribosomes
2. competition between functional (wt) and non-functional RP, thus either limiting the assembly of ribosomal particles, or giving rise to a mixture of fully functional and "sick" ribosomes with lower effciency of translation
3. RP haploinsuffciency could negatively affect rRNA maturation, causing slower production of ribosomes (either all functional, or again a mixture).

Previous findings of our team revealed that the majority of DBA patients had a decreased level of translation regardless of the presence of an RPS19 gene mutation [144]. This supported the interpretation that DBA is a ribosomal disorder and other RP genes are involved in the DBA pathogenesis.

RPS19 and RPS24 are both localized close to each other on the surface of small subunit, in the head region involved in the eIF-2 binding [188]. We assumed that patient RPS19 variants that were capable of the incorporation into ribosome might finally abolish the recognition of a vital translation initiation factor, and thus decrease

the overall rate of translation. We therefore reasoned that other proteins that have been mapped to this region might be affected in other DBA patients.

3.2.2 Results

In the vicinity of RPS19 and RPS24 reside also RPS3a, RPS13, RPS16 and RPS17. All these four RPs were reported to interact with eIF-2, all have homologs in both lesser and higher eukayrotes and are all essential in yeast. Therefore, we decided to sequence their corresponding genes in the 24 DBA patients from the Czech DBA Registry.

We identified a heterozygous point mutation in the RPS17 gene in one patient, a 31 year-old male. In addition to anemia, the patient also has a flat thenar and facial dysmorphy, and his growth was markedly slower. He is now being successfully treated by a low dose of steroids.

The mutation encoded a substitution c.2T>G that abolished a natural translation initiation codon ATG. Although the mutated allele was transcribed, it wasn't translated into a protein, because the next downstream start codon located at position +158 is followed by a near stop codon.

RPS17 is essential for yeasts and mutations of its gene lead to *Minute* phenotype in Drosophila. Eukaryotic RPS17 has no homolog in prokaryotes [190], therefore, little is known about its specific role. A direct binding between RPS17 and eIF-2 has not been confirmed, but interestingly, RPS17 is phosphorylated by a kinase S6K1 [191] that participates in the regulation of translation of all RP mRNAs.

Identification of a third ribosomal protein gene as a causal gene for DBA further supported the hypothesis that this syndrome is a ribosomal defect. Our findings were published in 2007 in Human Mutation (see **Appendix I.**).

- **Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia.** Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D., *Hum Mutat.* 2007 Dec;28(12):1178-82.

3.3 Part 2.: Identification of novel mutations in RPL5 and RPL11

3.3.1 Background

Chromosomal end regions are more susceptible to breaks, deletions, or other aberrations. In 2008, the team of North American DBA registry reported in two DBA patients small deletions in chromosome 3q, leading to the loss of one alelle of the *RPS35a* gene. Further screening of additional 148 DBA patients in the registry revealed three additional cases of point mutations in this large ribosomal subunit gene [138].

The team further continued with sequencing other RP genes, including those mapped to the chromosome 1 (RPL5, RPL11, RPL22, RPS27, RPS8), because a pericentric inversion of this chromosome was previously reported in one DBA patient [192]. Later in the same year, they identified mutations in RPL5 and RPL11, as well as several rare sequence variants of unknown importance in several other RP genes [133].

In addition to their structural roles in ribosome, all three large ribosomal subunit proteins (RPL35a, RPL5, RPL11) are also required for the pre-rRNA maturation. It is worth mentioning that RPL5 and RPL11 participate in the ribosomal stress response, i.e. in the activation of p53 pathway in reaction to defect in ribosome biogenesis. Similar role plays also RPL23, therefore, we screened our cohort of patients in addition to the above mentioned RP genes also for mutations in its gene.

3.3.2 Results

We screened 28 patients from the Czech DBA Registry for mutations in genes encoding RPL5, RPL11 and RPL23 and found novel mutations in RPL5 and RPL11 genes in six, resp. two patients; no mutations in a RPL23 gene were found. We also observed that all eight Czech DBA patients with a mutation in either of these two genes displayed growth retardation and somatic anomalies, especially thumb anomalies. No such correlation has been reported for other DBA causal genes and this observation hasn't been explained yet.

The percentage of Czech DBA patients with mutation in either of these RP genes is markedly higher than the percentage reported before us in the American registry (namely 21.4% a 7.1% for RPL5 and RPL11, respectively, in our group, in contrast to 10% and 6.5%, reported by Gazda et al. [133]). However, this can be explained by small number of patients in our DBA registry.

In six out of eight cases, mutations were predicted to cause frameshift with a premature termination. The affected mRNAs are thought to be degraded and the mutations therefore cause deficiency of a large subunit protein. In one case, mutation created a novel exon/intron splice site, and thus production of non-functional mRNA. Only in one case was the mutation expressed on protein level, encoding Leu20His variation which very likely abrogates the N-terminal nuclear export signal.

RPL5 and RPL11 both interact with 5S rRNA and they assist in its processing and incorporation into the pre-ribosomal 90S particle, and both of them ale also thought to participate in the processing of the precursor of the pre-28S rRNA. Haploinsufficiency of either of these two large ribosomal subunit proteins result in a defect in large pre-ribosomal subunit maturation, and to the accumulation of unprocessed precursors and free RPs in nucleolus, and to the induction of ribosomal stress response.

In summary, these findings contributed to the recognition of common mechanisms of DBA pathogenesis and also brought valuable insight into genotype-phenotype or genotype-treatment correlations. Our results were published in 2009 in Human Mutation (see **Appendix II.**).

- **Identification of mutations in the ribosomal protein L5 (RPL5) and ribosomal protein L11 (RPL11) genes in Czech patients with Diamond-Blackfan anemia.** Cmejla R, Cmejlova J, Handrkova H, Petrak J, Petrtylova K, Mihal V, Stary J, Cerna Z, Jabali Y, Pospisilova D., *Hum Mutat.* 2009 Mar;30(3):321-7.

3.4 Part 3.: Identification of novel sequence variant in PRMT3

3.4.1 Background

All genes implicated so far in DBA encoded a protein component of ribosome, however, according to yeast or animal models, different defects in ribosome biogenesis pathway can be also caused by mutations affecting some of the hundreds of non-ribosomal proteins participating in this process. Because sequencing of all these non-RP genes would be task beyond the capacity of our lab, we started with a selection of the most promising candidate genes.

The decidion process was the following: (i) RPS19 gene was by far the most frequently mutated one, (ii) wt-RPS19 interacts with ribosomal protiens RPS2 and RPS3, but patient RPS19 variants don't (Petrak et Cmejla, unpublished results), (iii) RPS2 is posttranslationally methylated on arginines[3] and this modification is vital for the assembly of small and large ribosomal subunits together. DBA was so far linked to defects in maturation of both subunits. Therefore, we decided to test whether a defect at a later stage, namely joining of the two subunits together, can also lead to DBA.

Methylation of RPS2 is catalyzed by a dedicated, highly conserved protein arginine methyltransferase 3 (PRMT3). The deletion of gene encoding homologous methyltransferase Rmt3 in fission yeast was reported to cause a decrease in 40S and 80S pool and phenotype with a slower growth, PRMT3-null mice show retardation of prenatal development and smaller size of embryos. These characteristics were similar to the *Minute* phenotype of several RP gene knockouts [4]. We therefore reasoned that PRMT3 mutations could lead to a DBA-like phenotype in humans, and decided to sequence the PRMT3 gene in a cohort of patients in the Czech DBA registry.

[3] Later, RPS3 was reported to be methylated on arginines too, and this PTM is important for its import into the nucleolus and for the small ribosomal subunit assembly [30].

[4] the common features include: smaller growth and developmental anomalies, although the presentation differs from gene to gene and also varies among different organisms.

3.4.2 Results

We screened 30 patients in the Czech DBA registry for mutations in both PRMT3 and RPS2 genes and identified one variant encoding the Tyr87Cys substitution in PRMT3 in one patient, and the mutated alelle was expresed at the mRNA level. The RPS2 gene was unaffected in all DBA patients enrolled in the study.

The patient PRMT3 variant didn't belong to common polymorphisms listed for this gene in SNP databases, nor was identified in 58 healthy controls. However, screening of the family members of the proband excluded the causal link between this variant and DBA. Nevertheless, we decided to assess the impact of the Tyr87Cys substitution on PRMT3 function.

The Tyr87Cys substitution affects a highly conserved tyrosine in a substrate binding domain, that is localized on the surface and was predicted to be phosphorylated [193]. We decided to address (i) the impact of this substitution on the PRMT3-RPS2 affinity and on the enzymatic activity of PRMT3, and (ii) to model the impact of the putative phosphorylation of Tyr87 on PRMT3 properties.

To study the impact of the substitution, we constructed plasmids expressing wt or Tyr87Cys variant, and to mimic the role of hypothetical Tyr87 phosphorylation, we constucted also Tyr87Glu and Tyr87Phe variants, glutamate mimicking negatively charged phosphotyrosine and phenylalanine mimicking nonpolar nonphosphorylated tyrosine. All PRMT3 variants were expressed as C-terminal fusion proteins with 3xFLAG oligopeptide tag, and RPS2 was expressed with N-terminal myc tag. All PRMT3 variants and its substrate RPS2 were expressed as recombinant proteins in cervical tumor cell line HeLa. This cell line was chosen because it excels with rapid growth, is easily transfectable and is stable in culture for a high number of passages.

All four 3xFLAG-PRMT3 variants displayed cytosolic localization and none of them showed rapid degradation. Next, we compared the methyltransferase activity of all 3xFLAG-PRMT3 variants towards RPS2, and found that variants Tyr87Cys and Tyr87Glu had significantly ($p<0.01$) lower activity than wt and Tyr87Phe variants. This predicted that if the Tyr87 was phosphorylated, it would have negative effect on the PRMT3 activity.

Tyr87 resides in a substrate binding domain of PRMT3, therefore we expected that the weakened interaction with the substrate would be the most plausible explanation of the observed reduction of catalytic activity. We proved this to be the case and the decrease in enzymatic activity of Tyr87Cys and Tyr87Glu variants was in good agreement with the lowered affinity of these two proteins. However, we didn't confirm the predicted phosphorylation of Tyr87, alhough we observed previously reported phosphorylation on two N-terminal serines.

In conclusion, we reported in one DBA patient an unusual variant in the PRMT3 gene. Protein arginine methyltransferase encoded by this gene methylates RPS2 and was in yeast and mouse models shown to be important for the assembly of small and large ribosomal subunit together. However, the identified sequence variant wasn't causal for DBA and we don't have enough data to speculate whether it has at least any effect on the severity of the phenotype. Nevertheless, we predict that mutations in genes encoding other non-ribosomal proteins involved in ribosome biogenesis can be found in some DBA patients, either as sole causal genes, or as factors modulating the severity of phenotype. For details, see our publication from Biochim Biophys Acta from 2011 (**Appendix III.**).

- **Tyrosine 87 is vital for the activity of human protein arginine methyltransferase 3 (PRMT3).** Handrkova H, Petrak J, Halada P, Pospisilova D, Cmejla R., *Biochim Biophys Acta.* 2011 Feb;1814(2):277-82.

4. Summary of results

The goals of this work were:

- select probable candidates for novel DBA causal genes
- study the role of such candidates in molecular pathogenesis of DBA
- screen the patients from Czech DBA registry for mutations in known causal genes

Our achievements:

- we were first to report a mutation in the *RPS17* gene in one DBA patient from the Czech DBA registry; this was at that time the third known DBA causal gene
- we also reported mutations in the *RPL5* and *RPL11* genes in Czech DBA patients and observed a higher incidence of thumb defects and other somatic anomalies in the affected individuals
- no mutations were found in other 17 screened *RP* genes in Czech DBA patients, nonetheless, they might be causative in other DBA patients
- since all known mutations affect ribosome biogenesis and function, we further focused on a possible involvement of non-ribosomal proteins in the molecular pathogenesis of DBA
- we identified a rare variant of protein arginine methyltransferase PRMT3 encoding the Tyr87Cys substitution
- this enzyme catalyze modificication of RPS2 necessary for small and large ribosomal subunit interaction; however, although the patient variant of the PRMT3 protein was not fully functional, its causative role in DBA was not confirmed

In conclusion, our data confirm the hypothesis that DBA does not stem from a loss of a specific function of several ribosomal proteins but rather from a more general defect in ribosome biogenesis. Ribosomal stress and p53 activation seem to be crucial for the pathogenesis of DBA. Therefore, we incline to the hypothesis that non-RP genes might also be causal for DBA.

5. Discussion

This work focuses on the study of molecular mechanisms of a rare congenital aplastic anemia, Diamond-Blackfan anemia, and on the identification of novel causal genes. Our main motivation was providing better care for DBA patients, most importantly, contributing to the development of more accurate diagnostics and genetic counseling for the affected families, and to design more specific and more efficient therapy in order to improve the quality of life of the affected individuals.

At the beginning of this project, *RPS19* was the only known DBA causal gene [4]. The product of this gene was identified as a component of the small ribosomal subunit, ribosomal protein (RP) S19. This was the first report of an RP gene mutation in human disease and the association of a ribosomal component with anemia was surprising at first. However, screening for mutation in *RPS19* in other DBA patients confirmed that this gene was mutated in 1/4 of all DBA patients [132, 133, 118].

RPS19 is an essential ribosomal protein and as such, it is ubiquitously expressed in all tissues. The only known function of RPS19 was at that time its structural role within ribosome. Since the decreased number and/or reduced activity of ribosomes had at a first glance no connection to anemias, RPS19 was originally expected to play an erythroid-specific extraribosomal role. However, this hypothesis has never been confirmed and the attention turned to a function of RPS19 within ribosome. In support for this possibility, findings of our lab indicated that irrespective of the presence or nature of the *RPS19* mutation, cells from DBA patients displayed decreased translational rate [144].

Reduced capacity of protein synthesis would be expected to affect all cells in the body, although to a different degree, since the translational requirements differ

markedly among tissues. Since the erythroid lineage has extreme demands for rapid protein synthesis, this argument seemed sufficient to explain why is the DBA phenotype restricted almost exclusively to erythroid progenitors, while other blood lineages and other adult tissues are unaffected [194]. The hypothesis that DBA is a ribosomal syndrome was further supported by the report of a second DBA causal gene, *RPS24* [134]. However, in contrast to *RPS19*, this gene was affected only in a handful (3/214) of DBA patients.

Reports indicating the involvement of RPS19 and RPS24 in the binding of an essential translation initiation factor eIF-2 indicated [188] that inefficient translation initiation might explained the reduced translational capacity in cells from DBA patients. This feature was observed also in cells negative for *RPS19* mutations. Therefore we reasoned that ribosomal proteins physically (residing close to RPS19 within ribosome) and functionally (probably also interacting with eIF-2) associated with RPS19 might be affected in some other DBA patients. We performed screening of genes encoding such ribosomal proteins (namely, genes encoding RPS3a, RPS13, RPS16, RPS17 and RPS24) and we identified a mutation in *RPS17* in one proband [135].

