mRNA translation regulation in Diamond Blackfan Anaemia

by

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ISBN 978-80-8126-075-9

Cover: Tim Thomas
Layout: Egied Simons
Printing: VIENALA, s.r.o., Košice

mRNA TRANSLATION REGULATION IN DIAMOND BLACKFAN ANAEMIA

mRNA translatie regulatie in Diamond Blackfan Anemie

PROESCHRIFT

ter verkrijging van de grad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof. dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 8 mai 2013 om 13:30 uur

door

Rastislav Horos
geboren in Košice, Slowakije

PROMOTIECOMMISSIE

Promotor:	Prof. dr. I.P.Touw
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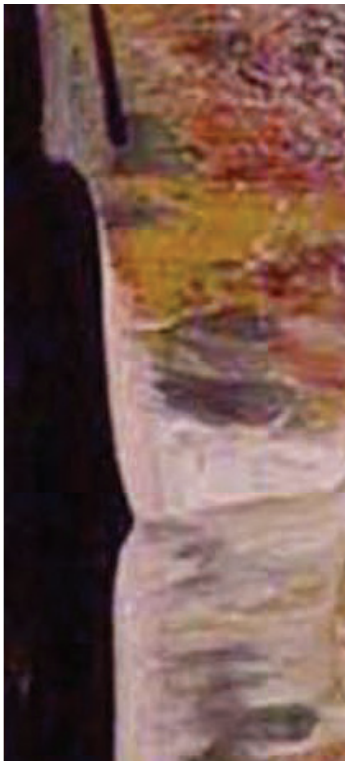

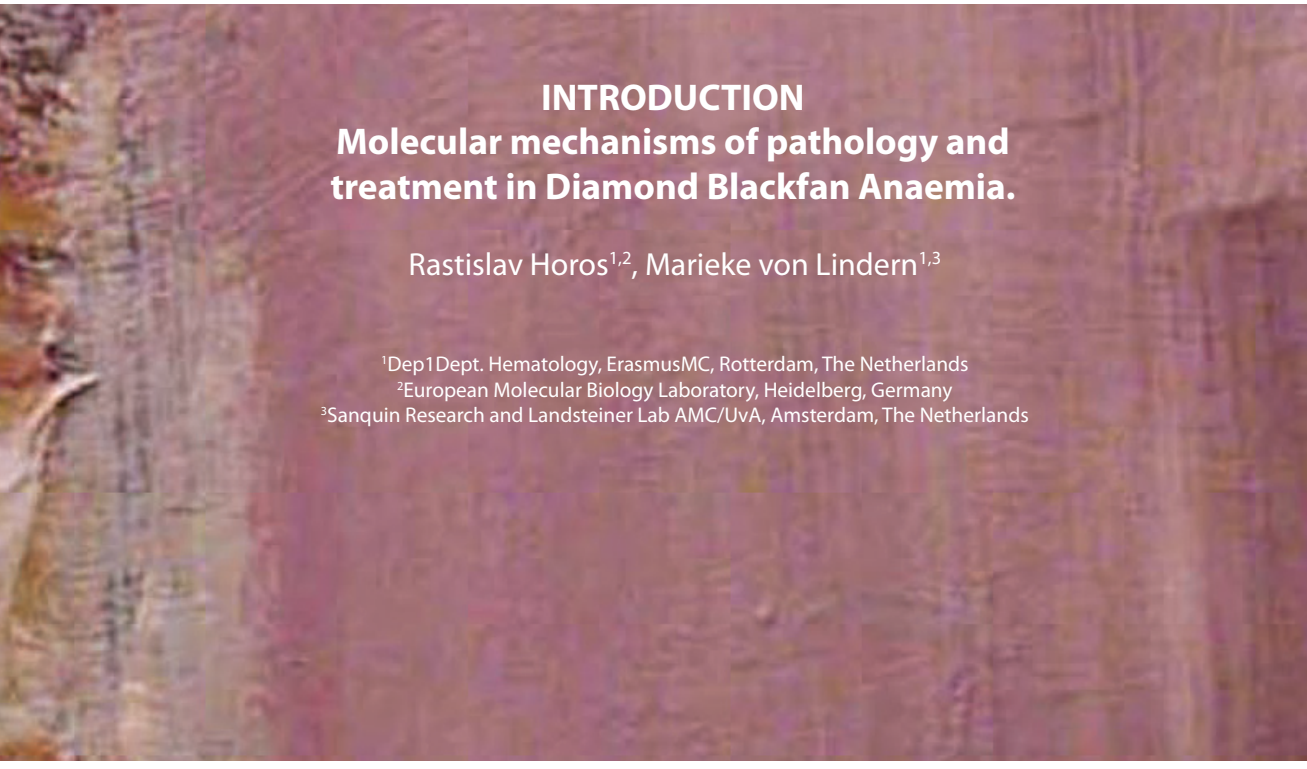

INTRODUCTION
Molecular mechanisms of pathology and treatment in Diamond Blackfan Anaemia.

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British Journal of Haematology 2012
Dec;159(5):514-27

Haematopoiesis and erythropoiesis

Haematopoiesis (from Greek "blood production") is the continuous process that generates mature blood cells. In adults, all blood cell lineages are produced in the bone marrow from haematopoietic stem cells (HSC)[1], and HSCs can repopulate and restore haematopoiesis in mouse transplantation experiments[2]=. HSC have both the capacity for self-renewal and multipotent differentiation when adopting an asymmetric division into one daughter HSC and a fate-restricted progenitor cell. At the very top of the haematopoietic hierarchy (Figure 1) reside long-term (LT-HSC) and short-term reconstituting HSCs (ST-HSC)[3] together with multipotent progenitor cells (MPP). They are characterized by expression of Sca1 and c-KIT and absence of any restricted lineage markers (Lin-), therefore also designated as the "LSK compartment"[4]. MPP give rise to two separate common progenitors – lymphoid (CLP), responsible for formation of B, T and NK cells, and myeloid (CMP), which further differentiate into granulocyte-monocytic progenitors (GMP) and megakaryocytic-erythroid progenitors (MEP). The GMPs are the predecessors of granulocytes and monocytes/macrophages lineages. MEPs are bipotential and differentiation generates the megakaryocytic and erythroid cells[5].

Figure 1

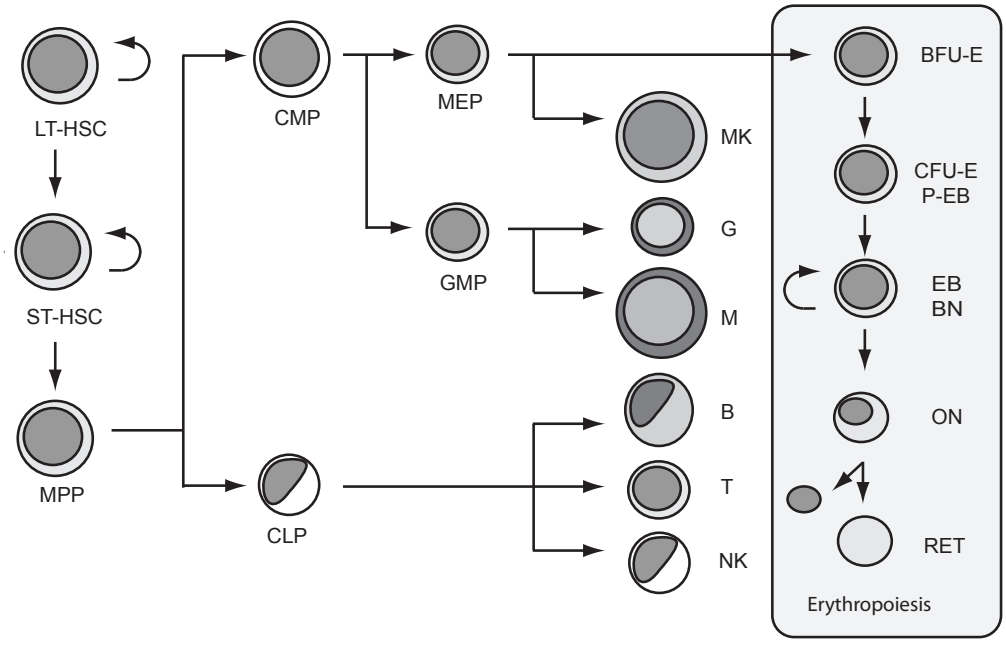

Figure 1. Haematopoiesis and erythropoiesis. Simplified schematic drawing of haematopoiesis and erythropoiesis. LT-HSC, long term haematopoietic stem cell; ST-HSC, short term HSC; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocytic-erythroid progenitor; GMP, granulocyte-macrophage progenitor; MK, megakaryocyte; G, granulocyte; M, monocyte; B, B-cell; T, T-cell; NK, natural killer cell; BFU-E, burst forming unit-erythroid; CFU-E, colony forming unit-erythroid; P-EB, proerythroblast; EB, erythroblast; BN, basophilic normoblast; ON, orthochromatic normoblast; RET, reticulocytes. Adapted from [5].

