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Molekulární mechanismy Diamondovy-Blackfanovy anémie

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Contents

1 Annotation

Diamond-Blackfan anemia (DBA) is a rare congenital syndrome that presents with normochromic macrocytic anemia and reticulocytopenia (a selective deficiency of erythroid precursors). Approximately half of the patients display additional somatic anomalies and growth retardation. The therapy is mostly symptomatic and is dominated by corticosteroids.

At the beginning of this work, only two DBA causal genes were known, encoding ribosomal proteins (RP) S19 and S24, 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 both putative and confirmed DBA causal genes in the Czech DBA registry.

To date, over a half of DBA patients have been reported to carry a mutation in one of nine RP genes, including RPS17, RPL11 and RPL5, that were identified by our team and are reported in this dissertation. We further studied a rare sequence variant in one non-ribosomal protein participating in ribosome biogenesis, 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.

2   Anotace

Diamondova-Blackfanova anémie (DBA) je vzácný vrozený syndrom, jehož hlavními příznaky je normochromní makrocytická anémie a retikulocytopenie (selektivní deficience erythroidních prekurzorů). Přibližně polovina pacientů vykazuje další somatické anomálie a zpomalený růst. Terapie je symptomatická, ve většině případů jsou podávány kortikosteroidy.

Na počátku této práce byly známy jen dva geny kauzální pro DBA, a sice geny kódující ribosomální proteiny (RP) S19 a S24, a jejich mutace byly nalezeny u čtvrtiny pacientů s DBA. Cíli mého výzkumu bylo popsat důsledky těchto kauzálních mutací na úrovni buňky, navrhnout pravděpodobné kandidáty na další geny kauzální pro DBA, a provést screening mutací v potvrzených i domnělých kauzálních genech u pacientů v českém registru DBA.

Doposud byla u více než poloviny pacientů nalezena mutace v jednom z devíti genů kódujících ribosomální proteiny, včetně RPS17, RPL11 a RPL5, které byly popsány naší skupinou a jsou předmětem této disertační práce. Dále jsme studovali význam jedné vzácné sekvenční varianty neribosomálního proteinu, který se účastní biogeneze ribosomů, protein arginin methyltransferasy 3 (PRMT3). Varianta PRMT3 nalezená u jednoho z našich DBA pacientů nebyla plně funkční, ale její kauzální roli pro DBA jsme nepotvrdili.

Naše poznatky přispěly k rozšíření spektra známých genů kauzálních pro DBA a podpořily hypotézu, že mechanismus patogeneze tohoto onemocnění spočívá v ribosomálním defektu.

3 Review of literature

3.1 Ribosomes, their composition and biogenesis

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). They are not only the most abundant macromolecular complexes but also one of the largest ones, with about 200 Å in diameter.

Composition of ribosomes

Ribosomes consist of two subunits, small and large, that are designated according to their sedimentation coefficients as 40S and 60S in eukaryotic ribosome; the completed eukaryotic ribosomes being sometimes referred to as 80S. Eukaryotic ribosomes contain four molecules of ribosomal (r) RNA and 79–81 ribosomal proteins (RPs) [1]; see Fig. 1.

Large ribosomal subunit contains three rRNAs (5S, 5.8S, and 28S), small ribosomal subunit harbors one (18S) rRNA. All four rRNAs are subjects to many posttranscriptional modiffications that facilitate the proper rRNA folding during ribosomal assembly and are required for a correct interaction with ribosomal proteins, and these modifications are also functionally important for the catalysis of peptide bond formation.

There are 32 human small subunit RPs and 47 large subunit RPs, however, the *exact* number of RPs per ribosome (slightly) differ even between individual ribosomes, because some RPs can under certain cellular conditions dissociate from mature ribosomes [3]. The size of RPs ranges from over 5 to almost 50 kDa, and most RPs are very basic, with a pI>10, which is in concordance with their ability to bind the acidic backbone of ribonucleic acids [1]. Ribosomal proteins are subjects of a rich spectrum of posttranslational modifications (PTMs). Some of them are vital for ribosome biogenesis [4], others affect the process of translation or play other roles [5].