Soon afterwards, Farrar et al. reported a mutation in a fourth DBA causal gene, affecting a large ribosomal subunit protein RPL35a [138]. This finding was wery interesting, since the large subunit does not interact with eIF-2 and joins the small subunit later after the translation initiation complex is assembled. This finding thus disfavored our original assumption of a reduced effectivity of translation initiation. However, it supported the hypothesis that DBA is caused by a ribosomal defect.

In order to be able to predict other promising candidates for causal genes, it was necessary to look for another characteristics that would be common for all of these four RP genes. All three previously identified DBA causal genes (RPS19, RPS24 and RPS17) were reported to participate in different steps of the maturation of ribosomal RNA and thus ribosome biogenesis [195, 169]. While such role is common for all small ribosomal subunit proteins, only some of the large subunit proteins assist in the maturation of large subunit RNA precursors. Farrar et al. also brought evidence that haploinsufficiency of RPL35A resulted in processing defects of large subunit rRNA precursors [138].

The common outcome of these defects was aberrant biogenesis of ribosomal subunits and decreased pool of mature ribosomes. The resulting lower rate of protein synthesis and slower growth and cell cycle progression delay were expected to be tolerated by most cells, except for those with extreme demands for a rapid production of large quantities of proteins, such as in maturating erythrocytes [194, 75]. However, this hypothesis didn't predict which other RP genes might be mutated in the remaining fraction of DBA patients (almost 3/4), because almost all RPs are essential for the function of ribosome, and the majority of them is also involved in ribosome biogenesis.

Given that human ribosomes contain 80 ribosomal proteins and some of them have multiple isoforms that are expressed only in some tissues [20, 17, 22], it was necessary to limit screening only to the most probable candidates. While *RPS19* is affected in 1/4 of DBA patients, mutations in the remaining three DBA causal genes are extremely scarce.

DBA is a very rare syndrome and because the Czech DBA registry contained at that time only approximately two dozens of patients, there was a considerable drawback in searching for other causative genes. The key to finding other DBA causal genes seemed to be the recognition of RP genes more prone to mutations. Examples of such DNA regions with higher susceptibility to deletions are for example the ends of chromosomes. Gazda et al. performed mutation screening of ribosomal protein genes residing in such locations, and identified mutations in *RPL5*, *RPL11* and *RPS7* [133]. We complemented their findings by providing the report of the incidence of mutations in these genes in the Czech DBA patients and, notably, we described a correlation between these causal genes and distinct somatic anomalies. All these three ribosomal proteins participate in ribosomal stress response, and similar role has also been confirmed for another large ribosomal subunit protein, RPL23. Therefore, we also screened our cohort of DBA patients for mutations in a *RPL23* gene, but with negative results [137].

To date, 9 RP genes were reported to have causal role in DBA, some of them only in a single DBA patient or one family, and together, mutations in RP genes have been found in over a half of DBA cases [157, 196]. Although rare sequence variants of unknown significance were also reported in several other genes, RP genes are still

the only genes with the confirmed causative role for the disease. None of these 9 RPs has an erythroid-specific extraribosomal role, but all of them are essential for the pre-rRNA processing and ribosome assembly [86]. Since ribosome biogenesis requires in addition to RPs also hundreds of non-ribosomal proteins (non-RPs), we speculated that mutations affecting these proteins might also cause ribosomal assembly defects and thus lead to a similar phenotype.

Since screening for mutations in all of them would be beyond the capacity of our lab, it was necessary to select for sequencing only some of them. Our major criteria were that such non-RP gene should be indispensable for ribosome biogenesis and its deletion should therefore result in similar phenotype as heterozygosity for RP genes. The participation in ribosome biogenesis should also be the major cellular role of such a protein; ideally, it shouldn't be involved in the modification or processing of any other RNA or protein.

Our approach further took advantage of our preliminary co-immunoprecipitation experiments, showing that patients' variants of RPS19 fail to interact with the small ribosomal subunit proteins RPS2 and RPS3 (Petrak et Cmejla, unpublished data). Although we didn't find any mutation in those two genes in the Czech cohort of DBA patients, we continued in searching for other proteins that might influence the interaction of RPS2 and RPS3 with RPS19, and/or their role in ribosome biogenesis and function.

Bachand et al. reported that RPS2 was methylated on multiple arginines by a protein arginine methyltransferase 3 (PRMT3) [197]. This modification occurs during ribosome biogenesis and is important in the joining of small and large ribosomal subunits to functional 80S ribosome. Since the deletion of PRMT3 gene homologs in model organisms negatively influenced their growth and altered the ribosomal profile [197, 33, 32], we reasoned that PRMT3 might be a promising candidate for DBA causal gene. Interestingly, we identified a rare sequence variant of the *PRMT3* gene in one DBA patient with yet unknown causal gene. We showed that the Tyr87Cys-PRMT3 variant encoded by the mutated alelle had lower enzymatic activity as a result of diminished affinity to RPS2. However, since the mutation didn't segregate with the disease in the family, it couldn't be regarded as causative for DBA [198].

Recently, methylation of RPS3 has also been reported to be important in ribosome biogenesis [30]. This modification is catalyzed by a different enzyme, PRMT1, and arginine methylation of RPS3 is indispensable for the transport of this ribosomal protein to nucleolus. PRMT1 is the predominant cellular methyltransferase responsible for at least 85% of all arginine methylations in human cells [199]. PRMT1 hasn't yet been associated with any human disease. *PRMT1*-null murine cells displayed signs of spontaneous DNA damage, delayed progression of cell cycle and checkpoint defects [200]. Mice carrying homozygous knockout of the *PRMT1* gene die early in gestation, heterozygous animals appeared normal [200]. Because the spectrum of PRMT1 roles is very broad and the PRMT1+/- mice show no resemblance to animals carrying RP gene mutations, we decided not to sequence the PRMT1 gene in DBA patients.

PRMT3 is not the only protein implicated in ribosome maturation that has been studied in respect to human diseases. Inefficient or aberrant RNA modifications have been reported in several other rare inherited syndromes, together classified as ribosomopathies [86, 201]. This term may be misleading, since the products of affected genes do not participate in ribosome biogenesis only. The clinical manifestation of these syndromes is very heterogenous, based on the diversity of their respective causal genes. Except for DBA and 5q- syndrome[1], the underlying mutations affect also other classes of RNAs, such as telomerase elements.

Despite the substantial heterogeneity, ribosomopathies share some common characteristics, such as growth retardation, skeletal anomalies and bone marrow aplasia [204, 109, 205]. This suggest that their mechanism of pathogenesis might be (to a certain degree) similar, too. Since the aberrant rRNA maturation and compromised ribosome biogenesis is the only defect that is common to all ribosomopathies, it seems reasonable to take it as a starting point for our further hypotheses.

In general, a block in ribosomal subunit assembly leads to the accumulation of defective subunit precursors and free RPs in nucleolus. One result is the above mentioned

[1] DBA and 5q-, which are both caused by haploinsufficiency in an RP gene that affects only *ribosomal* RNA maturation [202, 203]. While DBA is a congenital syndrome, 5q- is an acquired disorder and only hematopoietic tissue is affected, although causal genes are in both disorders ribosomal protein genes.

decreased pool of mature ribosomes, and the second, probably more serious, is the induction of the p53 stress pathway by free RPs that accumulate in nucleolus [69, 70]. This so called *ribosomal stress response* is rather complex and the roles of distinct RPs in this process vary [73, 206]. The dominating mechanism is inhibition of the negative regulator of p53, protein Mdm2, by several free ribosomal proteins of both subunits, including RPL5, RPL11 and several other DBA causal proteins [72]. Upon activation, p53 influences various cellular pathways, most importantly causes cell cycle delay and increased susceptibility to apoptosis [82, 207, 71].

It should be emphasised that a block in *some step* of ribosome biogenesis, and thus consequent ribosomal stress and reduced pool of mature ribosomes per cell, is common for *all* ribosomopathies. These outcomes would be expected to affect all cells, nevertheless, the clinical manifestation of ribosomopathies is very specific and seem to affect only some tissues. Which of these consequences (cellular stress or limited translation capacity) is more important in molecular pathogenesis of these syndromes, and how can we explain the observed tissue-specific defects?

To answer these questions, it may be useful to compare the phenotype of the following groups of disorders: syndromes caused by purely translation defects (i.e. syndromes caused by mutations in translation initiation factors or in tRNA-amino acid ligases [93]), and diseases associated with delayed cell cycle progression and/or increased apoptosis (due to various cellular stresses, such as DNA damage or cell cycle checkpoint defects).

Disorders caused by mutations affecting (cytosolic) translation usually manifest with various neural defects. For example, mutations in genes encoding factors essential for translation initiation (eIF2B complex) and in genes encoding aminoacyl-tRNA synthetases (Gly-tRNA and Tyr-tRNA synthetases) have been associated with syndromes of vanishing white matter, hypo- or demyelination, childhood ataxia and other neuropathies.

In contrast, the activation of p53 and cell cycle defects are a hallmark of several other inherited anemias that strongly resemble DBA, not only by hematologic findings, but also by somatic anomalies and/or growth and developmental defects. The best known example is Fanconi anemia, which is caused by mutations compromising the

crosslinked DNA strand repair [111]. A handful of cases of rare inborn anemias have also been associated with mutations in the gene for geminin [115] (another protein dedicated to DNA repair), or in genes for codanin [112, 113] and checkpoint kinase 1 [114] (both monitoring the cell cycle progression). The activation of p53 and subsequent cell cycle arrest and/or the induction of apoptosis can also be a result of various types of biological, chemical or physical stress, such as radioactive radiation or chemotherapy. However, given that these conditions should (theoretically) affect all tissues, why are the defects restricted almost exclusively to hematopoietic, especially erythropoietic cells?

Although the exact reasons are not fully known yet, the probable explanation is that hematopoietic progenitors are (i) hypersensitive to various types of stress [204, 109, 205], and (ii) they are especially prone to apoptosis [83, 208]. A lower treshold of cellular stress required for p53 activation is observed in all rapidly proliferating cells, such as hematopoietic progenitors, gastrointestinal epithelia, hair follicles, embryonal tissues, etc. However, while in some cell type leads the p53 activation to the induction of apoptosis, in others, p53 acts as a survival factor by promoting cell cycle arrest. The apoptotic response seems to serve as a protective mechanism that prevents multiplication of the population of potentially mutated cells, and is further restricted to cells that are not vital for the integrity of the tissue, i.e., cells that do not have connective functions, and that can be replenished from pluripotent stem cells. On the other hand, the p53-induced cell cycle arrest is typical for connective tissue and epithelial cells, and this evasion from apoptosis protect the integrity of these tissues (reviewed in [83]).

In conclusion, the activation of p53 appears to be more important for the phenotype of DBA and 5q- syndrome than the reduced ribosomal pool and thus decreased translational capacity [209, 210, 145]. It is not clear yet whether is the so-called ribosomal stress triggered only in erythroid progenitors, or in other tissues as well.

Hematopoietic tissue is indeed more sensitive to cellular stress, and it might be that within this tissue, *erythroid lineage* is especially sensitive to ribosomal stress since these cells have extreme requirements for ribosome biogenesis. In addition, maturating erythroid progenitors might be affected by other types of cellular stress as well, due to

the processing of toxic heme and iron metabolites. Another explanation for the DBA phenotype would be that ribosomal stress response is triggered in all tissues, but cells have different treshold levels at which they react on stress, and the outcome of p53 activation is specific, too.

Mechanisms involving the p53 response have also been suggested for two putative DBA causal proteins, namely FLVCR1 and Pim-1. FLVCR1 is a heme exporter which is a causal gene in feline erythroleukemia, and the likely mechanism of pathogenesis includes the accumulation of excess heme in cells [180]. Free heme is highly toxic and it is therefore believed to induce p53-mediated apoptosis in affected cells [179]. Pim-1 is a serine/threonine kinase that plays an important role in cell cycle control [211]. Lower Pim-1 levels are associated with cell cycle delay and although a homozygous deletion of this gene in mouse is asymptomatic, simultaneous knockout of its two close orthologs, Pim-2 and Pim-3, results in smaller phenotype and anemia [185]. In both cases, rare sequence variants have been reported in several DBA patients, none being a causal one.

More studies are needed to clarify this issue, since most of the available data regarding the tissue-specific response to stres were obtained from γ-irradiation experiments [116, 212]. Nevertheless, using this approach, it has been demonstrated that although a *permanent* suppression of p53 inevitably leads to a rapid development of tumors in irradiated animals, a *temporal* inhibition of p53 prevented the development of bone marrow aplasia following the irradiation [83]. Most recently, it has been suggested that a specific inhibitor of p53, pifithrin, might be advantageous for DBA patients [145]. The use of p53 suppressing drugs in DBA treatment is not entirely new. Glucocorticoids, which are at present the therapy of choice for DBA treatment [157], improve the phenotype by the stimulation of stress erythropoiesis, i.e. via overcoming the p53-induced apoptosis and promoting cell differentiation programme [158, 110]. A similar mechanism of action have androgens that were used for the treatment of some cases of Fanconi anemia [213].

The development of novel drugs is motivated mainly by debilitating side effects of currently used preparations and/or the unresponsiveness of some patients. Of such alternative therapies, leucine is regarded as a very promising remedy. The beneficial

effect of this essential amino acid on translation of DBA-derived cells was first reported by our team [144]. After its administration, one patient from Czech DBA registry entered remission [162]. As a natural amino acid, leucine doesn't cause any adverse side effects at used doses, and is now being clinically tested on a larger cohort of DBA patients. Leucine positively regulates a central controller of cell growth, mTOR complex 1, and thus stimulates ribosome biogenesis and a number of other anabolic pathways [161]. Via mTOR, it also promotes cel cycle progression and negatively regulates p53, which seems in the light of current understanding a very important, if not the major mechanism of its action.

Concluding remarks and future prospectives

In conclusion, opinions on DBA pathogenesis have undergone dramatic changes over the last five years (2006–2011). From a putative erythroid-specific role of a handful of ribosomal proteins, the general view shifted to a ribosomal defect and therefore compromised translational capacity. Most recently, the DBA phenotype is viewed as a result of the ribosomal stress response. At each time, each of these hypothesis seemed to be very strongly supported by experimental data and I believe that to a certain degree, each of them still seems to have some points over the others and shouldn't be entirely discarded.