Figure 2

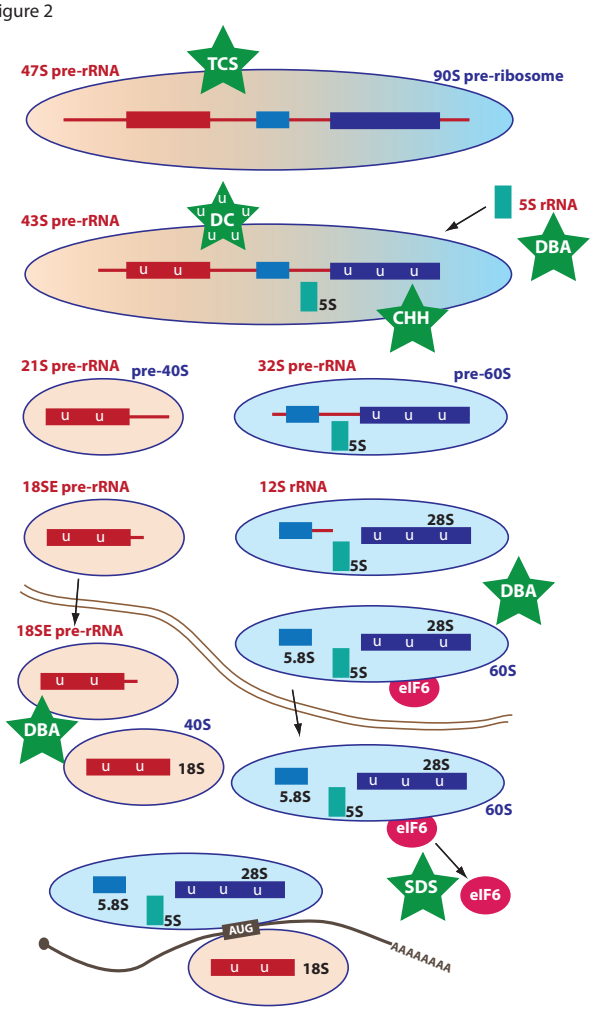

Figure 2. Ribosome biogenesis and ribosomopathies. Ribosome biogenesis starts with transcription of the 47S pre-rRNA by DNA polymerase I, which is impaired in Treacher Collins Syndrome (TCS; mutations in Pol-I subunits). Many ribosomal proteins and ribosome synthesis factors associate with the 47S pre-rRNA to form the 90S pre-ribosome. Processing of the pre-rRNA starts with cleavage of the external regions which results in the 43S pre-rRNA. During processing the rRNA undergoes many chemical modifications such as uridynilation (u) which requires proteins mutated in Dyskeratosis Congenita (DC). The 5S rRNA is transcribed in the nucleus and is transported to the 90S pre-ribosome by Rpl5 and Rpl11, two ribosomal proteins found to be mutated in Diamond Blackfan Anaemia (DBA). The 43S pre-rRNA is further cleaved to produce 21S and 32S fragments. Cleavage of the mitochondrial pre-rRNA requires proteins that are mutated in Cartilage Hair Hypoplasia (CHH). After the formation of 21S and 32S, the pre-90S changes into the pre-40S containing 21S pre-rRNA and the pre-60S containing the 32S pre-rRNA. The 21S is cleaved to 18SE after which the pre-40S is translocated to the cytoplasm. In the cytoplasm the 18S E is cleaved to yield the mature 18S rRNA. The 32S pre-rRNA is first cleaved in a mature 28S rRNA and a 12S pre-rRNA that is processed to the mature 5.8S rRNA. Ribosomal proteins mutated in DBA are involved in maturation of the ribosomal subunits, although the mechanism is not yet clear. The 60S ribosomal subunit translocates to the cytoplasm, which requires a transport complex that includes eukaryote Initiation Factor 6 (eIF6). This transport complex needs to be dissociated in a process that requires the protein mutated in Schwachman Diamond Syndrome (SDS), to allow for the association of the 40S and 60S ribosomal subunit at an AUG startcodon of a mRNA. This cartoon is a highly simplified image of ribosome biosynthesis. For a more complete description, please see [190-192].

The differentiation stages of the early erythroid lineage arising from the CMP are classically defined by their colony-forming potential when cultured *in vitro* in methylcellulose. The earliest common precursor cells are the colony forming unit – granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). These give rise to a bipotential progenitors that are restricted only to a erythroid/megakaryocyte lineage and expresses the Epo receptor[6,7]. Further lineage specification leads to the earliest erythroid-only restricted stage called burst forming unit-erythroid (BFU-E). This cell typically generates large and diffuse colonies of many thousand cells, and expresses the EpoR at the low levels[8,9]. The next stage is the colony forming unit-erythroid (CFU-E) cell that forms small, hemoglobinized colonies consisting of up to 64 cells. CFU-E expresses the EpoR at a higher level and form small, distinct colonies that eventually become red in the center due to the hemoglobin synthesis. The CFU-Es (proerythroblasts and their transition state to erythroblasts) can be cultured in vitro in suspension cultures in the presence of Epo, stem cell factor (SCF) and glucocorticoids[10,11]. They are characterized by a high proliferative potential and expression of the transferrin receptor (CD71), remodeling cytoskeletal proteins and adhesion molecules. They also represent the majority of erythroid precursors residing in the bone marrow and appear as large nucleated erythroid cells. Upon induction of differentiation by a high concentration of erythropoietin (Epo) they divide 3-4 times using a cell cycle regulation that is uniquely not accompanied by cell-size control[11]. During terminal differentiation towards reticulocytes, the cells produce large amounts of hemoglobin and eventually expel their nucleus in a process termed enucleation.

Inherited bone marrow failures

Inherited bone marrow failure syndromes (IBMFS) are hematological disorders characterized by a deficiency in one or several of the heamatopoiteic lineages. Initially patients may suffer from the lack of a specific lineage, but the disease may progress to marrow failure hallmarked by aplastic anemia. These conditions are often associated with other somatic abnormalities, increased risk of cancer development and are typically diagnosed in childhood[12]. Genes mutated in IBMFS are involved in basic cellular processes such as chromosome biology, translation, signal transduction and cytoskeletal organization of cells.

Fanconi anemia (FA) is caused by mutations in one of the many of Fanconi anemia genes that are involved in DNA damage repair[13]. Affected cells are hypersensitive do DNA crosslinking agents and display a high frequency of chromosomal abnormalities. Patients progress to overall marrow failure and cancer.

Patients suffering from **Severe congenital neutropenia** (SCN) display profound neutropenia which often progresses to myelodysplasia and leukaemia. Two thirds of the patients have mutations in neutrophil elastase (*ELA2*)[14], which cause accumulation of non-functional ELA2 protein in the endoplasmatic reticulum and triggers an unfolded protein response[15]. Mutations in other genes such as *HAX1*[16], *GFI1*[17] and *WASP*[18] had been identified in a minority of patients and lead to perturbations in actin remodeling. A mutation in the G-CSF receptor (CSF3R) is often found in the leukemic phase of the disease and contributes to unrestricted signaling upon ligand binding [19].

Congenital amegakaryocytic thrombocytopenia (CAMT) is characterized by absence of platelets and megakaryocytes and aplastic anemia with no associated physical abnormalities. Patients bear inactivating mutations in the *c-MPL* gene (which encodes the thrombopoietin receptor)[20] and is important for HSCs maintenance and megakaryocytic differentiation.

Several IBMFS are characterized by mutations in genes that are involved in ribosome function and biogenesis. Though not having a hematological phenotype, the **Treacher Collins Syndrome** (TCS) is of interest for this thesis because it is also a disorder of ribosome biogenesis. Patients born with TSC bear mutations in the *TCOF1* gene[21], which protein product *treacle* is implicated in rRNA processing. It interacts with upstream binding factor (UBF) and facilitates rRNA methylation[22].

One third of **Dyskeratosis congenita** (DC) patients carry mutations in the dyskerin gene (*DKC1*) that is important for pseudourylination of ribosomal RNA, but also associates with telomerase[23]. Typical features of DC are abnormal skin pigmentation, nail dystrophy, aplastic anemia and a high risk to develop cancer. DC is caused by a defect in any of the core proteins of the H/ACA box snoRNA associated proteins DKC1, NOP10 and NHP2[24,25], all involved in ribogenesis. H/ACA box snoRNAs also associate with the telomerase complex. Telomerase consists of two important units, the telomerase RNA (TERC) that contains a H/ACA box domain, and TERT, the telomerase reverse transcriptase[25-27]. Mutations in TERC or TERT have been found in DC and are autosomal dominant[24,27]. TERC and TERT are responsible for the maintenance of the telomeres and telomere length and a reduced activity of the telomerase complex was found in all patients with DC, regardless of their genetic background. Autosomal dominant mutations in TERC and TERT cause a less severe phenotype compared to mutations in genes involved in both ribogenesis and telomerase activity. Moreover, most TERC mutations do not immediately cause disease but presentation and onset of the disease increases in successive generations[24].

Interestingly, induced pluripotent stem (iPS) cells generated from DC patient fibroblasts harboring *DKC1* and *TERC* mutations can fully restore telomerase activity and telomere length[28]. *DKC1* is an X-linked gene, whilst *NOP10* and *NHP2* are autosomal recessively inherited. The dyskeratin defect in DC patients has no effect on general protein synthesis, but causes an impairment in translation of IRES containing transcripts from both endogenous and viral origin, suggesting a defect in the function of the ribosome[26,29,30]. It would be interesting to study whether mutations of the other H/ACA snoRNP proteins also specifically cause defects in IRES translation. This, however, still needs to be investigated.