The catalysis of the peptide bond formation is carried out by the longest rRNA of the large ribosomal subunit. To maintain a highly
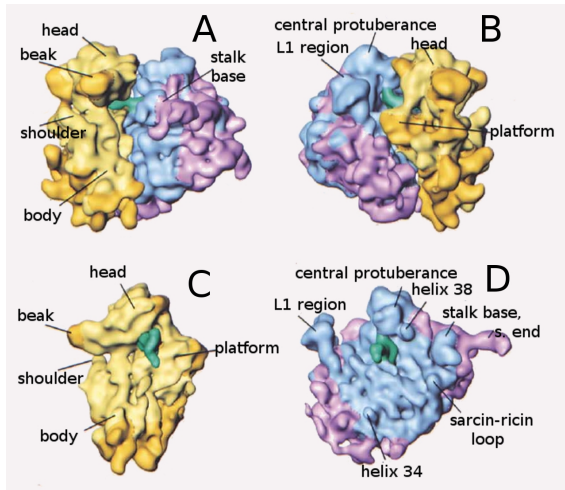
Figure 1: Eukaryotic ribosome and its separate subunits.
Illustration of 80S ribosome based on the cryo-electron microscopic analysis of the yeast ribosome. Yellow: small subunit, blue and violet: large subunit components, green: catalytic centre. Dark yellow and violet denote proteins that don't have homologs in prokaryotes. Adapted from [2]

organized 3D structure of the catalytic centre, the flexible ribonucleic acid backbone is fixed by interactions with other rRNAs and ribosomal proteins. RPs are vital for rRNA maturation during ribosome biogenesis, and for the interaction with proteins involved in translation and nascent protein folding. Some RPs also play extraribosomal roles; for details, see [6].

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 [7, 8, 9].

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). In this large precursor particle, various posttranscriptional modifications of pre-rRNAs and also first pre-rRNA cleavage steps occur.

In next step, the 90S particle divides into the precursor of the small (pre-40S) and large (pre-60S) ribosomal subunit. Most small subunit RPs and also some large subunit RPs are required for the processing of the pre-RNAs of the respective subunit and for the subunit assembly. Biogenesis of the large subunit is much more complex and requires a large number of non-ribosomal components (small RNAs and proteins).
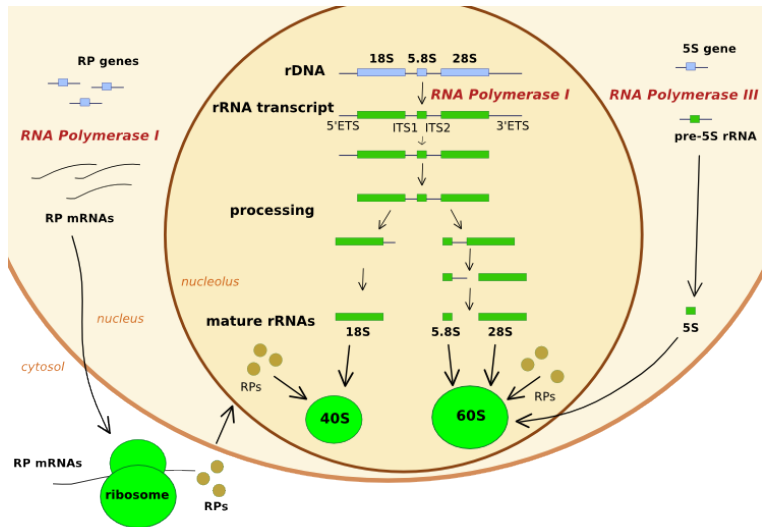

Figure 2: Ribosome biogenesis.
Schematic view of ribosome biogenesis; see text for further description. Altered from `http://www.edk.fr`.

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 (reviewed in [3]). This last step

depends on the methylation of a small subunit component, protein RPS2, by a dedicated protein arginine methyltransferase 3 (PRMT3) [4, 10, 11]. As already mentioned, further changes in the structure and properties of ribosomes (introduction of PTM, dissociation or re-association of several non-essential ribosomal proteins) may occur post-assembly.

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.

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.

Mutations in RP genes and in other components of ribosome biogenesis pathway have two major consequences: first, limited rate of ribosome biogenesis, and thus limited translational capacity. Second outcome is accumulation of free ribosomal proteins in nucleolus, which in turn activate the protein p53 and induce so-called *nucleolar* or *ribosomal stress response* [12, 13].