To study such a complex disease, it is essential to be open to new paradigms instead of focusing blindly only on one explanation of an observation. What lessons should we learn from the previous hypotheses? Although ribosomal proteins are indeed important for all human tissues, hematopoietic and especially erythropoietic precursors seem to be especially sensitive to their haploinsufficiency [194]. Since no erythroid-specific role of RPs has been found, the opinion on DBA evolved into the hypothesis assuming an exceptional importance of ribosome biogenesis in erythroid lineage. This seemed to be satisfactorily explained by the high translational demands of this lineage, but other anomalies, such as thumb defects or apparently asymptomatic phenotype of other rapidly growing cells, couldn't be explained by such hypothesis.

In the view of current understanding, it is tempting to consider re-classifying DBA as a syndrome resulting from the p53 activation, since it seems to be closer to defects

such as Fanconi anemia than to ribosomopathies as such [145, 201]. The strongest argumentation is that this hypothesis explains the additional somatic anomalies: activation of p53 in embryogenesis would cause cell cycle blocks or apoptosis, and thus cause abnormal development. Somatic anomalies are observed not only in DBA, but also in several other bone marrow disorders associated with the activation of p53. Apoptosis restricted (predominantly) to hematopoietic tissue was observed upon such variable stimuli as DNA damage (either as a result of irradiation, or caused by defects in the DNA repair in Fanconi anemia or in other rare anemias), cytotoxic stress (accumulation of excess heme in cells carrying FLVCR1 mutations), cell cycle arrest (chemotherapy, mutations in cell cycle regulating genes) and last but not least, ribosomal stress response (mutations in RP genes or other mutations affecting ribosome biogenesis).

Although the current hypothesis that emphasize the role of p53 activation is in good agreement with the clinical manifestation of DBA, more experiments are necessary to either confirm or reject this causative connection between ribosomal stress and the molecular pathogenesis of DBA. In the nearest future, we expect that the focus in DBA research will turn to the evaluation of the role of p53 in DBA pathogenesis. This would also open up the way for the design of more efficient therapeutic approaches with lesser risks and fewer adverse side effects. Theoretically, these modalities might be based on a short-term suppression of p53 (for example, by pulse doses of pifithrin), or on specific inhibition of p53 in hematopoietic tissues.

There are still many other questions regarding the molecular pathogenesis of DBA that remain to be resolved. For example, what causes the phenotypic heterogeneity of among DBA patients? Why do they differ in the response to therapy? And most importantly, what mechanisms are responsible for the remission and for relapses? We succeeded in the identification of causal genes in another quarter of DBA patients in the Czech DBA registry, and we also reported some cases of genotype-phenotype correlations. Together, observations of our team presented in this thesis support the view of DBA as a ribosomal syndrome, but this should not be taken only as a conclusion, but rather as a new beginning for further studies.

6. Appendices

6.1 Appendix I.

Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D., *Hum Mutat.* 2007;28(12):1178–82.

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RAPID COMMUNICATION

Ribosomal Protein S17 Gene (RPS17) Is Mutated in Diamond-Blackfan Anemia

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Diamond-Blackfan anemia (DBA) is a congenital erythroid aplasia characterized as a normochromic macrocytic anemia with a selective deficiency in red blood cell precursors in otherwise normocellular bone marrow. In 40% of DBA patients, various physical anomalies are also present. Currently two genes are associated with the DBA phenotype—the ribosomal protein (RP) S19 mutated in 25% of DBA patients and RPS24 mutated in ~1.4% of DBA patients. Here we report the identification of a mutation in yet another ribosomal protein, RPS17. The mutation affects the translation initiation start codon, changing T to G (c.2T>G), thus eliminating the natural start of RPS17 protein biosynthesis. RNA analysis revealed that the mutated allele was expressed, and the next downstream start codon located at position +158 should give rise to a short peptide of only four amino acids (Met-Ser-Arg-Ile). The mutation arose de novo, since all healthy family members carry the wild-type alleles. The identification of a mutation in the third RP of the small ribosomal subunit in DBA patients further supports the theory that impaired translation may be the main cause of DBA pathogenesis. Hum Mutat 28(12), 1178–1182, 2007. **© 2007 Wiley-Liss, Inc.**

KEY WORDS: ribosomal protein S17; RPS17; small ribosomal subunit; translation; Diamond-Blackfan anemia

INTRODUCTION

Diamond-Blackfan anemia (DBA; MIM# 205900) is a congenital erythroid aplasia that usually manifests during the first year of life. The disease affects five to seven children per million live births; most cases (80%) are sporadic, with both sexes affected equally. The hallmarks of the disease are normochromic macrocytic anemia; normocellular bone marrow with a selective deficiency in red blood cell precursors; and profound reticulocytopenia [Freedman, 2000; Gazda and Sieff, 2006]. Serum erythropoietin level is usually increased. Erythrocytes often express several fetal markers, and display an increased adenosine deaminase level [Glader et al., 1983; Glader and Backer, 1988]. Besides altered hematological parameters, about 40% of patients suffer from various congenital anomalies, including craniofacial dysmorphism, thumb and neck anomalies, congenital heart defects (mainly ventricular and septal defects), urogenital malformations, and mental retardation [Willig et al., 1999a]. Prenatal or postnatal growth retardation independent of steroid therapy is also often present.

Blood transfusion is the first step in DBA management, followed by corticosteroid treatment, to which approximately 80% of patients respond. About a third of these patients are able to achieve a tolerable dose while remaining in remission. Steroid-resistant patients enter a regular program of red blood cell transfusion [Freedman, 2000].

Regarding the genetic background, the ribosomal protein S19 (RPS19) has been for several years the only gene associated with DBA pathogenesis, and is found to be heterozygously mutated in 25% of DBA patients [Draptchinskaia et al., 1999; Willig et al., 1999b]. RPS19 is a component of the small ribosomal subunit (40S subunit), and in addition to being a building block of ribosomes, it seems to play a specific role during ribosome biogenesis [Leger-Silvestre et al., 2005; Choesmel et al., 2007; Flygare et al., 2007] and in the initiation of translation [Bommer et al., 1988, 1991; Lutsch et al., 1990].

In our recent work we demonstrated that the level of translation was lowered in DBA patients irrespective of a mutation in the RPS19 gene [Cmejlova et al., 2006a]. These results indicated that inefficient translation could be the general molecular mechanism of DBA. Consequently, mutations in other proteins involved in the process of translation besides RPS19 would be anticipated in the remaining 75% of DBA patients. Indeed, mutations in RPS24 were identified in three out of 215 (~1.4%) of DBA patients [Gazda et al., 2006], supporting the hypothesis of impaired translation in DBA.

In our study we focused on RPS17 (MIM# 180472). RPS17 lies in a distinct region of the "head" of the small ribosomal subunit near one end of the eukaryotic initiation factor eIF-2 binding site, in close vicinity to three out of five RPs (RPS13, RPS16, and RPS19) that are involved in eIF-2 binding [Bommer et al., 1988]. Though RPS17 has not been confirmed to be directly involved in this process, we reasoned that its unique position next to the three eIF-2-binding RPs made it a good candidate to be a third

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DBA-associated gene. Here we report the identification of a mutation in the RPS17 gene in a DBA patient.

MATERIALS AND METHODS

Mutation Nomenclature

The RPS17 gene is located on human chromosome 15. To design PCR primers and to show the structure of the RPS17 gene, *Homo sapiens* chromosome 15 reference assembly NC_000015.8 was used. For RNA analysis, the RefSeq mRNA sequence NM_001021.3 was used. The A of the ATG translation initiation start site was considered as +1.

DBA Patients

Mutational analysis of the RPS17 gene was performed in 24 DBA patients from the Czech National DBA Registry. In six of them, a heterozygous mutation in the RPS19 gene has been previously identified [Cmejla et al., 2000; Cmejlova et al., 2006b].

DNA Isolation and Analyses

After informed consent, buccal swab, nails, or nucleated blood cells were used as the source of genomic DNA. Peripheral blood mononuclear cells (PBMNC) from DBA patients and healthy controls (n = 71) were isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) gradient centrifugation. Genomic DNA was isolated from all tissues by a salting-out method [Miller et al., 1988]. Individual RPS17 exons were PCR amplified using the following sets of primers: 1F (5′-AGCGGGGAGCGGAAAGGG)+2R (5′-GGGCGGCAGA GCACACAAAC) for exons 1 and 2; 3F (5′-TGAATAGAGAGT GAGAAACCAAGG)+4R (5′-AGAAGCAGCCAAGAGAGAAA AG) for exons 3 and 4; and 5F (5′-CTGGAAAAGTGGGAGGCA TTG)+5R (5′-CAGGGAAGCAGGGTGGAGC) for exon 5. Corresponding PCR products were isolated from an agarose gel by a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and DNA was used for direct sequencing on an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

To confirm the mutation independently, purified PCR products comprising exon 1 were also subjected to restriction analysis using NlaIII restrictase (Fermentas, Burlington, Ontario, Canada). Due to the presence of one NlaIII recognition site in the amplified wild-type sequence, the PCR product (729 bp) is cleaved producing two fragments, 222 bp and 507 bp long. Since the mutation eliminates the NlaIII restriction site, the PCR product from the mutated allele cannot be cut by the NlaIII restrictase.

Expression Analysis

PBMNC were lysed in 0.5 ml RNA-Bee (TEL-TEST, Friendswood, TX), and total RNA was isolated according to the manufacturer's manual. To avoid possible DNA contamination, samples were treated with DNase I (Ambion, Austin, TX) for 15 minutes at 37°C, and the enzyme was then inactivated at 75°C for 5 minutes. Isolated RNA was used for reverse transcription using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) primer; the control reaction without reverse transcriptase was run in parallel. Forward 5′-CTC TTTTACCAAGGACCCGCC and reverse 5′-AGGTTGGACAG ACTGCCGAAG primers were used to amplify the mutated region. The reverse primer was used to sequence the PCR product.

RESULTS

DNA Analysis of the RPS17 Gene

The gene for RPS17 is located on chromosome 15, and consists of five exons spanning 3,705 bp (Fig. 1A). All exons with flanking exon/intron boundaries have been PCR-amplified and sequenced in 24 patients with confirmed DBA diagnosis. In one patient (Patient CZ02), a heterozygous mutation changing T to G (c.2T>G) in the translation initiation start codon has been identified (Fig. 1A). This mutation leads to the elimination of the natural start site for RPS17 protein biosynthesis.

To test the nature of the mutation, a study in family members and tissue analyses in the proband were performed. For this purpose, the NlaIII restriction endonuclease was used, since the c.2T>G mutation abolishes the NlaIII recognition site CATG that overlaps the translation initiation start codon. Figure 1B shows that only the proband exhibited an abnormal NlaIII restriction pattern in comparison with his apparently healthy brother and parents, indicating that the mutation arose de novo, and is likely associated with the disease. The presence of the mutation has also been confirmed in all tissues tested (PBMNC, buccal swab, and nails; Fig. 1C). All findings were also verified by sequencing.

To exclude the possibility of a polymorphism, 71 healthy controls were also screened for the mutation. As would be expected for this type of mutation, no alterations were identified within the control group. A search in the Single Nucleotide Polymorphism database (dbSNP; www.ncbi.nlm.nih.gov/SNP) also did not show the existence of a SNP at this position.

Expression Analysis

The c.2T>G mutation affects the translation initiation start codon, preventing the RPS17 protein from being translated from its natural start (Fig. 2). The next downstream initiation start codon is located at position +158, but if used for translation initiation, a peptide of only four amino acids, Met-Ser-Arg-Ile, would be produced. We therefore asked whether the mutation is also expressed at the mRNA level. As is shown in Figure 2, the mutated allele is expressed; however, mutated mRNA seems to be less stable than wild-type mRNA, as estimated from the T/G peak height proportions.

Patient Characteristics

Patient CZ02, now a 31-year-old man, was born to healthy nonconsanguineous parents from the second uncomplicated pregnancy (birth weight 2,600 g, height 48 cm), and he has one healthy brother. The diagnosis of DBA with typical macrocytic anemia was established after bone marrow examination at the age of 6 months (2.8% of erythroblasts in bone marrow). Another typical DBA feature, an increased activity of erythrocyte adenosine deaminase (eADA), was also observed in the patient, contrary to his parents and brother who have normal eADA levels.

The patient has a flat thenar and facial dysmorphy; his growth curve was under the third percentile (short stature). During the first year of life the patient received several transfusions. Steroid therapy was initiated at the age of 3 years, and the patient is still steroid-dependent, being treated by a low dose of steroids on alternate days. Due to moderate iron overload, low-dose iron chelating therapy was started at the age of 8 years.

DISCUSSION

For nearly 8 years, RPS19 has been the only gene associated with DBA pathogenesis in 25% of patients [Draptchinskaia et al., 1999; Willig et al., 1999b]. Recently, the gene for RPS24 has been identified to be mutated in ~1.4% of DBA patients [Gazda et al., 2006]. Here we report the mutation in yet another RP gene, RPS17.