Shwachman-Diamond syndrome (SDS) is characterized by neutropenia, exocrine pancreatic insufficiency and metaphyseal dystosis[31]. SDS is caused by a homozygous mutation in the Shwachman Bodian Diamond syndrome gene (*SBDS*)[32]. The yeast homologue Sdo1 has been shown to be necessary for the release of the 60S ribosomal unit from the Tif6 protein that transports the subunit from the nucleus to the cytoplasm. The human Tif6 homologue *EIF6* is also associated with the 60S subunit. Because binding of Tif6/*EIF6* and the 40S subunit to the 60S subunit are mutually exclusive, Tif6/*EIF6* has to be dissociated from the 60S subunit before the 80S ribosome

can be formed[33]. EIF6 is a rate limiting protein in export of the 60S subunit[34], failure to release EIF6 will block the further export of 60S subunits, while cytoplasmic 60S subunits bound to the EIF6 are not available for translation.

In fibroblasts derived from SDS patients the amount of 60S is not altered[35,36]. Yet, all rRNA production is reduced as well as general translation[37]. Loss of SBDS has a negative effect on the expression of many ribosomal genes including some that are mutated in DBA (*RPL5*, *RPS7*, *RPS17*). Many other genes important in ribogenesis and mRNA translation are downregulated in cells from SDS patients, including DKC1, NOP58 and fibrillarin, proteins involved in the modification of rRNA[38]. *SBDS* knockout mice are embryonically lethal[39] and knockdown with morfolinos of the *SBDS* homologue in zebrafish causes a phenotype comparable to the human disease[40]. It has been demonstrated that deletion of *Sbds* in mouse osteoprogenitor cells induces bone marrow dysfunction with myelodysplasia[41], suggesting a role of deficient microenvironment in bone marrow failure progression. However, this observation may be also accounted to the fact that conditional knock out of *Sbds* will disturb gene expression and thereby cellular function in most cell types. Further light into the role of Sbds was uncovered in a recent publication from Warren's lab. The authors showed that Sbds mediates GTP hydrolysis by the GTPase elongation factor-like 1 (Efl1) on the 60S subunit. This is required for the release of eIF6 from 60S subunit[42].

Diamond Blackfan Anemia

Diamond Blackfan Anemia (DBA) was first described in 1936 by Joseph. In 1938 Diamond and Blackfan discriminated the clinical phenotype of DBA from other disorders. DBA is a rare pure red cell aplasia affecting around 7 individuals per million live births. In 40% of patients it is associated with other clinical abnormalities, such as craniofacial, thumb, kidney and heart malformations and growth retardation[43]. Classically it presents within the first year of life, but non-classical cases of DBA are emerging, usually in a parent after the diagnosis of DBA in the probands. The basis for DBA diagnosis is macrocytic anemia with no other significant cytopenias, reticulocytopenia and normal bone marrow cellularity with a paucity of the erythroid precursors. Supporting criteria may be the positive history of the disease in the family, elevated erythrocyte adenosine deaminase (eADA) activity or congenital abnormalities that are usually associated with DBA[44]. Predisposition to cancer (including myelodysplastic conditions and AML) was reported to be 4%; which is less than in other IBMFs. The treatment of choice in DBA is corticosteroid administration. Close to 80% of the patients respond to steroids with an increase of hemoglobin levels within a month after initiation of the treatment[44]. There are, however, limitations to steroid usage in infants, because it can alter the individuals' growth and seriously inhibits the activity of immune system. Interestingly, the maintenance dose of steroids in DBA therapy is highly variable and does not correlate with the type of the identified mutation. Some patients even enter a symptom-free phase or may require only very low doses of steroids. Patients that do not respond to corticosteroids require regular blood transfusions with all associated risks. To avoid complications due to iron overload associated with regular blood

transfusion, iron chelators are used[45]. The only curative treatment of DBA is haematopoietic stem cell transplantation, which restores the hematological indices to normal levels[46,47]. However this treatment is limited by HLA matched donor availability and the age of the patients. Several alternatives are being tested including treatment with the essential amino acid leucine[48] which was administered as a part of the patient's diet.

The most enigmatic feature of DBA is the fact that around half of the patients bear a mutation in one of the structural protein of ribosome, an essential component in the protein synthesis machinery.

Mutations in genes encoding ribosomal proteins

Mutations in *RPS19* were first identified by Dahl and colleagues in a DBA patient carrying a congenital chromosomal translocation (X;19)[4]. Further screening indicated that a quarter of the DBA patients carry a mutation in *RPS19*. The mutations varied greatly and include non-sense, frameshift and splice variations that all result in haploinsufficiency[50,51]. Correction of Rps19 expression restored in vitro erythropoiesis, which further implicated Rps19 in DBA [52].

The complete 80S eukaryotic ribosome consists of the small, 40S subunit and the large, 60S subunit. Each of them contains ribosomal RNA (rRNA) and structural ribosomal proteins. RPS2 to RPS29 are structural proteins in the small subunit, RPL3 to RPL41 contribute to the large subunit[53]. Mutation analysis for all ribosomal proteins in DBA patients without *RPS19* mutations revealed mutations in genes encoding *RPS24*[54], *RPS17*[55], *RPL35A*[56], *RPS7*, *RPL5*, *RPL11*[57], *RPS10*, *RPS26* or *RPL26*[58]. These mutations mostly cause haploinsufficiency or production of mRNA variants identified that do not change the amino acid sequence but may change transcript stability or translation efficiency [57,58].

Genome-wide single nucleotide polymorphism arrays did not reveal mutations in novel genes, but indicated the presence of deletions targeting loci that were previously implicated in the pathophysiology of DBA. Notably deletions encompassing genes encoding ribosomal proteins were identified in 9 of 51 DBA samples studied[59]. In 4 patients the deletion affected *RPS17*, a duplicated gene of which both copies can be expressed. DBA samples carried a deletion of both tandem *RPS17* genes[59]. The Japanese register reported 3 *RPS17* deletions in 27 DBA patients[60]. A list of all mutated genes and the frequency at which they occur is given in Table I.

Mutations in *RPL5* and *RPL11* may be associated with a more severe phenotype than mutations in *RPS19*, particularly with respect to skeletal deformations[57]. In general, however, there is little or no correlation between the mutation and the severity of the disease.

Despite genome wide exome sequencing, the mutations underlying DBA remained uncertain in approximately 40% of patients. It has been hypothesized that these patients may carry mutations in genes encoding non-structural proteins involved in ribogenesis, but so far such mutations in this group of genes have not been found[58,59]. Instead, two siblings with corticosteroid dependent DBA and one unrelated patient were recently shown to carry a mutation in the erythroid transcription factor *GATA1*[61]. The mutations impaired splicing and induced expression of a short isoform of GATA1. Mutations in *GATA1* were previously shown to cause distinct haematological

phenotypes, including anemia and thrombocytopenia[62]. Interestingly, both siblings had a history of lower neutrophil counts and one patient had lower platelet counts, which is not common in DBA, and the patients did not suffer from skeletal abnormalities. With such few patients, it is unclear whether the patients with GATA1 mutations are true DBA patients or whether these patients and possibly part of the other patients that lack mutations in ribosomal proteins should be classified as variant CDA (Congenital Dyserythropoietic Anaemia) similar to patients with mutations in the transcription factor KLF1[63].

Table 1. Mutations of ribosomal protein genes in Diamond Blackfan Anaemia

Gene	Subunit	Frequency*	Reference
RPS19	40S	25	[193]
RPS24	40S	2	[54]
RPS17	40S	5[#]	[55,59]
RPL35A	60S	2	[56]
RPL5	60S	7	[57]
RPL11	60S	6.5	[57]
RPS7	40S	1	[194]
RPS10	40S	2.5	[194]
RPS26	40S	6.5	[58]

*frequency may depend on the cohort size
[#] including gene deletions

Defective ribosome synthesis in DBA

It was a great surprise when mutations in *RPS19* were found in DBA[49]. How could a molecular process that is so central to all cells predominantly cause a failure to produce erythrocytes? This initially led to the hypothesis that extra-ribosomal functions of RPS19 are involved. For instance, it was reported that murine Rps19 binds to the serine/threonine kinase Pim1, which is important to recruit Pim1 to polysomes[64]. It is difficult, however, to reconcile specific extra-ribosomal functions of ribosomal proteins with the observations that DBA can be caused by haploinsufficiency of a range of ribosomal proteins. It could also be envisioned that the ribosomal proteins involved in DBA are located at a specific surface of the ribosome. Crystallography of the ribosomal subunits showed that this is not the case: ribosomal proteins engaged in DBA are scattered all over the surface of the ribosome [65,66].

The first indication that ribosomal proteins mutated in DBA affect ribosome biosynthesis (ribogenesis) came from experiments in yeast that indicated that reduced expression of Rps19 led to a defect in the processing of the precursors of rRNA[67]. The role of DBA associated ribosomal proteins in ribogenesis has been confirmed in mammalian cells[68,69]. Insight into the synthesis and export of ribosomal subunits is essential to understand DBA and related syndromes with defects in ribosome synthesis such as TCS, DC, and SDS. Together these syndromes are indicated as ribosomopathies[70].