The capability to activate p53 pathway has so far been reported for several RPs, and the mechanism how individual RPs trigger p53 stabilization and activation differs. The most common one includes a specific binding of RPs to the ubiquitin ligase Mdm2 and thus blocking its enzymatic activity [12, 14]. Inhibition of Mdm2, a major negative regulator of p53, opens up the way to p53 accumulation and activation.

The outcomes of p53 pathway are diverse, most notably downregulation of anabolic pathways (including ribosome biogenesis and translation), delay or block in cell cycle progression, and/or increased susceptibility to apoptosis (Fig. 3). Mutations in RP genes develop in model animals a distinct phenotype known as *minute*, manifesting with smaller

body size and various somatic anomalies. Defects in ribosome biogenesis, caused either by mutations in RP genes or in other genes vital for the process, are associated with congenital syndromes termed *ribosomopathies.*
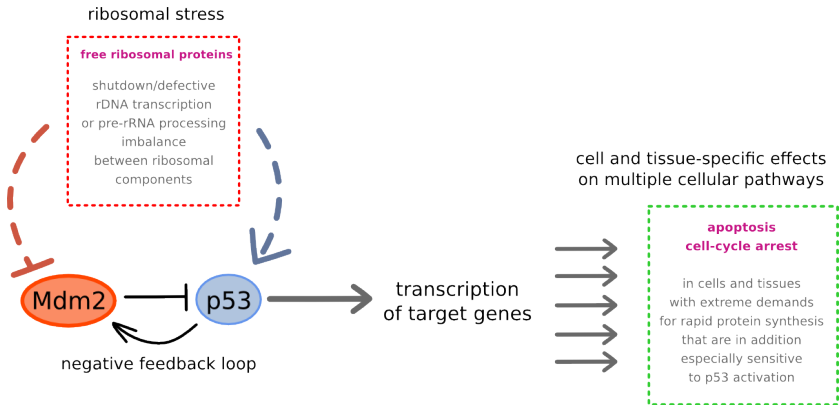
ribosomal stress

free ribosomal proteins

shutdown/defective
rDNA transcription
or pre-rRNA processing
imbalance
between ribosomal
components

cell and tissue-specific effects
on multiple cellular pathways

Mdm2   p53   transcription of target genes

negative feedback loop

apoptosis
cell-cycle arrest

in cells and tissues
with extreme demands
for rapid protein synthesis
that are in addition
especially sensitive
to p53 activation

Figure 3: Ribosomal stress response.
Defects at any stage of ribosome biogenesis lead to activation of p53, which in turn triggers various tissue-specific responses.

Ribosomopathies

Ribosomopathies [15] 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. The spectrum of causal genes is very diverse, ranging from RP genes, genes vital for rRNA maturation, or other genes encoding non-RPs participating in ribosome assembly, and in most cases, the products of these genes play also other cellular roles. Therefore, the underlying mechanisms of pathogenesis is very complex.

All ribosomopathies affect mainly tissues extremely sensitive to the ribosomal stress, i.e, rapidly growing and proliferating cells. Prenatally, the ribosomal stress causes abnormal development of embryonal tissues and leads to physical anomalies and growth retardation. Postnatally, the

most profound defects are seen in hematopoietic tissue and in epithelia. The 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.

3.2   Diamond-Blackfan anemia

Diamond-Blackfan anemia (DBA, OMIM: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) and profound reticulocytopenia (erythroblasts represent less than 5% of nucleated cells in the bone marrow). Almost half of DBA patients are born small for gestational age and display slower growth. Every other patient also display additional congenital malformations, mostly affecting head, upper limbs, genitourinary tract and heart [16, 17, 18].

The majority of DBA cases are sporadic, familiar cases show autosomal dominant inheritance. The incidence is 5–7 cases in one milion of live births, and both sexes are affected equally and no ethnic predominance was observed so far.

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) [19]. Most of the patients are infants or small children, which must be also taken into account in the selection of the therapeutic approach.

Causal genes

The first DBA causal gene to be identified was a gene encoding small ribosomal protein (RP) S19 [20], and this gene is mutated in approximately 25% of DBA patients. Later, several other RP genes encoding components of both ribosomal subunits were identified (*RPS* genes and *RPL* genes), and their mutations are found in over additional 30% of DBA patients (the statistical data from different DBA registries vary).