Human Mutation DOI 10.1002/humu

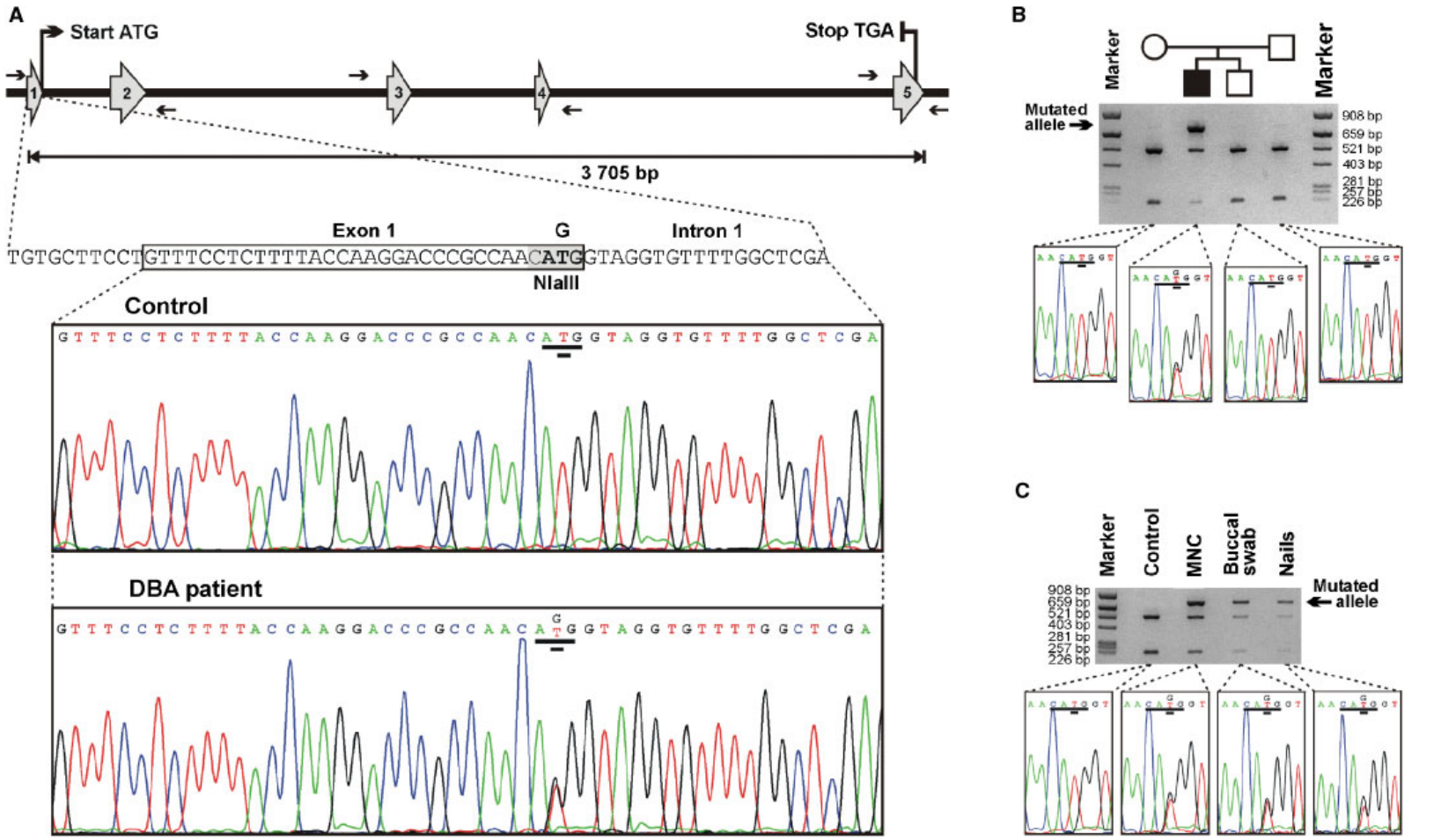

FIGURE 1. DNA analysis of the RPS17 gene. **A:** The human RPS17 gene is 3,705 nt long, and consists of five exons. Arrows indicate PCR primers used for amplification and sequencing of individual exons. The detail of exon 1/intron 1 with corresponding chromatograms is depicted in the middle. Exon 1 (boxed) is 32 nt long, and the last three nt represent the translation initiation start codon ATG of the RPS17 protein (in bold). The chromatogram shows a heterozygous mutation T to G (c.2T >G) in the start codon (underlined) that was identified in a DBA patient. This mutation abolishes the CATG recognition site for the NlaIII restrictase (shaded). The chromatogram for the wild-type sequence is shown for comparison. **B:** Sequencing and restriction analysis of the RPS17 gene exon 1 in family members. The PCR product (729 bp) comprising the wild-type exon 1 of the RPS17 gene contains only one NlaIII restriction site (CATG; underlined in the chromatograms) that overlaps the translation initiation start codon. After restriction with the NlaIII restrictase, the PCR product is cut, producing two fragments, 222 bp and 507 bp long. On the contrary, the mutation T to G (c.2T >G) in the start codon eliminates the NlaIII restriction site, preventing the PCR product of the mutated allele to be cleaved. Sequencing and NlaIII restriction analysis confirmed a heterozygous mutation T to G (c.2T >G) in the start codon only in the proband; both healthy parents and brother carry the wild-type alleles. **C:** The DBA patient carries the mutation in the RPS17 gene in various tissues. Genomic DNA was isolated from PBMNC, buccal swab, and nails. PCR products comprising exon 1 of the RPS17 gene were sequenced or restricted by the NlaIII restrictase in the same way as in B. A heterozygous mutation T to G (c.2T >G) in the translation initiation start codon was identified in all tissues tested. In the chromatograms, the NlaIII restriction site is underlined. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

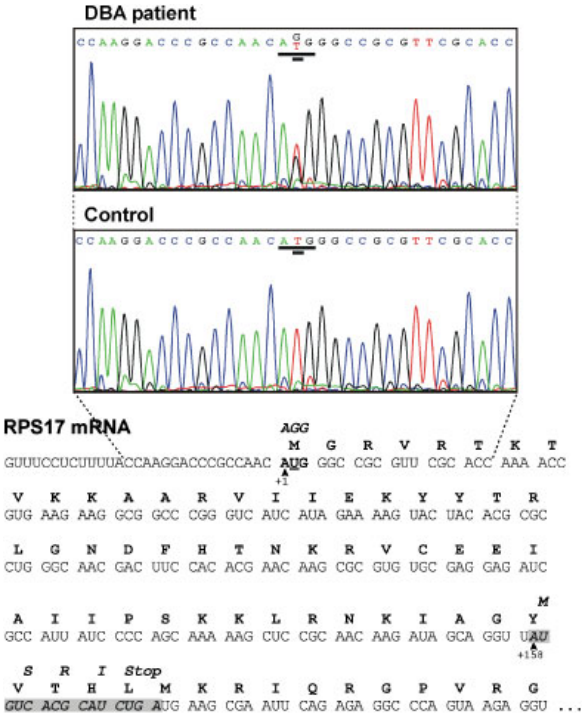

FIGURE 2. **Mutated allele is expressed at the mRNA level. Total RNA isolated from PBMNC was used as a template for RT-PCR. Sequencing of the PCR product comprising the translation initiation start codon for the RPS17 protein revealed that the mutated allele was expressed in the DBA patient, though to a lesser extent than the wild-type allele. The c.2T>G mutation eliminates the natural translation initiation start codon; the next start codon is located at position +158, presumably giving rise to a short peptide of only four amino acids Met-Ser-Arg-Ile (shaded). The chromatogram for the wild-type sequence is shown for comparison. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

The gene for RPS17 is located on chromosome 15, and consists of five exons. RPS17 is a basic protein (pI 9.85) 135 amino acids long with a calculated molecular weight of 15.5 kDa. In yeast, RPS17 is an essential RP, and belongs among six RPs unique to eukaryotes [Dresios et al., 2006].

The c.2T>G mutation found in the DBA patient obliterates the natural translation initiation start codon. Since mutated mRNA is transcribed, the next downstream start codon can theoretically initiate proteosynthesis of a short peptide of only four amino acids. In this regard, RPS17 expression from the mutated allele should be completely lost, and the DBA patient should be haploinsufficient in the production of the RPS17 protein. This is in a good agreement with our previous finding that the level of basal translation in this DBA patient was 39 to 47% of control values (Patient CZ02 in Cmejlova et al. [2006a]), indicating that this supposed haploinsufficiency in the RPS17 production can be indeed the cause of the half translational rate in this patient. Moreover, screening of the family members showed that the mutation in the proband arose de novo, and is present in all tissues tested, indicating that the mutational event occurred at the germline level during gametogenesis. The dbSNP database and healthy controls were also screened to exclude natural sequence variation at this site. As expected for a sequence alteration eliminating the translation initiation start codon, no alterations were found in 71 healthy controls, and there are also no data related to this position in the dbSNP database. Altogether, these findings indicate that the c.2T>G mutation in the RPS17 gene is connected with the DBA pathogenesis in the proband, and RPS17 should be considered a novel DBA disease gene.

What do RPS19, RPS24, and RPS17 have in common? All three RPs function as structural components of the small ribosomal subunit, and all of them have been studied for their ability to take part in the initiation of translation by binding to eukaryotic initiation factor eIF-2. While RPS19 and RPS24 have been concluded to be involved in eIF-2 binding, RPS17 has not, though it lies in close vicinity to RPS19 [Bommer et al., 1988, 1991; Lutsch et al., 1990]. In addition, RPS19 has been shown to play an important role in 18S rRNA maturation and thus in ribosome biogenesis [Leger-Silvestre et al., 2005; Choesmel et al., 2007; Flygare et al., 2007]. An interesting question is why mutations in RPS19 are present in a quarter of DBA patients, while mutations in RPS17 and RPS24 occur only sporadically. The answer is unclear at present, and more DBA candidate genes need to be identified. Based on current knowledge, mutations in other RPs or proteins associated with the translational apparatus are expected to be found [Cmejlova et al., 2006a; Flygare and Karlsson, 2007].

In conclusion, the identification of a mutation in the third RP of the small ribosomal subunit in DBA patients further supports the theory that impaired translation may be the main cause of DBA pathogenesis, and that substances enhancing the level of translation could be beneficial for DBA patients. Indeed, the use of the amino acid leucine, which is known to activate translation, has been recently documented to be curative in a DBA patient without side effects [Pospisilova et al., 2007]. New studies involving more patients are required, however, to evaluate all aspects of leucine administration not only in DBA patients, but possibly also in patients with inherited bone marrow failure syndromes (dyskeratosis congenita, cartilage-hair hypoplasia and Shwachman-Diamond syndrome), whose pathogenesis is believed to be connected with various aspects of ribosome synthesis [Liu and Ellis, 2006].

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6.2 Appendix II.

Identification of mutations in the ribosomal protein L5 (RPL5) and ribosomal protein L11 (RPL11) genes in Czech patients with Diamond-Blackfan anemia. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Petrtylova K, Mihal V, Stary J, Cerna Z, Jabali Y, Pospisilova D., *Hum Mutat.* 2009;30(3):321–7.

RAPID COMMUNICATION

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Identification of Mutations in the Ribosomal Protein L5 (RPL5) and Ribosomal Protein L11 (RPL11) Genes in Czech Patients With Diamond-Blackfan Anemia

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ABSTRACT: Diamond-Blackfan anemia (DBA) is a congenital red blood cell aplasia that is usually diagnosed during early infancy. Apart from defects in red blood cell maturation, the disorder is also associated with various physical anomalies in 40% of patients. Mutations in the ribosomal protein (RP) S19 are found in 25% of patients, while mutations in other proteins of the small ribosomal subunit—RPS17 and RPS24—have been found in a fraction of patients. Recently, mutations in RPL5, RPL11, and RPL35a of the large ribosomal subunit have also been reported in several DBA patients. Here, we present the identification of mutations in the RPL5 and RPL11 genes in patients from the Czech DBA Registry. Mutations in RPL5 were identified in eight patients from 6 out of 28 families (21.4%), and mutations in RPL11 in two patients from 2 out of 28 families (7.1%). Interestingly, all 10 patients with either an RPL5 or RPL11 mutation exhibited one or more physical anomalies; specifically, thumb anomalies (flat thenar) were always present, while no such anomaly was observed in seven patients with an RPS19 mutation. Moreover, 9 out of 10 patients with either an RPL5 or RPL11 mutation were born small for gestational age (SGA) compared to 3 out of 7 patients from the RPS19-mutated group. These observations may suggest that mutations, at least in RPL5, seem to generally have more profound impact on fetal development than mutations in RPS19. Since RPL5 and RPL11, together with RPL23, are also involved in the MDM2-mediated p53 pathway regulation, we also screened the RPL23 gene for mutations; however, no mutations were identified.
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KEY WORDS: ribosomal protein L5; RPL5; ribosomal protein L11; RPL11; ribosomal protein L23; RPL23; Diamond-Blackfan anemia; DBA

Introduction

In 1999, mutations were found in the ribosomal protein (RP) S19 gene (MIM# 603474) that encodes for one of the proteins of the small ribosomal subunit in 25% of patients with a hematological disorder known as Diamond-Blackfan anemia (DBA; MIM# 205900) [Draptchinskaia et al., 1999; Willig et al., 1999a]. Recently, mutations in two other RPs of the small ribosomal subunit—RPS17 and RPS24 (MIM#s 180472, 602412)—were found in a fraction of DBA patients [Cmejla et al., 2007; Gazda et al., 2006].

DBA refers to a congenital erythroid aplasia that usually manifests during the first year of life. The characteristics of the disease include normochromic macrocytic anemia, normocellular bone marrow with a selective deficiency in red blood cell precursors, and profound reticulocytopenia [Freedman, 2000; Gazda and Sieff, 2006]. Serum erythropoietin level is increased. Erythrocytes often express several markers of fetal erythropoiesis, and display increased adenosine deaminase levels [Glader et al., 1983; Glader and Backer, 1988]. In 40% of DBA patients, various congenital anomalies are also present, mainly craniofacial dysmorphism, thumb and neck anomalies, congenital heart defects, and urogenital malformations [Willig et al., 1999b]. Prenatal and/or postnatal growth retardation independent of steroid therapy is also often observed. The disease affects both sexes equally with an incidence of five to seven children per 1 million live births.

Steroid administration represents the frontline treatment, to which the majority of DBA patients respond. Patients that are

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steroid-resistant enter a regular program of red blood cell transfusions [Freedman, 2000].

Ribosomes contain one molecule each of 79 RPs [Perry, 2007], suggesting that a limited amount of a particular RP will also limit the number of functional ribosomes, and consequently the efficiency of protein synthesis. Indeed, a decreased level of translation has been documented in DBA patients regardless of the presence of a mutation in the RPS19 gene, indicating that inefficient translation could be the general mechanism leading to DBA, and other RPs or proteins related to translation were also predicted to be found mutated in DBA patients [Cmejlova et al., 2006]. Apart from the identification of RPS17 and RPS24 as additional DBA-associated genes, this assumption was further confirmed by recently reported findings of mutations in the genes for three RPs of the large ribosomal subunit—RPL5 (MIM♯ 603634) in 14 out of 138 DBA probands (10.1%); RPL11 (MIM♯ 604175) in 9 out of 138 DBA probands (6.5%) (both reported frequencies refer only to patients without mutations in RPS19 or RPS24) [Gazda et al., 2007], and RPL35a in 5 out of 150 DBA probands (3.3%) [Farrar et al., 2008].

Based on these findings, we performed analyses of the RPL5 and RPL11 genes in Czech DBA patients. Here, we report the identification of RPL5 mutations in 6 out of 28 probands (21.4%) and RPL11 mutations in 2 out of 28 probands (7.1%). We also provide the first comparison of the RPL5-mutated group of patients with patients carrying an RPS19 mutation, relating clinical findings and treatment outcome.

Aside from being components of ribosomes, RPL5 and RPL11 have also been shown to take part in the p53 pathway regulation through MDM2 protein inhibition, along with yet another RP of the large ribosomal subunit, RPL23 (MIM♯ 603662) [Dai and Lu, 2004; Zhang et al., 2003; Bhat et al., 2004; Dai et al., 2004, 2006; Jin et al., 2004]. For this reason, we also screened the RPL23 gene for mutations, but none were identified.

Materials and Methods

Mutation Nomenclature

The RPL5 and RPL11 genes are located on human chromosome 1, and the RPL23 gene is located on human chromosome 17. PCR primers for RPL5 and RPL11 were designed on the basis of *Homo sapiens* chromosome 1 reference assembly NC_000001.9. Chromosome 17 reference assembly NC_000017.9 was used for the design of RPL23 PCR primers. For RNA analysis, the RefSeq mRNA sequences NM_000969.3 and NM_000975.2 were used for RPL5 and RPL11, respectively. The A of the ATG translation initiation start site was considered as +1. Protein RefSeq sequences NP_000960.2 and NP_000966.2 for RPL5 and RPL11, respectively, were used to describe identified mutations at the protein level.