Ribosome biogenesis – connections to "ribosomopathies"

Ribogenesis involves over 170 different ribogenesis factors, around 80 ribosomal proteins and more than 100 snoRNAs[71,72]. Because ribogenesis consumes a vast amount of the cell's energy, it is tightly co-regulated with cell cycle progression, growth and proliferation[73]. Mammalian cells have hundreds of copies of tandemly repeated ribosomal DNA (rDNA) that are simultaneously transcribed to pre-ribosomal RNA (pre-rRNA) in the nucleolus. The 47S pre-rRNA is transcribed by RNA polymerase I (Pol-I) to be processed to mature 28S, 18S and 5.8S rRNA. The 5S rDNA is transcribed by RNA polymerase III (Pol-III) in the nucleus[74]Swann Building, Kings Buildings, Mayfield Road, Edinburgh EH9 3JR, Scotland.</auth-address><titles><title>Identification of prote. Transcripts for ribosomal proteins are transcribed by RNA polymerase II (Pol-II) in the nucleus, translated in the cytoplasm and the ribosomal proteins subsequently translocate to the nucleolus to assist in ribosome assembly.

Transcription of the 47S pre-rRNA requires binding of upstream binding factor (UBF) and promoter selectivity factor (SL1) to the promoter region. Subsequently several other factors including Pol-I associate with transcription initiator factor 1A (TIF1-A/Rrn3) which are then recruited to initiate gene transcription[75-77]. Genes encoding Pol-I subunits C and D have been found mutated in TCS, which results in suppression of rRNA transcription which causes an imbalance between rRNA availability and the synthesis of ribosomal proteins[78]. The 47S pre-rRNA construct comprises of two external transcribed spacers (ETS) at the 3' and 5'side, and the 18S, 5.8S and 28S rRNAs interspersed with two internal transcribed regions ITS1 and ITS2. This pre-rRNA is processed in a multiprotein complex, containing small nucleolar RNA (snoRNA), structural ribosomal proteins and non-ribosomal proteins, called pre-90S complex. In this complex the rRNA undergoes a series of endonucleolytic and exonucleolytic cleavages that are depicted in Figure 2.

Cells in which RPS19 expression is reduced contain less 18S rRNA, while the 18SE pre-rRNA is increased[68,69]. Not only *RPS19* haploinsufficiency, but also reduced expression of several DBA-related ribosomal proteins affects ribogenesis and maturation of the small and the large ribosomal subunits[79,80]. The 5S rRNA gene is transcribed by Pol III after binding of TFIIIA to the promoter region. Uniquely, TFIIIA binds not only the promoter region of the gene, but also the transcribed 5S rRNA. The complex of 5S and TFIIIA is translocated as a 7S RNP to the cytoplasm where 5S rRNA is released from TFIIIA and binds ribosomal proteins RPL5 and RPL11 to form RNP 5S. Ribosomal protein RPL5 contains nucleolar localization signals (NLS) on both the C and N terminus of the protein, which allows for translocation of RNP 5S to the nucleolus, where it is recruited to the pre-90S complex and used in the assembly of the 60S ribosomal subunit[81,82]. Although there is little or no genotype-phenotype relation in DBA, patients with mutations in *RPL5* and *RPL11* tend to show a more severe erythroid and non-erythroid phenotypes, which may be correlated with the fact that these two proteins have this specific function of including the 5S rRNA in the 60S subunit[83].

Maturation of ribosomal subunits not only requires the subsequent cleavage of rRNA and its association with ribosomal proteins, but also involves modification of rRNA by 2'O-ribose meth-

ylation and pseudouridylation[84]. Of note, mutations in the pseudouridylation enzyme dyskerin cause DC[23]. Modification and cleavage of rRNA depends on snoRNA associated proteins among which NOP10 and NHP2, two other proteins carrying mutations in a subgroup of DC patients, that are part of the same protein complex as dyskerin[24,25]. The Dyskerin complex is also required to modify the RNA component of the telomerase complex. Therefore, it is still controversial whether the haematological phenotype of DC is due to a ribosomopathy or a telomere problem in haematopoietic stem cells or both of these[85].

Once the 60S subunit has matured in the nucleolus it is transported to the cytoplasm in complex with eIF6 (Tif6 in yeast). Experiments in yeast showed that binding of Tif6 and the 40S subunit to the 60S subunit are mutually exclusive, Tif6 has to dissociate from the 60S subunit before the 80S ribosome can be formed[33]. eIF6 is a rate- limiting protein in export of the 60S subunit[34]. Failure to release eIF6 will block the further export of 60S subunits, while cytoplasmic 60S subunits are not available for translation. This release is mediated by SBDS which is mutated in approximately 95% of SDS patients[32,42].

Now that all ribosomal genes have been sequenced in a large cohort of DBA patients, mutations have not been detected in ribosomal proteins to which a specific function was assigned (apart of RPL5 and RPL11). This, together with defective maturation of rRNA in different ribosomal knockdowns[80], suggests that the function of mutated ribosomal proteins defective in DBA is mainly connected to the maturation of rRNA.

Qualitative versus quantitative differences of ribosomes in DBA

Whereas the distinct ribosomopathies give rise to different haematological phenotypes, they share common clinical features. Patients often display developmental abnormalities, such as growth retardation and skeletal defects, including craniofacial anomalies, an abnormal thumb, or absence of the radius. They also share an increased risk of neoplastic transformation. However, DBA, TCS, DC and SDS are distinct syndromes that show a very specific phenotype. This raises the question whether the deficiencies are due to quantitative defects in the ribosomes, or whether qualitative differences are involved as well.

Qualitative differences imply that distinct ribosomal proteins have specific functions in the context of the ribosome. This could be ribosome specific functions such as RPS15 interacting with eIF3 to recruit the 40S ribosomal subunit to the mRNA cap[86], or diverse functions of RPL22 or RPL23 which are positioned around the exit channel of the ribosome[87,88]. Ribosomal proteins may also interact with specific transcripts to regulate their translation. RPL13a, for example, functions to restrain IFNγ (interferon gamma)-induced gene expression[89]. Transcription of ceruloplasmin and V-EGF is induced by IFNγ. IFNγ-induced phosphorylation of RPL13a results in release of RPL13a from the 60S ribosomal unit. RPL13a subsequently binds to the so-called GAIT element in the 3'UTR of specific transcripts, which abrogates their translation[90,91]. Differential expression of ribosomal proteins in distinct tissues prompted Barna and coworkers to investigate the role of Rpl38 in mouse bone development, because Rpl38 expression is relatively high in skeletal tissue. Deletion of Rpl38 resulted in pronounced homeotic transformations of the

axial skeleton[92]. Deregulation of Rpl38 impairs translation of a specific set of Hox genes due to impaired IRES-dependent translation initiation. There are also examples of ribosomal proteins such as RPS13 in human and Rpl30, Rps40 and Rps24 in yeast, that can regulate splicing and turnover of their own transcripts[93]. Lastly, one of the most studied examples of extra-ribosomal function of ribosomal proteins is connected to p53 activation, which will be discussed below.

Lately, studies using profiling strategies unravel interesting functions for ribosomal proteins in various molecular processes. Using reporters of miRNA mediated translation repression, it was shown that knockdown of a plethora of ribosomal proteins relieves translational inhibition, thus causing de-repression[94]. This phenotype was the direct consequence of nucleolar stress, which points to p53 activation. Although this study left many open questions, it suggested that ribosomal proteins may function as inhibitors of polyribosome formation on miRNA-targeted mRNAs. The extent of the impact of ribosomal proteins on mRNA metabolism and translation is demonstrated by the protein composition of RNA granules formed in the cytoplasm, including stress granules, P bodies and others[95]. As many as 54 ribosomal proteins were identified as components of RNA stress granules. The stress granules contain non-translated mRNAs, therefore the presence of ribosomal proteins points towards their function as mRNA binding proteins[96]. The identification of proteins that were crosslinked to and purified on polyA RNA in HeLa cells under stringent conditions recently allowed for establishment of the so-called mRNA interactome[97], which also contains many ribosomal proteins. It has not been analysed whether the ribosomal proteins affected in DBA bind an overlapping set of (erythroid specific) transcripts.

Impaired erythropoiesis in DBA

The major characteristic of DBA is anaemia. In contrast to other bone marrow failure syndromes such as Fanconi Anemia, the anaemia is a pure red cell aplasia and not the result of general haematopoietic insufficiency and loss of haematopoietic stem cells. At diagnosis, mostly young children (<1 year of age) are presented at a hospital with critically low levels of erythrocytes, while white blood cells are less affected[98]. Haploinsufficiency of ribosomal proteins occurs in all cells of the haematopoietic system, however the bone marrow of DBA patients appears normocellular and colony forming units granulocytic-monocytic (CFU-GM) are not significantly different. The number of burst forming units-erythroid (BFU-E) is severely reduced or even absent in transfusion dependent DBA patients[99-101]. NOD/SCID mice repopulation studies with CD34+ cells from DBA patients showed that the stem cells failed to give rise to the erythroid lineage[99,100]. In two-stage in vitro cultures, it was specifically the Epo-dependent phase in which cells failed to expand[102]. Knock down of *RPS19* in human CD34-positive stem cells corroborated the findings with DBA-derived bone marrow. Rps19 was reduced by shRNA-mediated knock down in CD34+ haematopoietic stem and progenitor cells isolated from cord blood or bone marrow. Outgrowth of non-erythroid progenitors was not significantly affected by RPS19 knock down, but in vitro expansion of erythroid cells was severely reduced at the stage of BFU-E and CFU-E[103,104]. The pathophysiological mechanisms together with proposed and current treatments are depicted schematically in Figure 3.