Currently known DBA causal genes include *RPS19* [20], *RPS24* [21], **RPS17** [22] (see Publication 1), *RPS7* [23], *RPS10* and *RPS26* [24], **RPL5** and **RPL11** [23, 25] (see Publication 2), *RPL35a* [26]. Genes listed in **bold face** are subjects of articles that are part of this work.

Probable mechanism of DBA pathogenesis

The original hypothesis expected a specific role of RPS19 in the erytroid maturation, however, such role has never been confirmed. With the identification of mutations in additional RP genes in DBA patients, DBA began to be regarded as a ribosomal syndrome.

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.

Reduced capacity of protein synthesis can also result from mutations in other molecules involved in translation, however, the clinical manifestation of such mutations is dissimilar to DBA [27]. In contrast, DBA resembles in many aspects other congenital aplastic anemias (Fanconi anemia [28] or congenital dyserythropoietic anemia type I [29]). Although their respective causal genes are not involved in ribosome biogenesis or function, these anemias are also associated with p53 activation, and subsequent prolonged differentiation and/or increased apoptosis of erythroid precursors.

4 Aims of the work

Specific aims

- 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

Experimental design

At the begining of our work, mutations in *RPS19* gene were reported in 1/4 of the patients, and the only other known DBA causal gene was *RPS24*, being affected only in a few individuals. The mechanism of DBA pathogenesis was unknown.

Our original approach to find promising candidates for DBA causal genes was based on the hypothesis that proteins rarely act on their own, but rather in multiprotein complexes. We reasoned that a suitable candidate protein would interact with wild-type RPS19, but not its patient variants. We planned to identify such proteins and to sequence their respective genes in our cohort of DBA patients.

Secondly, 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 [30], we focused on ribosomal proteins functionally related to RPS19.

Thirdly, after the spectrum of known DBA causal genes enlarged with large ribosomal subunit genes, we were attracted by a hypothesis that non-ribosomal proteins vital for ribosome biogenesis and/or function might also be affected in some DBA patients. We identified an unusual sequence variant of one such protein (PRMT3) in a DBA patient and decided to study its role in detail.

5 Materials and methods

The biological roles of candidate proteins were studied in model cell lines HeLa and K562. To distinguish the studied protein variants from their wild-type endogenous counterparts and to facilitate their detection, the proteins of interest were expressed as recombinant proteins with an oligopeptide tags (myc, 3xFLAG). Protein-protein interactions were studied using cross-immunoprecipitation of the protein complexes using the anti-myc and anti-3xFLAG antibodies. Methyltransferase activity of PRMT3 was assessed using an *in vivo* enzymatic assay using a radioactively labelled ^{3}H-methionine as a donor of methyl.

The genomic DNA for sequencing of the candidate genes in the cohort of patients of Czech DBA registry was isolated from peripheral blood mononuclear cells (PBMC). Individual gene exons were PCR-amplified using specific primers, and the agarose gel-purified fragments were then used for direct sequencing. In case a mutation was found, the expression of the affected alelle was examined, too, and the family members were also screened for the presence of the mutation. The cDNA for the expression analysis was obtained by reverse transcription of the RNA isolated from PBMC, using random hexaprimers and SuperScript II reverse transcriptase.

For further details regarding the materials and methods, please refer to the publications.

6 Results

We started our selection of promising candidates for DBA causal genes by the identification of proteins that are present in a multiprotein complex with wild-type RPS19 but fail to interact with its mutated variants.

In our pilot co-immunoprecipitation studies, we identified small subunit components RPS2 and RPS3 as proteins missing in complexes of mutated RPS19 protein. 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 38 patients from 33 families), it can't be excluded that RPS2 or RPS3 might be affected in some patients from other DBA registries.

Since 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 [30], we focused on other ribosomal proteins functionally related to RPS19. This protein resides in a close vicinity to the binding site for the eukaryotic initiation factor eIF-2 [31]. Interestingly, the second DBA causal gene to be reported, RPS24 [21] was also localized in this ribosomal region, and we reasoned that some of the other RPs residing in this area might be involved in DBA pathogenesis as well. We sequenced the five genes that encode these neighboring RPs (RPS3a, RPS13, RPS16, RPS17 and RPS24) in our patient cohort, and identified a mutation in RPS17 gene (see Cmejla et al., 2007, **Publication I.**).