DNA Isolation and Analyses

After informed consent, nucleated blood cells were used as the source of genomic DNA. Peripheral blood mononuclear cells (PBMCs) from DBA patients and healthy controls were isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) gradient centrifugation. Genomic DNA was isolated using a Genomic DNA Purification Kit (Fermentas, Burlington, Ontario, Canada). Individual RPL5, RPL11, and RPL23 exons with flanking regions were PCR-amplified (primer sequences on request), and corresponding PCR products were isolated from an agarose gel by a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The isolated DNA was used for direct sequencing on an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

To confirm each identified mutation, sequencing was also performed on a second independent PCR product, and when available, a different genomic DNA isolate was used as PCR template. In cases of deletions and insertions, respective PCR products were cloned to discriminate mutated and wild-type alleles.

Expression Analysis

PBMCs were lysed in 0.5 ml RNA-Bee (TEL-TEST, Friendswood, TX), and total RNA was isolated according to the manufacturer's manual. To avoid possible DNA contamination, samples were treated with DNase I (Ambion, Austin, TX) for 15 minutes at 37°C, and the enzyme was then inactivated at 75°C for 5 minutes. Isolated RNA was used for reverse-transcription using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexaprimers; the control reaction without reverse transcriptase was run in parallel. The cDNA for RPL5 and RPL11 was PCR-amplified using specific primers (primer sequences on request), and corresponding PCR products were isolated and sequenced.

Results

The Czech DBA Registry

The Czech DBA Registry was founded in 1996 thanks to the cooperation of a number of pediatric hematologists in the Czech Republic. The Czech DBA Registry currently contains 31 patients from 28 families born between 1959 and 2007. Most cases (25) are sporadic, while the remaining six patients are from three families. In seven patients (two patients belonging to the same family), a mutation in the RPS19 gene was identified (21.4%). A mutation in the RPS17 gene was found in one patient. The gene encoding RPS24 was also analyzed, but no mutations have been identified so far.

Analyses of the RPL5, RPL11, and RPL23 genes were performed in 23 DBA patients, in whom no mutation has been identified in the RPS17, RPS19, and RPS24 genes.

RPL5 Gene Analysis

The human RPL5 gene spans 9,888 bp on chromosome 1, and consists of eight exons. The RPL5 mRNA is 1,031 nt long, and codes for a protein of 297 amino acids. Figure 1A shows six mutations (21.4%) that were identified in eight DBA patients; four mutations were sporadic and two mutations were of familial origin. Four mutations (67%) were localized in exon 3 or its close vicinity, one mutation in exon 6 and one in exon 8. As for the type of mutations, we found one point mutation affecting a splicing acceptor (SA) site (family of Patient CZUH04), a duplication of one nucleotide (Patient CZUH032), three small deletions (Patients CZUH41, CZUH26, and CZUH40), and one point mutation (Family CZUH24).

In Patient CZUH04, a point mutation changing AG in the SA of intron 2 to AA (c.74–1G>A) was detected. The same mutation was also found in his affected mother, and the mutation was classified as familial. Since exon 3 begins with AG, we hypothesized that due to this mutation, AG at the beginning of exon 3 could be used as an alternative SA with an assumed frameshift p.Glu25GlyfsX2. However, the assumed splicing variant

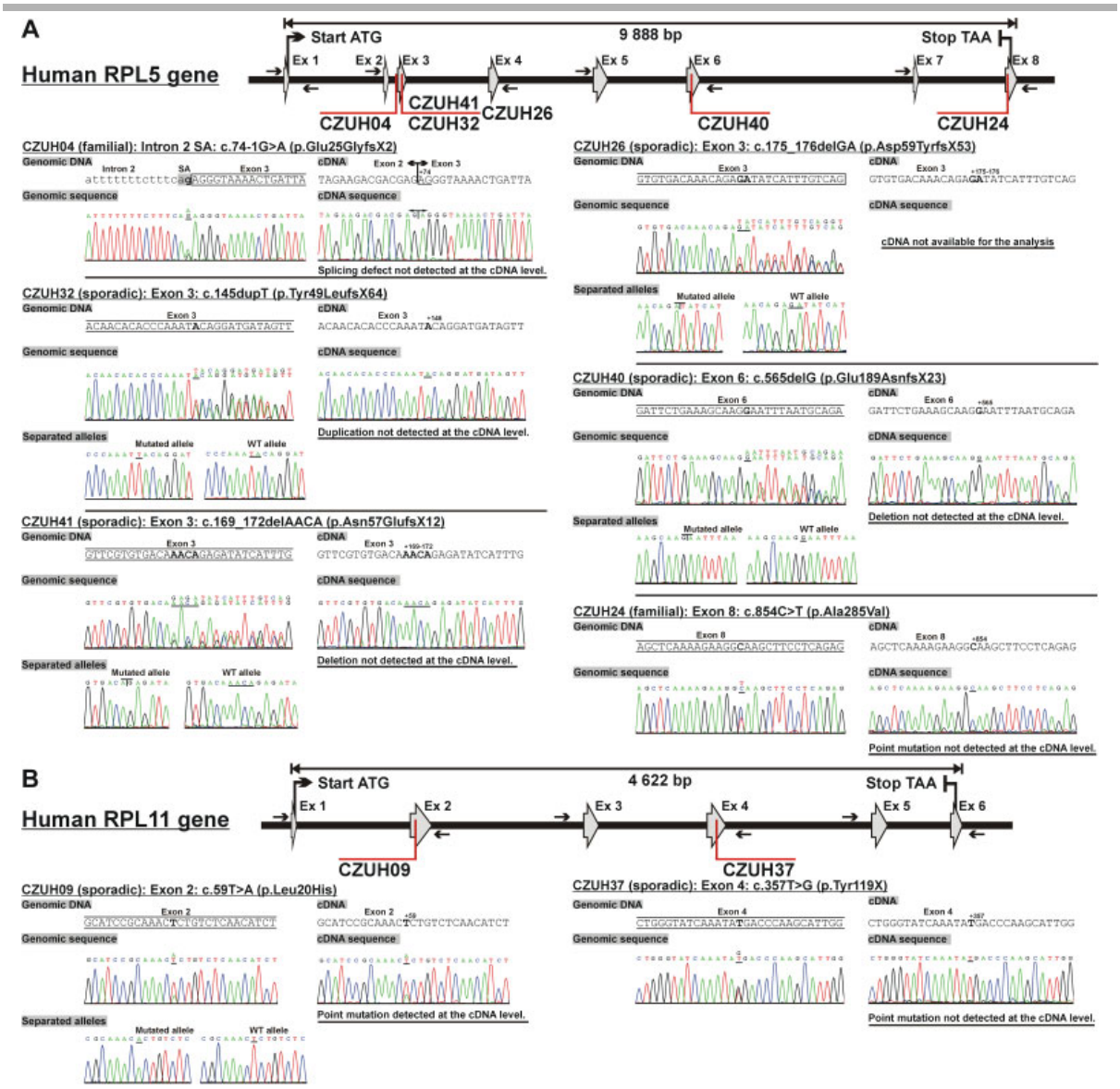

Figure 1. Mutations identified in the RPL5 and RPL11 genes in DBA patients. Arrows represent primers used for PCR amplification and sequencing of individual exons with flanking regions. **A:** Mutations in the RPL5 gene. In total, six mutations were identified in eight patients—one in a splicing acceptor (SA) site (family of Patient CZUH04), one duplication (Patient CZUH32), one point mutation (family of Patient CZUH24), and three deletions (Patients CZUH41, CZUH26, and CZUH40). Four of them were localized in exon 3 or its vicinity. To show the extent of the insertion and deletions, mutated as well as wild-type alleles were cloned and verified by sequencing. In five patients, cDNA was also available for expression analysis, but none of the mutations was detected at the cDNA level. GenBank reference sequences NM_000969.3 and NP_000960.2 were used for mRNA and protein, respectively. **B:** Mutations in the RPL11 gene. In total, two types of mutations were identified, one point mutation in Patient CZUH09 and one nonsense mutation in Patient CZUH37, but only in Patient CZUH09 was it also detected at the cDNA level. GenBank reference sequences NM_000975.2 and NP_000966.2 were used for mRNA and protein, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

was not detected in the proband at the cDNA level, nor we were able to detect any other aberrant splicing variants.

Three further mutations were identified in exon 3—a duplication c.145dupT in Patient CZUH32, a deletion of four nucleotides (c.169_172delAACA) in Patient CZUH41, and a deletion of two nucleotides (c.175–176delGA) in Patient CZUH26. All mutations are sporadic, and are predicted to cause a frameshift with a premature termination (Table 1). Expression analysis in Patients CZUH32 and CZUH41 showed that the mutated transcript could not be detected at the cDNA level. In Patient CZUH26, cDNA was not available for expression analysis, because the patient died 9 years ago.

A deletion of G at position 565 (c.565delG) was found in Patient CZUH40 in exon 6, and was described as sporadic. This deletion is predicted to lead to the frameshift p.Glu189AsnfsX23, but the mutated transcript was not detected at the cDNA level.

A point mutation changing C to T at position 854 (c.854C>T) in exon 8 was detected in Patient CZUH24 and also in his affected mother, but it was not detected in either of the mother's healthy

parents. This mutation is predicted to substitute alanine 285 with valine (p.Ala285Val); nonetheless, the mutation was repeatedly not detectable in the proband at the cDNA level in two independent blood samples taken for RNA isolation.

None of the mutations identified in the RPL5 gene in DBA patients could be found in the Single Nucleotide Polymorphism Database (dbSNP at www.ncbi.nlm.nih.gov/SNP). To further exclude the possibility of a rare polymorphism, 52 healthy controls were also screened for the mutations, but with negative results.

A complete summary of RPL5 mutations with their predicted outcome is shown in Table 1.

RPL11 Gene Analysis

The human RPL11 gene consists of six exons, and spans 4,622 bp on chromosome 1. The RPL11 mRNA is 607 nt long, and codes for a 178–amino acid protein. Two mutations in two DBA patients (7.1%) were identified, one in exon 2 and one in exon 4 (Fig. 1B).

In Patient CZUH09, a point mutation was detected at position 59 (c.59T>A), leading to p.Leu20His. This mutation was also observed at the cDNA level, and it should therefore be translated into protein. To understand the functional importance of leucine 20, we further compared the human RPL11 protein sequence with other species. Out of 79 organisms for which the RPL11 protein sequence was available for comparison, ranging from fungi, invertebrates to vertebrates, all have leucine 20 and a preceding lysine 19 (numbering according to the human RPL11 protein sequence; data not shown), implying that these two amino acids may be of extreme importance for RPL11 function, and therefore evolutionarily conserved.

In Patient CZUH37, a nonsense mutation c.357T>G was found, resulting in the stop codon (p.Tyr119X). This mutation was not detected at the cDNA level.

Table 1. Mutations Identified in the RPL5 and RPL11 Genes and DBA Patient Characteristics*

A. Mutations identified in the RPL5 and RPL11 genes

Patient (Inheritance)	Mutation	Predicted protein change	cDNA level	Conclusion
Mutations in the RPL5 gene				
CZUH04 proband; CZUH04 mother (familial)	Intron 2 SA: c.74–1G>A	Splicing defect; shift of SA 2 nt downstream; p.Glu25GlyfsX2	Not detected; NA	WT mRNA and protein haploinsufficiency
CZUH32 (sporadic)	Exon 3: c.145dupT	p.Tyr49LeufsX64	Not detected	WT mRNA and protein haploinsufficiency
CZUH41 (sporadic)	Exon 3: c.169_172delAACA	p.Asn57GlufsX12	Not detected	WT mRNA and protein haploinsufficiency
CZUH26 (sporadic)	Exon 3: c.175_176delGA	p.Asp59TyrfsX53	NA	WT mRNA and protein haploinsufficiency
CZUH40 (sporadic)	Exon 6: c.565delG	p.Glu189AsnfsX23	Not detected	WT mRNA and protein haploinsufficiency
CZUH24 proband; CZUH24 mother (familial)	Exon 8: c.854C>T	p.Ala285Val	Not detected; NA	WT mRNA and protein haploinsufficiency
Mutations in the RPL11 gene				
CZUH09 (sporadic)	Exon 2: c.59T>A	p.Leu20His	Detected	Mutation in conserved amino acid, loss of function
CZUH37 (sporadic)	Exon 4: c.357T>G	p.Tyr119X	Not detected	WT mRNA and protein haploinsufficiency

B. Patient characteristics

Patient (Sex)	Current age (years)	Associated anomalies	Current treatment
Mutations in the RPL5 gene			
CZUH04 proband (M)	21	SGA; flat thenar; short stature	Remission
CZUH04 mother (F)	49	SGA; flat thenar; microphthalmia; short stature	Remission
CZUH32 (F)	22	SGA; flat thenar; tetralogy of Fallot	Remission
CZUH41 (M)	8	SGA; flat thenar; facial dysmorphism	Transfusions; iron chelators
CZUH26 (F)	Died at 19	SGA; flat thenar; bifid thumb	Steroids; irregular transfusions; died after SCT
CZUH40 (M)	1	Flat thenar	Transfusions; iron chelators
CZUH24 proband (M)	20	SGA; flat thenar, short stature	Steroids; irregular transfusions; iron chelators
CZUH24 mother (F)	42	SGA; flat thenar, short stature	Remission
Mutations in the RPL11 gene			
CZUH09 (F; sporadic)	31	SGA; flat thenar; atypical thumb	Transfusions; iron chelators
CZUH37 (F; sporadic)	20	SGA; flat thenar; bifid thumb	Steroids; transfusions; iron chelators

*GenBank reference sequences for RPL5: NM_000969.3 for mRNA; NP_000960.2 for protein. GenBank reference sequences for RPL11: NM_000975.2 for mRNA; NP_000966.2 for protein.

WT, wild type; NA, not available for expression analysis. SCT, stem-cell transplantation; SGA, small for gestational age.

Screening of respective exons in 52 healthy controls revealed no alterations, nor was any record found in the dbSNP.

A summary of both mutations found in the RPL11 gene in DBA patients is given in Table 1.

RPL23 Gene Analysis

Several reports have been published suggesting that RPL23 has the same extraribosomal function as RPL5 and RPL11: it modulates the p53 pathway through MDM2 regulatory feedback [Dai et al., 2004; Jin et al., 2004]. We were therefore interested whether this RP is also mutated in DBA patients.