Figure 3

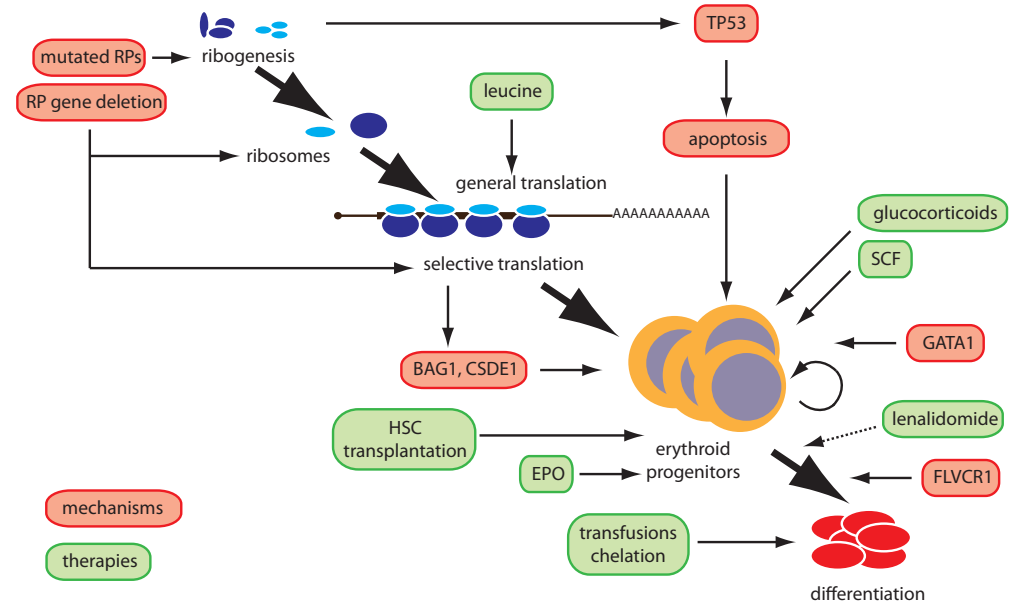

Figure 3. Pathophysiology and treatment in DBA. Ribosomal proteins function in ribosome biosynthesis and become a structural part of mature ribosomes. The availability of ribosomes controls mRNA in general, but some transcripts are hypersensitive to ribosome availability. FLVCR1, BAG1 and CSDE1 are among the proteins that are specifically deregulated in erythroblasts of DBA patients. Reduced expression of Bag1 and Csde1 in mouse erythroblasts impairs their differentiation capacity. FLVCR1 is a haem transporter and affects iron overload in transfusion dependent DBA. Haploinsufficiency of a ribosomal protein causes a disbalance between ribosomal proteins and rRNA, which activates p53 and may lead to apoptosis and loss of erythroblasts. When DBA patients do not carry a mutation in ribosomal proteins, they may carry other mutations that affect erythropoiesis such as Gata1, although this variant DBA is not a ribosomopathy. Therapies in DBA aim to replenish erythrocytes (*i*) by transfusion, which requires concurrent treatment with iron chelators, or (*ii*) by transplantation of haematopoietic stem cells (HSC). Treatment with glucocorticoids increases the compartment of early erythroid progenitors, while lenolidomide may increase the ability of erythroblasts to differentiate. Leucine supplements may be a well tolerated treatment that enhances general protein synthesis. Treatment with EPO and/or SCF may be less effective because EPO levels are already high, and SCF enhances undesired mast cell proliferation.

To mimic haploinsufficiency in vivo, it was attempted to generate mice carrying a single mutated allele, but mice carrying a knock-in mutation in all cells appeared to be non-viable. Recently, conditional mouse models were generated in which a mutation in Rps19 was introduced by coexpression with a CRE-recombinase allele, or in which a shRNA able to reduce Rps19 expression could be induced from a doxycyclin-inducible promoter[105,106]. In concordance with the human disease, bone marrow of these mice contained normal levels of CFU-GM, but a severely reduced number of BFU-E and CFU-E.

The failure to generate mice with a constitutive haploinsufficiency of ribosomal proteins precludes the comparison of haematopoietic and non-haematopoietic defects in DBA models. To this end, zebrafish models have been very useful, because they show a variety of developmental defects (see below, role of p53 in DBA).

Bone marrow failure syndromes are associated with an increased risk to develop cancer. Although the cancer risk in DBA is low in comparison to other bone marrow failures such as

Fanconi Anemia, there is a significantly increased risk for solid tumors. Among 608 patients of the North American register, who had not received a bone marrow transplant, there were 15 patients that developed solid tumors, 2 developed acute myeloid leukaemia's, and 2 myelodysplastic syndrome at a median age of 41 years[107]. Notably, zebrafish in which expression of ribosomal proteins was reduced developed solid tumors at high incidence[108,109]. Therefore the zebrafish model seems ideal to study the mechanism of carcinogenesis in ribosomopathies.

Ribosomal stress: activation of p53

Ribosome synthesis can require up to 25% of a cell's energy. As a major guardian of cell cycle, growth and apoptosis, p53 and its downstream pathway is activated upon defective ribosome biogenesis[110]. When a disturbed balance in the synthesis of rRNA and ribosomal proteins results in an excess of ribosomal proteins, the free ribosomal proteins bind MDM2/HDM2 to shield p53 from ubiquitination and degradation. Particularly RPL5 and RPL11 were identified to bind Mdm2 and cause p53 stabilisation[111,112]t. Such a mechanism seems plausible for haploinsufficiency of e.g. RPS19, however, it does not explain why haploinsufficiency of RPL5 or RPL11 itself has a relatively severe phenotype. Recently, mutations in yet another ribosomal protein gene which regulates the activity of p53 via its interaction with MDM2, RPL26, was identified in a DBA patients[58]. Currently, the phenotype of ribosomopathies is mainly ascribed to activation of p53, but how p53 is activated in the context of DBA is not fully resolved[113].

The hypersensitivity of erythroid progenitors to p53 has been described in the context of Friend erythroleukaemia, where loss of p53 is a requirement for leukemic progression[114]. Detection of p53 in primary erythroid progenitors, however, is difficult. This is further complicated by the fact that p21, the classical p53 target gene that is often used to report p53 activation, is a Stat5 target gene in haematopoietic cells[115]. In erythroid cells, however, p27 and not p21 inhibits cell cycle progression[116].

Reduced expression of ribosomal proteins in zebrafish induced several developmental defects including impaired erythropoiesis. The role of p53 activation in the developmental defects seemed well established, while in erythropoiesis it is so far a matter of debate[117-119]. On the other hand, in a mouse model lacking Rps19, abolition of p53 could rescue erythropoiesis[106].

mRNA translation in ribosomal protein deficiencies

Following the identification of a mutated ribosomal protein in DBA, analysis of mRNA translation in cells of DBA patients became of interest. Measuring the rate of protein synthesis in lymphoid cells from peripheral blood of DBA patients indicated variable (48-73%) reduction of global translation in RPS19 mutated DBA patients as well as in patients without known mutations[120]. This indicated that reduced protein synthesis capacity is a general feature of DBA.

Conventional RNA expression profiling indicated a reduced expression of a large cluster of transcripts encoding ribosomal proteins as well as translation factors and tRNA transferases in either CD34+ haematopoietic stem cells, lymphocytes or fibroblasts of DBA patients[83,121]. This indicated that mutation of a single ribosomal protein results in reduced translation capacity due

to a general downregulation of the protein synthesis machinery. Indeed, knock down of a large number of distinct ribosomal proteins of the 40S subunit is associated with decreased expression of other ribosomal proteins for the same subunit, as well as with a decrease in the total amount of mature subunit, 80S complex and polysomes[80]. Thus, there may not be a specific function for distinct ribosomal proteins in ribosome biogenesis, but rather the reduced availability of any ribosomal protein changes the rate of ribosome biogenesis.

It is generally accepted that the conventional mRNA profiling does not necessarily represent actual protein expression and that the translation efficiency is the predominant regulator in gene expression[122]. Indeed, the "crosstalk" between the expression of the subunits components is interesting: it was shown that in mouse hepatic cells depleted of Rps6, *Rpl11* mRNA is actively recruited to the polyribosomes and translated[123]. The observation that protein synthesis is also decreased in lymphoid cells and fibroblasts, and that this phenomenon is not restricted to erythroid cells, is in accordance with general developmental defects in many DBA patients such as small stature and a reduced number of haematopoietic stem cells[120,121]. However the pronounced erythroid defect suggests that erythroid progenitors may express specific RNAs that are hypersensitive to the decreased translation capacity. Previously, we showed that the expansion of proerythroblasts relies upon the growth factor dependent activation of mTOR and subsequently S6 kinase (S6K) and 4E-BP via a defined signalling cascade[124]. We have shown that this leads to the preferential translation of an array of mRNAs, which are essential for the proliferative phenotype of the late erythroid compartment. One of these genes, alpha4 (also known as Igbp1), is one of many regulatory subunits of PP2A phosphatase that specifically inhibits the phosphatase activity towards mTOR targets 4EBP and S6kinase[125].