Identification of a mutation in a large ribosomal subunit protein, namely RPL35a [26], in a DBA patient brought a strong support to the hypothesis that DBA is a ribosomal defect. Later, mutations affecting two other 60S subunit proteins, RPL5 and RPL11, were reported [32]. Interestingly, RPL5 and RPL11 were known to stimulate the p53-stress response. 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, **Publication 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 initial experiment in which we identified RPS2 and RPS3 as candidate proteins. Both of them are methylated on arginines [4, 33], and this modification is important in the small ribosomal subunit assembly in eukaryotes. Methylation of RPS2 is important for the association of the small and large ribosomal subunits, while 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. The RPS3-specific methyl-transferase PRMT5 has not been sequenced yet. 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, **Publication III.**).

7 Conclusions

- We identified a novel DBA causal gene *RPS17*

- We reported a correlation between mutations in *RPL5* or *RPL11* and thumb defects

- We completed screening of 38 DBA patients for mutations in all confirmed DBA causal genes

- We further identified a rare sequence variant in a non-ribosomal protein PRMT3 in one patient, however, we excluded the causality of this gene for DBA

- Our data support the hypothesis that DBA is caused by a defect in ribosome biogenesis

8 Discussion

Diamond-Blackfan anemia is a rare congenital syndrome that presents with anemia and selective deficiency of erythroid precursors, and almost half of the patients display additional somatic anomalies and/or growth retardation. All eight currently known DBA causal genes encode a protein of cytosolic ribosomes, but the causal genes are still to be identified in approximately 40% of DBA patients.

The opinions on DBA pathogenesis have undergone dramatic changes during the course of this study (years 2006–2011). At the begining of this project, RPS19 was the only known DBA causal gene, and since it was mutated in 1/4 of all patients, it was supposed to have an erythroid-specific role. With the identification of rare mutations in several other small subunit RP genes (including RPS17, first reported by our group in [22]), the general view shifted to an explanation based on lower ribosomal pool and inefficient translation.

Later, mutations in large ribosomal subunit proteins have been reported (RPL35a [26], and RPL5, RPL11 [23] and [25]), which further confirmed the classification of DBA as a ribosomal defect. This pointed to the possibility that non-ribosomal proteins vital for the ribosome assembly might be causative for DBA, too. In one DBA patient, we identified a rare sequence variant in one such protein, PRMT3 (see our publication [34]). However, we didn't prove any causal connection between this protein variant and DBA.

Current oppinions on the mechanism of DBA pathogenesis are based on a ribosome biogenesis defect, and consequent inefficient translation and activation of ribosomal stress response [35, 36, 37]. Although ribosomes are indeed important for all human tissues, the most striking manifestation of human RP gene mutations is anemia. Since no erythroid-specific role of RPs has been found, this seemed to bee due to the high translational demands of maturating red blood cells in comparison to the demands of other lineages [38].

However, mutations in other human genes vital for protein synthesis (initiation or elongation factors, tRNA charging enzymes, etc.) aren't associated with anemias [27]. In contrast, mutations in other house-keeping genes (responsible for DNA repair or cell cycle progression control) are

causal for several types of congenital anemias [28, 29] that are also associated with additional somatic anomalies. The best studied examples are Fanconi anemia, as well as several types of congenital dyserytropoietic anemia. The common mechanism of pathogenesis of these inherited anemias include the activation of p53 pathway.

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. Hematopoietic and especially erythroid progenitors are (i) hypersensitive to various types of stress [39, 40, 41], and (ii) they are especially prone to apoptosis [42, 43]. A lower treshold of cellular stress required for p53 activation is also observed in other rapidly proliferating cells, including embryonal tissues. The additional somatic anomalies of DBA patients thus can be caused by aberrant p53 activation during embryogenesis.

To summarize, the activation of p53 appears to be more important for the phenotype of DBA than the reduced ribosomal pool and thus decreased translational capacity, and it is tempting to consider reclassifying DBA as a syndrome resulting from the p53 activation [37, 44]. 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.

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?

In conclusion, 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.

9   List of publications

1. 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.

   Impact factor (2007): 6.273

2. 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.

   Impact factor (2009): 6.887

3. 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.

   Impact factor (current, by 28th June, 2011): 2.480

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