The human RPL23 gene consists of 5 exons, and is located on chromosome 17, spanning 3,733 bp. The RPL23 mRNA is 577 nt long, and encodes for a 140–amino acid protein. All exons with flanking regions were analyzed for mutations, but none were discovered in 23 DBA patients.

Patient Characteristics

Characteristics of all patients for whom a mutation in the RPL5 or RPL11 gene was found are summarized in Table 1B. Out of six mutations in the RPL5 gene identified in eight patients, two were of familial origin, and segregated with the disease. Mutations were distributed equally between males (four; 50%) and females (four; 50%). Both mutations identified in the RPL11 gene were found in females and were sporadic.

In the family of Patient CZUH04, the proband inherited the mutation from his affected mother. The patient also had an older sister that died at the age of 8 years from complications from prehepatal portal hypertension (genetic material for analyses not available). She suffered from chronic erythroblastopenia, and shared the same type of associated anomalies with her brother—both were small for gestational age (SGA) and had short stature and flat thenar. The mother of both affected children had anemia, short stature, flat thenar, and microphthalmia. Her older brother had anemia and short stature, and died 20 years ago at the age of 35 years (genetic material for analyses not available). Both the mother and son were treated with steroids in the past, and they are currently in remission.

In the family of Patient CZUH24, the mutation segregates with the disease, and was inherited from the affected mother to her son. The mutation arose de novo in the mother, since neither of her healthy parents carries the mutation. Both the proband and his mother have short stature, chronic anemia, flat thenar, and were SGA. The mother was treated with steroids until the age of 12 years, and at present she is in remission having a mild form of macrocytic anemia without the need for treatment. The son is steroid-dependent, being treated by a low dose of steroids on alternate days with occasional need for transfusions.

Owing to the fact that seven patients (three males, four females) from the Czech DBA Registry carry a mutation in the RPS19 gene, we were able to compare this group of patients with the group of eight patients with a mutation in the RPL5 gene. The two groups do not differ in sex ratio, treatment outcome, or the course or severity of the disease. Differences were, however, noticed in birth weights and in the manifestation of associated anomalies.

Seven patients (87.5%) with an RPL5 mutation were born SGA, and only one patient (Patient CZUH40) (12.5%) had a borderline birth weight. In contrast, three patients (42.9%) with an RPS19 mutation were born SGA, and four patients (57.1%) had a normal birth weight.

The second difference between the group of DBA patients with an RPS19 mutation and those with an RPL5 mutation was the presence of one or more associated anomalies. In particular, flat thenar with or without bifid thumb was noticed in all patients with an RPL5 mutation, while in the RPS19-mutated group thumb anomalies were not observed. Fisher's exact test indeed showed a highly significant difference between these two groups of patients ($P < 0.0005$). Interestingly, flat thenar was also observed in both patients with an RPL11 mutation, in one patient with an RPS17 mutation, and in two patients with no mutation in known DBA-associated RPs. Other physical anomalies observed in patients with an RPL5 mutation include tetralogy of Fallot, microphthalmia, facial dysmorphism, and short stature.

Discussion

Last year, preliminary results were reported describing the identification of mutations in proteins of the large ribosomal subunit in DBA patients: 14 mutations in the RPL5 gene, nine mutations in the RPL11 gene [Gazda et al., 2007], and recently five mutations in the RPL35a gene [Farrar et al., 2008]. Here, we provide a description of another six mutations in RPL5 (21.4% of unrelated probands) and two mutations in RPL11 (7.1% of unrelated probands) found in patients from the Czech DBA Registry.

The frequency of RPL11 mutations in Czech DBA patients is higher compared to reference [Gazda et al., 2007]: 9.5% vs. 6.5%. However, considering the frequency of RPL5 mutations, the discrepancy between frequencies is even higher: 28.6% of Czech DBA patients vs. 10.1% in reference [Gazda et al., 2007] (to be comparable with reference Gazda et al. [2007], only patients without mutations in RPS19 and RPS17 were counted). At this time, it is unclear what could be the cause of this large difference in the high frequency of RPL5 mutations, which is currently the same as the frequency of RPS19 mutations in Czech DBA patients.

As for mutations, 6 out of 8 mutations detected were predicted to result in a premature stop codon. Since prematurely-terminated mRNAs are rapidly degraded by nonsense-mediated decay mechanisms, we were interested in whether the mutations could also be identified at the cDNA level. As expected, the nonsense mutation and all of the frameshift-inducing mutations for which expression analysis was performed could not be detected at the cDNA level. Unfortunately, a biological sample for RNA isolation was not available for Patient CZUH26, but we speculate that the mutated transcript would be also degraded similarly to that of Patient CZUH32, because the 2-nt deletion in Patient CZUH26 leads to the same frameshift with the same stop codon as in Patient CZUH32.

Interestingly, the missense mutation c.854C>T in exon 8 in Patient CZUH24 was also not detected in two independent RNA isolates. We suggest the following explanation: the wild type allele sequence is AGGCAAG, while the mutation leads to the sequence AGGTAAG, creating a novel consensus donor splice site AG‖GTRAG (‖ denotes the exon-intron boundary) [Burset et al., 2000]. Exactly the same sequence can be found within the RPL5 gene (9,888 bp long) in two locations—at the end of exon 3 and exon 5—where it functions as a natural donor splice-site. It is therefore very likely that this newly formed donor splice-site is used by the cellular splicing machinery, resulting in an aberrant splicing and rapid degradation of the transcript.

Thus, the only mutation detected also at the cDNA level was c.59T>A in RPL11 in Patient CZUH09 leading to the substitution of the evolutionary-conserved, uncharged, hydrophobic leucine 20 with a charged hydrophilic histidine, suggesting its strict requirement for the function of RPL11. Indeed, leucine 20 lies

in the center of a putative nuclear export signal (NES; amino acids 15–26) [Zhang et al., 2003], and it is very likely that the substitution results in the loss of function.

Though the Czech DBA Registry is small, we compared the group of patients with RPS19 mutations (n = 7) with the group of patients with RPL5 mutations (n = 8), and noticed two differences. Patients with RPL5 mutations were generally born SGA (87.5%), while in the RPS19-mutated group the frequency was lower (42.9%). For comparison, the frequency of SGA in full-term newborns in the general Czech population is less than 3%. The second difference is even more striking—all patients with RPL5 mutations have one or more associated anomalies. All have flat thenar and some also have bifid thumb, while no thumb anomalies are observed in patients with RPS19 mutations. This is also in agreement with a recently published review of genetic and clinical data for 104 patients with RPS19 mutations, in which only 31% of patients have associated anomalies: nine patients (8.7%) were reported to have thumb anomalies, and only one had flat thenar (1%) [Campagnoli et al., 2008]. Interestingly, flat thenar was also observed in both patients with an RPL11 mutation and in one patient with an RPS17 mutation, indicating that these RPs are in some way more important for proper thumb development than RPS19. Taking into account the fact that 9 out of 10 Czech DBA patients with either RPL5 or RPL11 mutation were also born SGA, these observations may together suggest that mutations at least in RPL5 seem to have more profound impact on fetal development than mutations in RPS19. For definitive conclusions, however, a larger cohort of patients is needed.

The identification of mutations in the RPL5 and RPL11 genes in DBA patients is also interesting from another point of view. Both proteins have been reported to be implicated in the activation of p53 through their interaction with the MDM2 protein, suppressing its E3 ubiquitin ligase function that otherwise directs the rapid degradation of p53 [Dai and Lu, 2004; Zhang et al., 2003; Bhat et al., 2004; Dai et al., 2006]. It is noteworthy that yet another RP of the large ribosomal subunit, RPL23, has been described as having the same function [Dai et al., 2004; Jin et al., 2004]. We were therefore interested whether RPL23 is also mutated in DBA patients. No mutations in RPL23 were identified, however, suggesting that the DBA phenotype is caused rather by the haploinsufficiency of RPL5 and RPL11 in ribosome biogenesis and/or translation than by their insufficient function in p53 pathway regulation.

Indeed, the level of translation has previously been measured in Patients CZUH04 and CZUH24 carrying a mutation in the RPL5 gene (Patients CZ12 and CZ14 in Cmejlova et al. [2006]). The level of basal translation was 61 to 79% and 25 to 42% of control values in Patients CZUH04 and CZUH24, respectively, confirming that RPL5 haploinsufficiency adversely affects the efficiency of translation.

Moreover, in yeasts, the depletion of RPL1, a mammalian RPL5 homolog, led to the disruption of 60S ribosomal subunit assembly, and the defect was localized to 27SB pre-rRNA processing (32S pre-rRNA in humans) [Deshmukh et al., 1993]. Another recent report indicates that processing of 27SB pre-rRNA requires a subcomplex consisting of Rpf2, Rrs1, 5S rRNA, RPL5, and RPL11, and each of the four protein components is required for production of 60S ribosomal subunits [Zhang et al., 2007]. It seems, therefore, likely that RPL11 similarly to RPL5 takes part in 27SB pre-rRNA processing as well. Their cooperation is also reflected in sharing the same location within the large ribosomal subunit, while RPL23 lies on the opposite side [Uchiumi et al., 1985; Spahn et al., 2001].

In conclusion, we present the identification of six and two new different mutations in the RPL5 and RPL11 genes, respectively, expanding the repertoire of known DBA-associated mutations, and provide the first comparison of an RPL5-mutated group of patients with patients carrying an RPS19 mutation, relating clinical findings and treatment outcome.

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6.3 Appendix III.

Tyrosine 87 is vital for the activity of human protein arginine methyltransferase 3 (PRMT3). Handrkova H, Petrak J, Halada P, Pospisilova D, Cmejla R., *Biochim Biophys Acta.* 2011;1814(2):277–82.

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Tyrosine 87 is vital for the activity of human protein arginine methyltransferase 3 (PRMT3)

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ABSTRACT

Protein arginine methyltransferase 3 (PRMT3) is a cytosolic enzyme that catalyzes the formation of mono- and asymmetric dimethyl arginines, with ribosomal protein (RP) S2 as its main in vivo substrate. The interplay of PRMT3-RPS2 homologs in yeast is important for regulating the ribosomal subunit ratio and assembly. *Prmt3–null* mice display slower embryonic growth and development, although this phenotype is milder than in mouse RP gene knockouts. Defects in ribosome maturation are the hallmark of Diamond-Blackfan anemia (DBA). Sequencing of the *PRMT3* gene in patients from the Czech DBA registry revealed a heterozygous mutation encoding the Tyr87Cys substitution. Although later analysis excluded this mutation as the cause of disease, we anticipated that this substitution might be important for PRMT3 function and decided to study it in detail. Tyr87 resides in a highly conserved substrate binding domain and has been predicted to be phosphorylated. To address the impact of putative Tyr87 phosphorylation on PRMT3 properties, we constructed two additional PRMT3 variants, Tyr87Phe and Tyr87Glu PRMT3, mimicking non-phosphorylated and phosphorylated Tyr87, respectively. The Tyr87Cys and Tyr87Glu-PRMT3 variants had markedly decreased affinity to RPS2 and, consequently, reduced enzymatic activity compared to the wild-type enzyme. The activity of the Tyr87Phe-PRMT3 mutant remained unaffected. No evidence of Tyr87 phosphorylation was found using mass spectrometric analysis of purified PRMT3, although phosphorylation of serines 25 and 27 was observed. In conclusion, Tyr87 is important for the interaction between PRMT3 and RPS2 and for its full enzymatic activity.

1. Introduction

Arginine methylation plays a role in many cellular processes, such as the regulation of gene transcription, signal transduction, mRNA splicing, DNA repair, and nuclear/cytoplasmic shuttling (reviewed in [1]).

Protein arginine methyltransferase 3 (PRMT3) is a cytosolic enzyme that catalyzes the formation of ω-mono- or asymmetric dimethylarginine [2] and has orthologs in most eukaryotes. PRMT3 contains a unique substrate binding N-terminal C_2H_2 Zn finger domain and a catalytic C-terminal domain homologous to other PRMTs [3]. PRMT3 associates with ribosomes in the cytosol, and the main *in vivo* substrate of PRMT3 is a component of the small ribosomal subunit, ribosomal protein (RP) S2 [4].

RPS2 is essential for rRNA maturation, namely 32S pre-RNA splicing, as well as for translational fidelity [5,6]. Upon gene depletion of the yeast ortholog *RpS2*, nucleolar export of the small ribosomal subunit precursors is blocked, leading to their rapid degradation [7]. *Rps2* gene deletion in *Drosophila* manifests as a specific ovarian defect [8], in addition to the general *Minute* characteristics (thin and reduced bristles, delayed development, and larval lethality) in common with other RP gene deletions.

PRMT3 methylates multiple arginines in the RG-rich N-terminal tail of RPS2 to asymmetric dimethylarginines [4], which is the only known posttranslational modification of RPS2. RPS2 is recognized by the N-terminal Zn finger domain of PRMT3, an interaction that is conserved among eukaryotes and is crucial for substrate specificity.

The biological significance of RPS2 methylation was first demonstrated in fission yeast. Deletion of *Rmt3*, the *Schizosaccharomyces pombe* homolog of the *PRMT3* gene, leads to the production of nonmethylated Rps2 and is manifested as a decrease in the 40S pool and an imbalance between ribosomal subunits [9], although pre-rRNA processing appears to proceed normally.

Prmt3–null mice show developmental delay during embryogenesis, and embryos are markedly smaller than wt embryos [10]. However, size at birth is normal in *Prmt3–null* mice, as is the ratio of ribosomal subunits and the translational rate. This suggests that the loss of PRMT3 is quite satisfactorily compensated for in most cell types

Abbreviations: PRMT3, protein arginine methyltransferase 3; RP, ribosomal protein; DBA, Diamond-Blackfan anemia; wt, wild-type; IP, immunoprecipitation

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but may become critical in cells (or under conditions) demanding extremely fast protein synthesis.

The developmental delay and slower growth in *Drosophila Minutes* are associated with mutations in RP genes or genes involved in ribosome biogenesis, transport, or in translation itself [11,12]. Because mutations in RP genes with concomitant defects in ribosome biogenesis are the hallmark of Diamond-Blackfan anemia (DBA), and PRMT3 seems to take part in this process, we decided to screen patients suffering from DBA for mutations in both the *RPS2* and *PRMT3* genes.

DBA (OMIM: 105650, reviewed in [13,15,16]) is a rare congenital hematological disorder characterized by normochromic macrocytic anemia and reticulocytopenia, and nearly 50% of patients have additional physical abnormalities. The disease is associated with a mutation in an RP gene in more than half of the patients.

Such RP gene mutations result in rRNA maturation defects, a reduced pool of mature ribosomes and thus a decreased level of translation, and also trigger a p53-mediated stress response that leads to cell cycle delay and increased apoptosis in the population of erythroid progenitors ([14], [15] and references within).