Therefore, detailed investigation of mRNA translation efficiency in the erythroblasts deficient for ribosomal proteins could indicate how impaired ribosome synthesis impact on the qualitative mRNA translation output in the diseased cells.

DBA treatment options

Glucocorticoids. Initially, the response of DBA patients to glucocorticoid hormones suggested that DBA was an autoimmune disease, similar to glucocorticoid sensitive aplastic anaemia. However, the finding that the erythroid defect in DBA is cell autonomous at the level of the BFU-E implicated that glucocorticoids may directly improve the fate of affected erythroid progenitors.

Glucocorticoid hormones enhance proliferation of erythroblasts and enhance the delay of differentiation in presence of SCF[126-128]. Suppression of the immune system by glucocorticoids requires the monomeric form of the glucocorticoid receptor (GR) to bind to transcription factors of the Jun/Fos family or of the NF-κB family. Such immunosuppressive effect requires pharmacological concentrations of glucocorticoids (~1 μM)[129,130]. Sustained erythropoiesis, however, requires dimerisation of the GR and its action as a bona fide transcription factor[128,131]. Target genes include genes crucial for proliferation of erythroblasts such as the SCF receptor c-Kit and the transcription factor Myb[127,128]. Among the target genes in erythroblasts are also genes known to be involved in apoptosis and cell cycle arrest of T-cells, such as *Tdag8* (T-cell death associated

gene 8, also known as *Gpr65*)[132]. Regulation of many apoptosis inducing genes, however, was counter regulated by SCF signalling[132]. A largely overlapping set of glucocorticoid target genes was also identified in human erythroblasts[103]. Also, in this study, part of the glucocorticoid target genes was counteracted by SCF signalling (such as the redox control genes *TXNIP* and *PON2*)[103]. Thus, the combined effect of SCF and glucocorticoids may be required to enhance expansion of the erythroid compartment, without the growth inhibitory effects that glucocorticoids impose on lymphoid cells or macrophages. In addition, the GR was shown to interact with the EpoR to modify Epo signaling, which specifically suppressed differentiation inducing signals initiated by the EpoR[133].

The role of glucocorticoids in erythropoiesis was controversial for a long time, partly because glucocorticoids act at physiological concentrations (5 nM). They may only be required to boost erythropoiesis upon major blood loss. Mice carrying a dimerisation deficient GR ($GR^{dim/dim}$) display normal erythrocyte numbers in peripheral blood. Upon induction of hypoxia, the spleen of wt mice rapidly enlarges, and becomes the site of stress erythropoiesis. The $GR^{dim/dim}$ mice, however, fail to show stress erythropoiesis in the spleen[131], which indicates that the glucocorticoid dependent expansion of erythroblasts in vitro mimics stress erythropoiesis in vivo.

The mechanism by which glucocorticoids can improve erythropoiesis in DBA is yet poorly understood. In vivo, mouse erythroblasts proliferate with a cell cycle time of 8,5 hours while they mature from a large proerythroblast to small reticulocytes[134]. In vitro, SCF delays differentiation of erythroblast, but the cells gradually get smaller and continue to differentiate into reticulocytes[11]. This loss of size control is typical for late erythroid differentiation and may pose an additional disadvantage for cells with reduced mRNA translation capacity. It is an attractive hypothesis that glucocorticoids control S-phase entry, similar to what has been observed in lymphocytes. Thus, glucocorticoids may not inhibit proliferation, but delay S-phase entry and maintain size control. This gives erythroblasts more time to synthesise sufficient protein and compensates the decreased mRNA translation capacity. Alternatively, the GR induces expression of genes that impact on mRNA translation. For instance, dexamethasone induces expression of the RNA binding factor Zfp36l2. Zfp36l2 is an essential gene for haematopoiesis, and a member of the Tristetraprolin family that binds an AU-rich sequence in the 3'UTR of mRNA to control mRNA stability and translation[132,135].

The human glucocorticoid receptor is highly polymorphic due to the occurrence of several single nucleotide polymorphisms (SNP) and alternative splicing. Mice only express the GR-α isoform, but human cells express the glucocorticoid responsive GR-α and the dominant negative GR-β isoform. The GR-β lacks exon 9, which changes the conformation of the GR resulting in constitutive nuclear localisation, but without ligand binding and transactivating capacity[136,137]. A nucleotide polymorphism (SNP) at position A3669G stabilises GR-β mRNA. The frequency of A3669G and expression of GR-β is increased in Polycythaemia Vera, but also in DBA[138,139]. Although there is a trend towards glucocorticoid resistance in DBA patients carrying the A3669G SNP, the presence of (*i*) the SNP, (*ii*) expression of the GR-β, and (iii) glucocorticoid responsiveness has to be investigated in a larger number of patients and suitable controls.

Stem cell factor. SCF causes proliferation of mast cells, which limits its clinical use. However, SCF promotes in vitro proliferation of erythroblasts derived from DBA patients and rescues BFU-E formation[140,141]. Notably, SCF prominently activates the phosphoinositide-3-kinase (PI3K) in mouse erythroblasts and is required to phosphorylate and activate S6K[125]. S6K, in turn, phosphorylates Rps6 which increases ribosome function[142,143]. In addition, PI3K activation phosphorylates UBF, which causes it to associate with RNA Pol-I and renders its activation for rRNA transcription[144,145]. It is not clear, however, whether increased rRNA transcription can compensate impaired rRNA processing.

Lenalidomide. Once a promising sleeping drug for pregnant women, thalidomides were banned 50 years ago for causing severe birth defects. Over the last 2 decades, however, thalidomides have proven their effectiveness as treatment for cancer and autoimmune disease. Lenalidomide is a more potent and less teratogenic derivative that appeared very effective to treat a subtype of myelodysplatic syndrome characterised by a deletion on chromosome 5q (5q- MDS). That deletion encompasses RPS14, and loss of RPS14 in CD34+ stem and progenitor cells was shown to cause a 5q- MDS phenotype, including severe anemia[146,147]. Whereas glucocorticoids increased the number of BFU-E colonies obtained from control, RPS14- or RPS19-deficient CD34+ cells, lenalidomide increased the number of CFU, moreover both drugs acted synergystically[148]. Lenalidomide also improved the hemoglobin and MCV of erythrocytes in *Rps6* heterozygously deleted mice, although the same was noted in the control mice[149]. The mechanism employed by lenalidomide is still elusive. It may serve as a kinase inhibitor of Cdc25c and pp2aCA[150], but it was also suggested to block cytokinesis[151]. Also, it can act as an agent intercalating in DNA and double strand RNA[152]. In 5q- MDS, lenalidomide suppresses the 5q- clone and restores erythropoiesis from wt stem cells[154]. In DBA, however, there are no wt clones to restore erythropoiesis. A clinical trial, executed with utmost care, will have to show whether lenalidomide can improve erythropoiesis in DBA patients, or whether it exhausts the erythroid compartment further by accelerating differentiation.

Leucine. Leucine was initially described to increase translation via the rapamycine sensitive pathway[155]. Subsequently this was shown to be mediated via mTOR activation of S6K and 4E-BPs[156]. Addition of L-leucine, one of the essential amino acids, to the lymphocytes from non-RPS19 mutated DBA patients was shown to increase the overall translation rates in vitro[120]. This prompted for a small trial where the daily intake of leucine in DBA patients was increased. One of four patients showed a consistent health improvement, including increased haemoglobin and MCV levels, as well as increased BFU-E numbers from bone marrow[48]. From the 4 patients in this first trial, one became transfusions independent, whereas in three other patients, appetite, growth and well-being were considerably improved. Leucine also showed promising effects in animal models of DBA. Administration of leucine improved the erythrocyte counts and haemoglobin in peripheral blood upon induction of *Rps19* knockdown in a mouse model[157]. Interestingly, the frequencies of stem and progenitor cells, and of the erythroblast population were decreased,

which suggested that leucine enhanced differentiation of erythroid progenitors. Administration of leucine to zebrafish embryos treated with morpholinos against *rps19* and *rps14* also increased the hemoglobinization as well as number of *gata-1* expressing erythroid cells[158]. Recently, it was shown that leucyl-tRNA-synthetase (LRS) senses increased intracellular levels of leucine, upon which it binds the Rag GTPase as its effector protein. Rag activates mTORC1, which leads to increased translation rates[159]. The activation of mTORC1 was insensitive to intracellular levels of amino acids when the leucine-binding motives on LRS were mutated. It is of interest to investigate whether this molecular pathway is intact in cells of DBA patients, or whether leucine concentration has to be raised to levels that effectively signal towards the ribosome[160].

The benefit of increased mTOR activity in DBA cells could include both increased translation rates, and enhanced rRNA transcription via S6K activation[161]. Increased mTOR activity stimulates translation of eIF4E-sensitive transcripts, which is required for transient expansion of the erythroid progenitor compartment[125]. However, translation of these transcripts is already relatively increased in DBA models[123,162]. Decreased IRES-dependent translation initiation will not be improved by enhanced mTOR activity. On the contrary, mTOR activity will shift the competition between cap- and IRES-dependent towards cap-dependent mRNA translation.

In conclusion, it remains to be investigated how leucine causes its beneficial effect and whether it directly interferes with pathways that are deficient in DBA or whether it causes a general health improvement independent on the pathogenic mechanism of DBA.

Iron overload. Iron overload is a serious problem for the large cohort of DBA patients that require frequent blood transfusion. Therapy with iron chelators relieves the iron overload, but it is nearly impossible to restore a healthy balance. Because of its high oxidative potential, iron is both essential and toxic to cells. It is complexed to iron transporters and within the cell it is stored in mitochondria. Iron metabolism involves many 'chaperones' and transporters and dysfunction of these proteins leads to anaemia (for review see[163]). A major haem exporter in erythroblasts was initially cloned as the feline leukaemia virus subgroup C cellular receptor (FLVCR1)[164]. *Flvcr1*$^{-/-}$ mouse exhibit a strikingly similar phenotype to DBA and, moreover, alternative splicing of *FLVCR1* leading to its decreased expression was found in immature erythroid cells from DBA patients[165,166]. As a consequence, iron accumulation in early erythroblasts may be enhanced in DBA patients, which could lead to mitochondrial damage, leakage of reactive oxygen species (ROS) to the cytoplasm and activation of haem-regulated kinase (HRI, or eukaryotic translation initiation factor 2 alpha kinase 1, EIF2AK1). HRI senses lack of haem or an increase in ROS and phosphorylates eIF2a which subsequently leads to decreased general translation output[167]. The activation of HRI adapts the erythroid gene expression program to protect cells from adverse conditions[168]. Particularly for transfusion dependent DBA patients, it is important to investigate haem and iron transport in DBA erythroblasts, and the effect of iron chelators in more detail.

Other treatments. Before the cause for DBA was known, several other treatments were tested. EPO was a logical treatment in the first place, but without success, as the serum EPO levels are

usually normal or elevated in DBA patients[169]. Despite beneficial effects of Epo and IL-3 on the in vitro expansion of DBA derived erythroid cells[170], successful treatment with these growth factors was only sporadic and did not give significant improvement over the steroid therapy [171,172].

Regulation of mRNA expression by translation initiation control

In *Eukaryotes*, protein translation occurs in three consecutive steps: Translation *initiation*, during which the pre-initiation complex consisting of translation initiation factors and 40S subunit is assembled on 5'UTR of mRNA; it scans (in most cases) the 5'UTR mRNA until the start codon is recognized and a complete ribosome with 60S subunit is assembled. Next, translation *elongation* takes place with a ribosome proceeding on mRNA and synthesizing a polypeptide. Finally, when the termination codon is encountered, the ribosomal subunits dissociate from the mRNA and release the synthesized peptide, a step designated as translation *termination.* Ribosomal subunits as well as translation initiation factors are then recycled for a further round of translation or are degraded in the autophagy pathway[173]l/. The importance of translation regulation is highlighted by recent studies, which show that translation efficiency is a dominant regulator in gene expression[174].

The widest layer of control mechanisms is employed during the translation initiation, where various interactive events of many translation initiation factors, ribosomal subunits and mRNA take place[175]. The largest areas of the regulation of translation initiation include: (i) the cap-binding event and formation of pre-initiation complex, (ii) cap-independent mechanism of internal initiation via the internal ribosomal entry site (IRES), and (iii) phosphorylation of the α subunit of translation initiation factor 2 (eIF2). These mechanisms will be shortly discussed below.

Cap-dependent translation initiation. Cellular mRNAs receive at the 5'-prime end a modified nucleotide methyl-7-guanylate (m^7G), also termed as the 'cap'. This chemical modification is required for the transport of mRNA from the nucleus to the cytoplasm, prevents mRNA from degradation and, most importantly, facilitates translation initiation[176]. m^7G is recognized by the cap-binding translation initiation factor 4E (eIF4E), which, upon binding, recruits eIF4 A helicase and eIF4G bridging factor, thus forming the eIF4F complex. eIF4G can then interact with the poly A binding protein (PABP) which binds the polyA-tail and forms therein the 'closed loop' of mRNA which then attracts the ternary complex for mRNA scanning and AUG recognition[177]. The formation of eIF4F complex on the mRNA is thus the rate-limiting step in the cap-dependent translation initiation mechanism. This step is regulated through eIF4E binding proteins (4E-BP), which compete with eIF4G for binding to eIF4E. The binding activity decreases with their phosphorylation status[178]. 4E-BPs are phosphorylated by activated mTOR kinase, which is the main regulator of cell growth and proliferation[179]. Both Epo and SCF signal through their receptors to the PI3K/AKT pathway which activates mTOR. Therefore, these events are indispensable for the proliferation of erythroid cells. Previously, we showed that the

overexpression of eIF4E in erythroblasts activates a subset of mRNAs with long unstructured 5'UTRs which promote erythroid expansion[124].

Internal initiation. mRNAs that bear IRES sequence in their 5'UTR can be translated without the requirement of forming closed loop mRNA through initiation factors binding followed by ternary complex scanning. IRESes were initially discovered in viral mRNAs and the translation initiation mechanisms employed by IRESes differ between the viral families. Type 1 and 2 IRESes from *Picornaviridae* employ the eIF4G and eIF4A complex to recruit the ternary complex to the mRNA[180]. Type 3 IRESes (e.g. from hepatitis C virus) use binding of the ternary complex to the mRNA[181]. Finally, type 4 IRESes from cricket paralysis virus can directly attract the 40S subunit to the mRNA without the need for any initiation factor[182]. The IRES mode of translation initiation bypasses the regulatory event through 4E-BPs and some can bypass eIF2α phosphorylation, too. This is exploited by virus mRNAs in order to escape cellular translational shutdown and to deviate the cellular translation machinery for production of viral proteins. Several cellular mRNAs have IRES activity in their 5'UTR. Typically, these mRNAs code for proteins implicated in the stress response, apoptosis, cell cycle and differentiation; events, under which the canonical cellular translation undergoes a temporarily decrease[183].

eIF2α phosphorylation**.** The limiting translation initiation factor eIF2 is a GTPase that forms a heterotrimeric complex with initiator tRNA bound to methionine ($tRNA_i^{Met}$)[184]. This complex binds to the 40S ribosomal subunit and is part of the complex that scans the 5'UTR of mRNAs for the first start codon[185]. When a favorable start codon is recognized, GTPase activity is activated, methionine is deposited to the protein synthesis site of the ribosome, and tRNAi and GDP-eIF2 are released into the cytoplasm[175]. To reassociate with $tRNA_i^{Met}$ and enter another round of translation initiation, GDP on eIF2 has to be converted to GTP by the exchange factor subunit eIF2B. However phosphorylation of Ser51 on the eIF2α subunit prevents recycling of eIF2 for translation initiation and causes a global translation shutdown[186]. The protein kinases that phosphorylates eIF2α Ser51 are: Eif2ak1 (also known as HRI), which phosphorylates the eIF2α under heme deficiency; Eif2ak2 (PKR), which senses the presence of double stranded RNA e.g. from viruses in the cytoplasm; Eif2ak3 (PERK), which signals to eIF2α when the unfolded protein response is activated in the endoplasmatic reticulum; and, finally, Eif2ak4 (GCN2), which is regulated by the presence of the essential amino acids in the cytoplasm[187]. Some transcripts can escape the regulatory level of phosphorylated eIF2 by using alternative start codons and/or shortable

t upstream open reading frames (uORFs)[188]. Through this very mechanism a feedback loop is ensured; the ORF of the activated transcription factor 4 (Atf4) is translated under these conditions. Atf4 then activates the genes for proteins that counteract the activity of eIF2 kinases[189].

Aim of the thesis

Diamond Blackfan Anemia is a bone marrow failure syndrome in which patients lack erythroid precursor cells in the bone marrow. Therefore, it is also designated as pure red cell aplasia, as other hematological indices are at normal levels. Around half of DBA patients bear an autosomal dominant mutation in one of the ribosomal protein genes which leads to decreased cellular ribosomal content. How mutations in such general proteins can result in an erythroid restricted phenotype is as yet unknown.

In this thesis we aimed to elucidate the pathological mechanisms of erythroid failure in DBA. We hypothesized, that decreased ribosomal content in erythroid precursor cells could cause a decrease in the translation of SCF-dependent transcripts, which we identified previously[125]. For this purpose, we set up shRNA mediated knockdown of ribosomal proteins in proerythroblasts followed by translational profiling (**Chapter 2**). After we have confirmed that translational de-regulation of erythroid-specific transcripts occurs in DBA patients too, we further investigated their function. We aimed to characterize erythroid in vitro phenotype of Bag1 knockout mice and investigate the molecular role of Bag1 in the unfolded protein response (**Chapter 3**). Further, we set out to delineate the function of Csde1 in translational regulation during erythropoiesis (**Chapter 4**). Together, these data are summarized and discussed in **Chapter 5** where we also propose further experiments which could help to decode the pathophysiology of DBA at the molecular level.