In our screen, we identified the heterozygous substitution Tyr87Cys in the *PRMT3* gene in one DBA patient, in whom no mutations had been identified in the currently known DBA causal genes *RPS10*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, *RPL5*, *RPL11*, and *RPL35a*. However, subsequent familial analysis excluded the PRMT3 mutation to be the cause of DBA, since it did not segregate with the disease in the affected family.

Nevertheless, we continued to focus on characterizing the importance of Tyr87 for PRMT3 functioning, since it resides in a highly conserved N-terminal domain [17] that has previously been shown to be both sufficient and necessary for substrate binding [4]. Since tyrosine 87 was considered a possible target for phosphorylation [3], we also studied Tyr87Glu and Tyr87Phe variants (mimicking phosphorylated and nonphosphorylated tyrosine, respectively) and analyzed the phosphorylation of PRMT3 directly by mass spectrometry.

2. Materials and methods

All the following procedures were done in accordance with the ethical standards stated by the Helsinki Declaration.

2.1. Patients, genetic analyses

All 10 patients enrolled met the diagnostic criteria for DBA. After informed consent, genomic DNA and total RNA were isolated from peripheral blood mononuclear cells. Primers for PCR (upon request) were designed to allow amplification and sequencing of all exons and exon/intron boundaries of the *RPS2* and *PRMT3* genes. Amplified PCR fragments were gel-purified and used as templates in a sequencing reaction. Sequences were analyzed on an ABI3130 Genetic Analyzer (Applied Biosystems, USA). When needed, cDNA was prepared according to a standard procedure using random hexaprimers.

2.2. Plasmid construction

To construct the myc-RPS2 vector, full-length cDNA of RPS2 was PCR-cloned behind the myc tag. Expression vectors carrying either wild-type (wt) PRMT3 or Tyr87Cys–PRMT3 were prepared by subcloning of full-length cDNA obtained from a healthy control or the affected DBA patient behind the 3xFLAG tag (Sigma-Aldrich, Czech Republic). Tyr87Glu and Tyr87Phe mutants were constructed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA).

2.3. Immunocytochemistry

HeLa cells were seeded on glass slides and allowed to grow to 50–60% confluency in D-MEM supplemented with 10% dialyzed FCS. Either the wt-3xFLAG–PRMT3, one of the mutant variants, or the empty 3xFLAG plasmid was then transfected into cells using Jet-PEI (Polyplus-transfection SA, France).

One day after transfection, cells were rinsed with PBS, incubated 20 min at room temperature with freshly prepared 4% paraformaldehyde (in PBS, pH 7.4), rinsed with PBS, and permeated by incubation in 0.5% Triton X-100 in PBS for 5 min, followed by another PBS wash. Fixed cells were then incubated for 1 h with a mouse anti-FLAG antibody (1:1000 in PBS, Sigma-Aldrich), then rinsed with PBS and incubated for 6 h with a FITC-conjugated anti-mouse antibody (1:300 in PBS, Jackson Laboratories Inc., USA), and again rinsed thoroughly with PBS.

Before mounting the cells in VectaShield medium (Vector Laboratories Inc., USA), the nuclei were stained by a DAPI solution (1 μg/ml in PBS, Sigma-Aldrich). Images were taken using an Olympus CKX41SF-2 fluorescent microscope.

2.4. Protein interaction assay

HeLa cells were grown in 60-mm dishes to 80–90% confluency and then co-transfected using Nanofectamine (PAA Laboratories GmbH, Austria) with either one of the 3xFLAG–PRMT3 variants and the myc-RPS2 plasmid, or the empty 3xFLAG and myc-RPS2 vectors as controls, or with the empty 3xFLAG and GFP plasmids as a visual control of transfection efficiency (in each case, 3 μg DNA of each plasmid was used, i.e., 6 μg of total DNA per dish).

A day after transfection, cells were rinsed three times with cold PBS and lysed with 400 μl of mild lysis buffer (150 mM NaCl,10 mM Hepes, 1% Triton, pH 7.4, Complete protease inhibitor cocktail (Roche, Czech Republic)). Lysates were then cleared by centrifugation (10 min, 14000 RPM, 4 °C) and the protein concentration was determined using the Bradford method (Bio-Rad, USA). Immunoprecipitation was performed using EZview anti-3xFLAG or EZview anti-myc agarose beads (Sigma-Aldrich). Cell lysates containing 750 μg of total protein were added to 20 μl of peletted agarose beads; the suspension was diluted to 1 ml and incubated 1 h at 4 °C on a rotator. The suspension was then centrifuged 1 min at 8600 RPM, and the pellet was washed three times with 1 ml of ice-cold lysis buffer. The immunoprecipitated proteins were eluted by 20 μl of 2× SDS-Laemmli buffer at 99 °C for 5 min. Eluates were then separated by SDS-PAGE (on a 10% gel), transferred to PVDF membrane, and proteins were detected using HRP-conjugated anti-FLAG and anti-myc tag antibodies (both 1:1000 in PBS, Sigma-Aldrich). Densitometric analysis was performed using Quantity One software (Bio-Rad).

The relative affinities of all four PRMT3 variants to RPS2 were determined as follows: the ratio of the band densities of myc-RPS2/3xFLAG–PRMT3 for the anti-FLAG immunoprecipitate and the ratio of 3xFLAG–PRMT3/myc-RPS2 for the anti-myc immunoprecipitate were calculated for each of the 3xFLAG–PRMT3 variants. Finally, these ratios were expressed relatively to that of the wt-3xFLAG–PRMT3/myc-RPS2 pair. The independently collected data from 4 anti-myc and 4 anti-FLAG immunoprecipitates for each of the 3xFLAG–PRMT3 variants were evaluated statistically (using an unpaired 2-tailed *T*-test, 95% confidence interval).

2.5. Methyltransferase assay

This assay was adapted from Refs. [18] and [4]. Cells were co-transfected with the myc-RPS2 and 3xFLAG–PRMT3 vectors as described above. One day after transfection, cells were rinsed with PBS and incubated for 30 min in methionine-free D-MEM (Sigma-Aldrich) supplemented with the proteosynthesis inhibitors

cycloheximide (100 mg/ml, Invitrogen, Czech Republic) and chloramphenicol (40 mg/ml, Sigma-Aldrich). The medium was then replaced with D-MEM containing 1 mCi/ml ^{3}H-Met (GE Healthcare, AP Czech, Czech Republic) as the sole source of methionine and proteosynthesis inhibitors as above. After incubation for 3 h at 37 °C, cells were lysed in the mild lysis buffer and lysates containing 1.5 mg of total protein were subjected to immunoprecipitation using 50 µl of pelleted EZview anti-myc agarose beads as described above. The protein was then eluted with 50 µl of elution buffer, and 5 µl of the eluate was used to quantify the relative amount of myc-RPS2 in each immunoprecipitate using densitometric analysis of the Western blot. The rest of the sample was dried at 37 °C and mixed with 5 ml of Ready Safe scintillator (Beckmann Coulter, Czech Republic). The activity of ^{3}H-Met in the samples was then measured on a Beckmann Counter LS 1801. After subtraction of background, the radioactivity was normalized to the amount of myc-RPS2 in the sample and expressed relatively to wt-3xFLAG–PRMT3. The data from 5 independent replicates were then evaluated statistically.

2.6. Mass spectrometry

Enzymatic in-gel digestion was performed as follows: an excised CBB-stained protein band was destained with 50 mM 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN) and the protein was reduced with 30 mM TCEP at 65 °C for 30 min and alkylated by 30 mM iodacetamide for 60 min in the dark. The gel was then washed with water, shrunk by dehydration in MeCN, and reswelled again in water. Gel pieces were then partly dried in a SpeedVac concentrator; rehydrated in a cleavage buffer containing 25 mM 4-ethylmorpholine acetate, 5% MeCN, and 100 ng trypsin (Promega, East Port, Czech Republic); and incubated overnight at 37 °C. The resulting peptides were extracted in 40% MeCN/0.1% TFA. Phosphopeptide enrichment using an Fe-IMAC microcolumn was performed as described previously [19].

Mass spectra were measured on an Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonics, Germany) equipped with a Smartbeam™ solid-state laser and LIFT™ technology for MS/MS analysis. The peak lists were searched using an in-house MASCOT search engine against the SwissProt 57.13 database subset of human proteins with the following search settings: peptide tolerance of 20 ppm, missed cleavage site value set to one, variable carbamidomethylation of cysteine, oxidation of methionine, protein N-terminal acetylation and phosphorylation on serine, threonine, and tyrosine.

3. Results

3.1. Identifying a mutation in the PRMT3 gene of a DBA patient

In total, 10 DBA patients with no known mutation in the *RPS10*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, *RPL5*, *RPL11*, *and RPL35a* genes were screened for mutations in the *RPS2* and *PRMT3* genes. No alterations were found in the *RPS2* gene; however, a heterozygous point mutation c.260A>G in exon 4 of the *PRMT3* gene was identified in one patient. This mutation changes the highly conserved tyrosine 87 to cysteine (Tyr87Cys) and is not yet referenced in the single nucleotide polymorphism database (www.ncbi.nlm.nih.gov/projects/SNP). To further exclude the possibility of a rare polymorphism, we tested 58 healthy donors of the same Caucasian origin for the presence of this mutation, but with negative results. RT-PCR followed by sequencing showed that both the wild-type and mutated alleles were expressed equally, indicating that mRNA stability is not affected.

Subsequent analyses showed that the mutation was also present in apparently healthy relatives, and therefore, this mutation is not the primary cause of DBA in this patient. Although the mutation is not causative, the Tyr87 residue seems to be important for PRMT3 function, since it resides in a highly conserved N-terminal domain that has previously been shown to be necessary for substrate binding and has been considered a possible target for phosphorylation.

3.2. The Tyr87Cys mutation disrupts the enzymatic activity of PRMT3

To determine the activity of mutated PRMT3 towards its substrate RPS2, we expressed both proteins as N-terminal fusion proteins with distinct short oligopeptide tags (3xFLAG–PRMT3 and myc-RPS2). Since Tyr87 has been predicted to be a potential site for phosphorylation [3], in addition to Tyr87Cys–PRMT3, we also studied the variants Tyr87Glu and Tyr87Phe (mimicking phosphorylated and non-phosphorylated tyrosine, respectively).

The *in vivo* methyltransferase assay was performed in HeLa cells overexpressing the recombinant myc-RPS2 protein as a substrate along with one of the 3xFLAG–PRMT3 variants (wt, Tyr87Cys, Tyr87Glu, or Tyr87Phe). Transfected cells were incubated in the presence of translational inhibitors and ^{3}H-Met as the sole source of methionine. Methylated myc-RPS2 was then immunoprecipitated and the radioactivity of incorporated ^{3}H corresponding to the methyltransferase activity of each of the 3xFLAG–PRMT3 variants was measured. We verified by Western blot that myc-RPS2 and all four 3xFLAG–PRMT3 variants were expressed at comparable levels (data not shown). The radioactivity of incorporated ^{3}H was normalized to the amount of immunoprecipitated myc-RPS2, which was assessed densitometrically from the Western blot. The results were then expressed relative to the activity of wt-3xFLAG–PRMT3.

As shown in Fig. 1, the methyltransferase activity of the 3xFLAG–PRMT3–Tyr87Cys mutant was about one-third ($28 \pm 17\%$) of the wt enzymatic activity and a similar decrease was also observed for the Tyr87Glu mutation ($21 \pm 19\%$); activities of both variants differed significantly from wt ($n = 5$, $p < 0.01$). The difference between the activity of the 3xFLAG–PRMT3–Tyr87Phe ($76 \pm 16\%$) and the activity of the wt enzyme was not statistically significant. Our data thus clearly demonstrate that Tyr87 is necessary for the full enzymatic activity of PRMT3. Since the acidic glutamate resembles the phosphorylated tyrosine in terms of size and charge, we predict that Tyr87 phosphorylation would decrease the activity of PRMT3. Because PRMT3 is the preferential methyltransferase for RPS2 [4], the Tyr87Cys mutation found in our DBA patient may result in a decrease in RPS2 methylation.

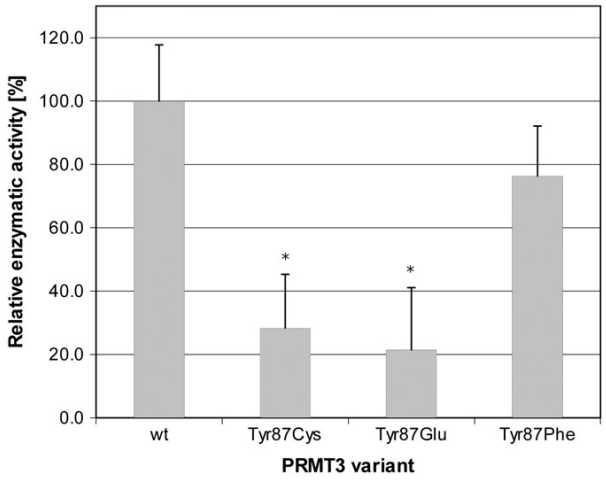

Fig. 1. Tyr87 is vital for the full enzymatic activity of PRMT3. The in vivo methyltransferase assay in HeLa cells overexpressing recombinant myc-RPS2 protein as a substrate, along with one of four variants of 3xFLAG–PRMT3 (wt, Tyr87Cys, Tyr87Glu, or Tyr87Phe). Data were collected from 5 independent biological replicates. Values that differ significantly from wt ($p < 0.01$) are marked by an asterisk.

3.3. Tyr87 alterations do not affect the cellular localization of PRMT3

Unlike most other methyltransferases, PRMT3 is a cytosolic enzyme [2]. We tested whether the observed decrease in enzymatic activity of the 3xFLAG–PRMT3 mutants might be caused by accumulation of the enzyme in different cellular compartments or in insoluble bodies.

Immunostaining of transiently transfected HeLa cells showed that the wild-type 3xFLAG–PRMT3 and all three studied mutants are localized to the cytosol and are distributed homogenously (Fig. 2). The observed decreases in the enzymatic activity of Tyr87Cys and Tyr87Glu mutants are therefore not caused by impaired enzyme localization.

3.4. Tyr87 is important for RPS2 binding

Cross-immunoprecipitation is a fast and elegant method for studying protein–protein interactions. The cell lysate is precipitated with one antibody (against the enzyme, for instance), and the eluate is then detected and relatively quantified with another antibody (against the substrate), and *vice versa*. This method is also suitable for comparing the relative affinities of different enzyme variants to one substrate: the relative quantity of the substrate that co-precipitates with the distinct enzyme variant is proportional to their binding strength, i.e., to their affinities to the substrate.

To assess the role of tyrosine 87 in the binding of PRMT3 with its substrate RPS2, we performed cross-immunoprecipitation from cells co-expressing myc-RPS2 together with one of the 3xFLAG–PRMT3 variants. Relative affinities were then calculated from 8 independent immunoprecipitations (4 with the anti-myc tag, 4 with the anti-FLAG antibody). The expression levels of both tagged proteins were comparable, as determined by densitometry of Western blots for each transfection (data not shown). Representative Western blots of both immunoprecipitates are shown in Fig. 3.

The higher protein levels of myc-RPS2 when co-expressed with 3xFLAG–PRMT3 variants in comparison to controls containing RPS2

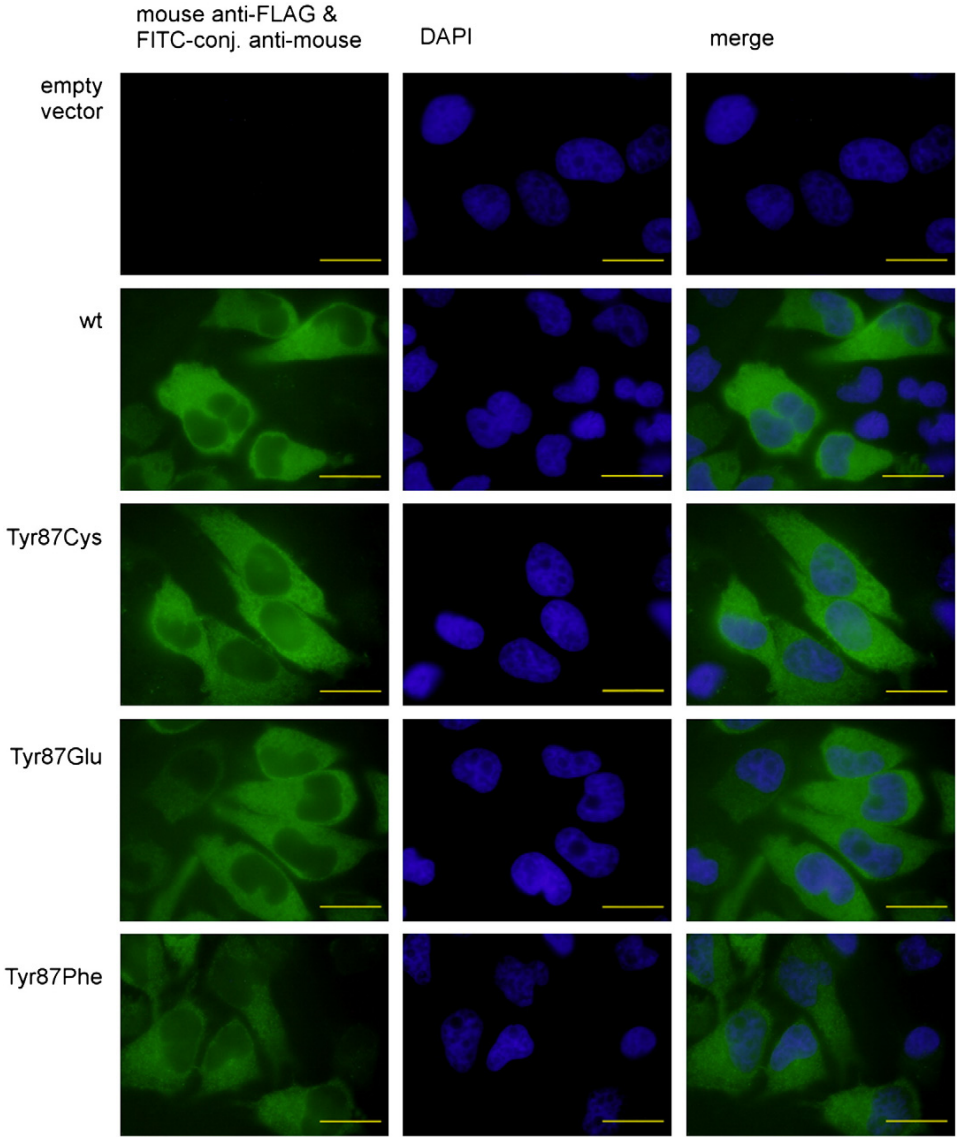

Fig. 2. Alterations of Tyr87 do not affect the cytosolic localization of 3xFLAG–PRMT3. HeLa cells transfected with either an empty 3xFLAG vector or one of the 3xFLAG–PRMT3 variants were fixed and stained with primary mouse anti-FLAG and secondary FITC-conjugated anti-mouse antibodies. The nuclei were visualized with DAPI. The scale bar indicates 20 μm.

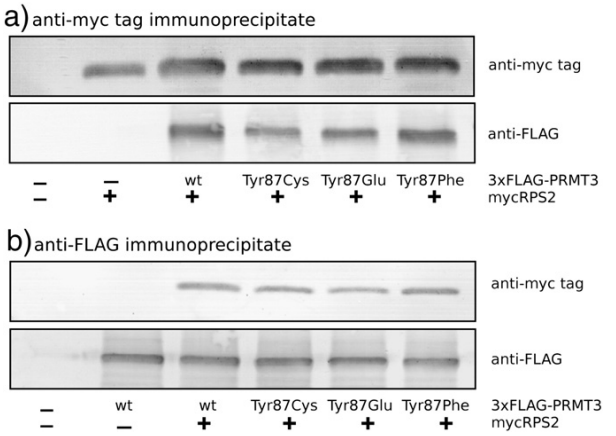

Fig. 3. Western blots of anti-myc and anti-FLAG immunoprecipitates. Complexes of myc-RPS2 with the respective 3xFLAG–PRMT3 variants were immunoprecipitated using either an anti-myc antibody (a) or anti-FLAG antibody (b). myc-RPS2 and 3xFLAG–PRMT3 were detected by anti-FLAG and anti-myc tag antibodies, respectively.

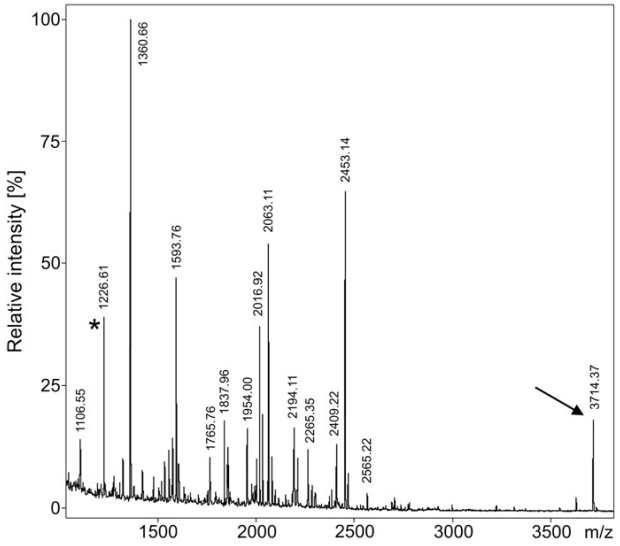

Fig. 5. The MALDI-TOF mass spectrum of the 3xFLAG–PRMT3 tryptic digest after Fe-IMAC phosphopeptide enrichment. The intense peak corresponding to the unmodified peptide ^{82}HGLEFYGYIK^{91} was found at m/z 1226.62 (labeled by an asterisk). No signal of this peptide with putative phosphorylation at Tyr87 was detected at m/z 1306.59. The only phosphorylation observed was at the doubly phosphorylated N-terminal peptide ^{13}GAVENEEDLPELSDSGDEAAWEDEDDADLPHGK^{45} (m/z 3714.37, marked by arrow). The identity of phosphopeptides was verified by MS/MS analysis.

alone were observed repeatedly. This is in full agreement with similar findings previously reported by Choi et al. [20], who demonstrated that overexpressed RPS2 was rapidly ubiquitinated in cells under normal conditions, but that simultaneous expression of PRMT3 reduced this ubiquitination and thus stabilized the RPS2 protein independently of the PRMT3 methyltransferase activity.

As shown in Fig. 4, relative affinities of 3xFLAG–PRMT3 variants to myc-RPS2 reflect the observed changes in enzymatic activities (see Fig. 1). The Tyr87Cys–3xFLAG–PRMT3 variant had a significantly decreased relative affinity to myc-RPS2 ($66 \pm 15\%$ of the wt-PRMT3, $n = 8$, $p < 0.01$). Even lower affinity to myc-RPS2 ($49 \pm 13\%$, $p < 0.01$) was observed for the Tyr87Glu mutant. On the other hand, the relative affinity of the Tyr87Phe mutant ($88 \pm 13\%$) was not significantly decreased.

Our results thus suggest that Tyr87 plays an important role in its interaction with RPS2, and therefore in the proper function of PRMT3. Since the glutamate mimics a phosphorylated tyrosine, our results also predict that the hypothesized phosphorylation of tyrosine 87 would negatively regulate this enzyme.

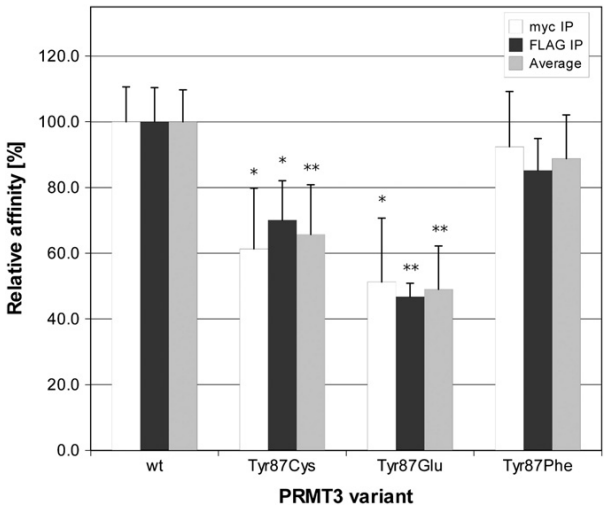

Fig. 4. Tyr87 is important for the RPS2–PRMT3 interaction. The 3xFLAG–PRMT3/myc-RPS2 complex was isolated in a cross-wise manner, using either an anti-myc antibody or an anti-FLAG antibody. The presence of the interacting protein partner was detected by anti-FLAG or anti-myc antibodies, respectively, and quantified densitometrically. The data were then normalized and expressed as relative values to the wt-3xFLAG–PRMT3/myc-RPS2 pair. The average values obtained from 4 anti-myc and 4 anti-FLAG immunoprecipitations are shown in the graph, along with average values calculated from all 8 imunoprecipitations. Values significantly different from wt at $p < 0.05$ and $p < 0.01$ are marked by one and two asterisks, respectively.

3.5. PRMT3 is phosphorylated in vivo on serines 25 and 27, but not tyrosine 87

Phosphorylation is a reversible posttranslational modification that allows fast and efficient modulation of enzymatic activity. Based on the results mentioned above, we predict that Tyr87 phosphorylation would negatively regulate PRMT3 methyltransferase activity. Therefore, our next goal was to identify putative phosphorylated residues in PRMT3 directly using mass spectrometry.

The stoichiometry of tyrosine phosphorylation is generally low; therefore, we performed phosphopeptide enrichment on an Fe-IMAC column prior to the MALDI-TOF analysis. We observed phosphorylation on serines 25 and 27 (both residues in one peptide), in accordance with a recent observation by Dephoure et al. [21]. However, there was no evidence of Tyr87 phosphorylation, although the peptide ^{82}HGLEFYGYIK^{91} was clearly detected in the mass spectrum (Fig. 5).

4. Discussion

Positively charged arginines are often involved in hydrogen bonds and interactions with aromatic residues of proteins or nucleic acids. Methyl groups attached to arginine not only act as a steric hindrance preventing hydrogen bond formation but also increase the hydrophobicity of arginine and thus favor non-polar interactions. The positive induction effect of methyl has only a minor effect on arginine basicity, and methylation thus impacts protein properties less than posttranslational modifications affecting amino acid charge, such as

phosphorylation or acetylation. Rather than acting as an on/off switch, arginine methylation modulates protein–protein and protein–RNA interactions and serves to fine-tune various cellular processes. The importance of protein methylation is also demonstrated by the high energy cost per transfer of one methyl group, which has been calculated to be 12 ATPs (reviewed in [2]).

Many ribosomal proteins have been reported to be methylated on either lysines or arginines, but the role of these modification remains unclear. Protein arginine methyltransferase 3 (PRMT3), which modifies the small ribosomal subunit protein RPS2, has been shown to be involved in the regulation of ribosome biogenesis. Developmental delays and ribosome maturation defects in yeast and mouse knockouts of the PRMT3 homolog resemble the *Minute* phenotype of ribosomal protein knockouts in *Drosophila* in having slower growth and mild developmental delay.

Here, we report a heterozygous point mutation in the *PRMT3* gene of one DBA patient leading to the Tyr87Cys substitution. Sequencing analysis of other family members excluded this mutation to be the cause of DBA in the patient.

Nonetheless, we decided to address the role of this Tyr87 mutation in more detail because it affects an evolutionarily conserved residue on the surface of the substrate-binding domain and, moreover, this tyrosine has previously been predicted to be phosphorylated.

In addition to the Tyr87Cys substitution and the wild-type enzyme, we therefore studied two additional PRMT3 variants, Tyr87Glu and Tyr87Phe, with glutamic acid mimicking a phosphorylated and phenylalanine a non-phosphorylated tyrosine, respectively.

Unlike most other methyltransferases, PRMT3 is localized in the cytosol. We showed that all three mutants, i.e., Tyr87Cys, Tyr87Glu, and Tyr87Phe, were also cytosolic and soluble, their distribution in the cytoplasm was homogenous and they did not associate with membranes or other cellular structures.

We further demonstrated that the Tyr87Cys mutation significantly reduced the enzymatic activity of PRMT3 towards its predominant substrate, RPS2. This decrease was caused mainly by the reduced affinity of mutated PRMT3 to RPS2. The substitution of the bulky aromatic tyrosine with a much smaller cysteine may eliminate the π or hydrophobic interactions involving this tyrosine. Similar behavior was also observed for the Tyr87Glu mutant, where the introduction of negatively charged glutamic acid likely had a strong impact on local surface properties. On the contrary, the interaction of the Tyr87Phe variant with RPS2 and its enzymatic activity did not differ from wild-type PRMT3, presumably because phenylalanine has similar size and physico-chemical properties as the substituted tyrosine. Our observations thus predict the possible impacts of Tyr87 phosphorylation.

Using mass spectrometry (MALDI-TOF) with metal-affinity (Fe-IMAC) enrichment of phosphorylated peptides, we did not observe any phosphorylation on Tyr87 of PRMT3, although we identified the phosphoserines 25 and 27 reported previously. The regulatory role of Ser25 and Ser27 phosphorylation on PRMT3 activity will be the subject of further studies.

In conclusion, Tyr87 is critical for the interaction between PRMT3 and RPS2 and for its full enzymatic activity.

Acknowledgments

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