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CHAPTER
2

Ribosomal deficiencies in Diamond-Blackfan-Anemia impair translation of transcripts essential for differentiation of murine and human erythroblasts

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Blood. 2012; 119(1):262-272

ABSTRACT

Diamond Blackfan Anemia (DBA) is associated with developmental defects and profound anemia. Mutations in genes encoding a ribosomal protein of the small (e.g. Rps19) or large (e.g. Rpl11) ribosomal subunit are found in over half of these patients. The mutations cause ribosomal haploinsufficiency, which reduces overall translation efficiency of cellular mRNAs. We reduced expression of *Rps19* or *Rpl11* in mouse erythroblasts and investigated mRNA polyribosome association, which revealed deregulated translation initiation of specific transcripts. Among these were *Bag1*, encoding a Hsp70 co-chaperone, and *Csde1*, encoding an RNA binding protein, both expressed at increased levels in erythroblasts. Their translation initiation is cap-independent and starts from an internal ribosomal entry site (IRES), which appeared sensitive to knock down of Rps19 or Rpl11. Mouse embryos lacking Bag1 die at embryonic day E13.5 with reduced erythroid colony forming cells in the fetal liver, and low Bag1 expression impairs erythroid differentiation in vitro. Reduced expression of Csde1 impairs proliferation and differentiation of erythroid blasts. Protein but not mRNA expression of *BAG1* and *CSDE1* was reduced in erythroblasts cultured from DBA patients. Our data suggest that impaired IRES-mediated translation of mRNAs expressed at increased levels in erythroblasts contributes to the erythroid phenotype of DBA.

INTRODUCTION

Diamond Blackfan anemia (DBA) presents as normochromic, macrocytic anemia with reduced erythroid precursors in bone marrow[1]. Approximately half of the patients have skeletal abnormalities such as thumb malformations, or growth retardation[2]. DBA is mostly diagnosed in infants less than 1 year of age, but in recent years non-classical cases of DBA are being diagnosed in adult patients[1].-

DBA is associated with mutations in genes encoding ribosomal proteins in 55% of patients[3]. The most prominently mutated gene (in 25% of patients) is *RPS19*[4], but mutations in *RPS7, RPS10, RPS17, RPS24 and RPS26* in the small ribosomal subunit, and in *RPL5, RPL11,*and *RPL35A* in the large ribosomal subunit were also found[3]. The mutations cause haploinsufficiency of ribosomal proteins and lead to loss of ribosome function, reducing general translation as observed in lymphocytes derived from DBA patients[5]. Knock down of RPS19 in hematopoietic progenitors either from human bone marrow or cord blood decreases colony forming capacity of erythroid progenitors while it affects colony forming capacity of myeloid progenitors to a far lesser extent[6]. Knock down of Rps19 in mouse fetal liver derived erythroblasts impairs their proliferation, but the differentiation of cells that survive the knockdown is not affected[7].

Because ribosome synthesis consumes up to 25% of a cell's energy, a disbalance in the synthesis of ribosomal proteins activates p53 and inhibits cell proliferation[8]. Free Rpl11 and Rpl5 bind and inhibit Mdm2, which reduces p53 ubiquitination and leads to its stabilization. Erythroid cells may be more sensitive to p53 activation than other hematopoietic lineages[9], which is also exemplified by the requirement to inactivate p53 in Friend virus-induced erythroleukemia[10]. In a zebrafish model of DBA, however, inactivation of p53 rescued the morphological abnormalities caused by morpholinos against Rps19, but not impaired erythropoiesis[11,12]. Therefore, it is possible that ribosomal insufficiency causes a specific defect in erythroblasts.

Expansion and maturation of erythroblasts depends on erythropoietin (Epo) and stem cell factor (SCF)[13,14]. This requires activation of the phosphotidylinositol-3 kinase (PI3K) pathway[15], which results in phsphorylation and activation of mammalian target of rapamycine (mTOR) that phosphorylates 4E-binding protein (4EBP), subsequently releases the cap-binding eukaryotic initiation factor 4E (eIF4E) and allows for association of the translation scanning complex[16]. We previously showed that expansion of erythroblasts depends on the availability of eIF4E and SCF-induced translation of specific transcripts with a complex RNA structure such as *Igbp1* and *Use1*[17]. The scanning complex involves the small ribosomal subunit, and the 60S subunit joins at the AUG start codon[16]. We hypothesized that reduced availability of ribosomal subunits in DBA, similar to availability of eIF4E, may affect translation initiation of specific transcripts important for erythroid development.

To investigate the role of translation in a DBA model independent of p53 activation, we reduced Rps19 and Rpl11 expression in the p53-deficient mouse erythroblast line I/11 and examined mRNA polyribosome association. Loss of Rps19 or Rpl11 suppressed translation of a specific set of transcripts, including *Bag1* and *Csde1*. Their mRNA expression was high in erythroblasts,

and their 5'UTR contains an internal ribosomal entry site (IRES). Importantly, protein levels of CSDE1 and BAG1 were also low in erythroblasts cultured from peripheral blood of DBA patients, while RNA expression was not affected. Mouse embryo's deficient in the Hsp70/Hsc70 co-chaperone Bag1 die at day E13.5 with a lack of definitive erythrocytes and a marked reduction of fetal liver erythroid progenitors. Reduced expression of the RNA-binding protein Csde1 inhibits both proliferation and maturation of erythroblasts. In summary, these data provide the first evidence for an erythroid specific mechanism that contributes to the anemia in DBA.

MATERIALS AND METHODS

Cell culture

I/11 erythroblasts, derived from p53-deficient mouse fetal livers, or primary fetal liver-derived erythroblasts were expanded in StemPro-34 medium (Invitrogen, Breda, The Netherlands) supplemented with 0.5 U/ml Epo (gift from Jansen-Cilag, Tilburg, The Netherlands), 100ng/ml of SCF (supernatant of CHO producer cells) and 10^{-6} M dexamethasone (Sigma-Aldrich, St Louis, MO)[15]. For differentiation, medium was supplemented with 5 U/ml of Epo and 0.5 mg/ml iron-loaded transferrin (Scipac, Sittingbourne, UK). Cell number and size were determined with electronic cell counter (CASY-1; Innovatis, Reutlingen, Germany). Hek293T cells were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) fetal calf serum (PAA, Pasching, Austria). After approval of Ethics Committee (Faculty Hospital, Palacky University, Olomouc) and informed consent, 30 ml of patient blood was harvested in heparin tubes and erythroblasts were cultured as described[18].

Hemoglobin content and cell morphology

Cell morphology was assessed by cytospins stained with histological dyes and neutral benzidine[17], using 40x magnification microscope (Leica, Wetzlar, Germany). Images were processed in Adobe Photoshop CS3, version 10.0 (Adobe, San Jose, CA).

Plasmids, transfections and bi-cistronic assays

The pβgal/CAT and pβgal-LamB1-CAT were kindly provided by W. Mikulits (Medical University, Vienna, Austria). 5'UTRs of *Bag1* and *Csde1* were amplified from mouse cDNA using Fidelity PCR kit (Roche, Basel, Switzerland). Amplicons were subcloned using TA cloning kit (Invitrogen), sequence verified and cloned in pβgal/CAT by *NheI* and *XhoI* sites. The CAT activity was determined 48 hours after the transfections using the CAT ELISA assay (Roche). Transfections of siRNA pools against Rps19, Rpl11 and scrambled control (Dharmacon, Lafayette, CO) were done with INTERFERin (Polyplus, Strasbourg, France) according to manufacturer's protocol.

Lentivirus production, titration and transductions

293HekT cells were transfected with pLKO.1-puro lentiviral construct containing shRNA sequences (MISSION® TRC-Mm 1.0 shRNA library; Sigma; Table S-IV), pMD2.G and pSPAX.2 packaging plasmids (gift of T. van Dijk, ErasmusMC, Rotterdam, The Netherlands) using LT-1 transfection reagent (Mirus, Madison, WI). Medium was concentrated by ultracentrifugation at 60 000×g for 2h at 4°C. Lentivirus titer was assessed by colony assays in 293HekT cells. Erythroblasts were transduced with a MOI of 3-5 and addition of 8 μg/ml polybrene (Sigma). Cells were selected with puromycine 24 hours post transduction.

Sucrose gradients and polyribosomal profiles

35×10^6 I/11 cells were incubated with 0.1 mg/ml cycloheximide (CHX) for 10 min at 37°C, washed with cold PBS containing 0.1 mg/ml CHX and lysed as described[17]. Polysomal RNA was separated on 10.5ml 7-46% linear sucrose gradients containing 10mM Tris-HCl, 12mM $MgCl_2$, 140mM NaCl and centrifuged at 190,000 g for 3 hours. Measurement of absorbance at 254 nm, visualization and fractionation were done by Econo system (Biorad, Hercules, CA). RNA structure was predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi)[19].

RNA isolation, cDNA synthesis, qRT-PCR

In vitro synthesized luciferase RNA was added to each fraction of the sucrose gradient to control for RNA isolation efficiency from the distinct fractions. RNA was isolated from gradient fractions as described[17] and further purified with LiCl[20]. Total RNA was isolated using RNeasy mini or micro (bone marrow cells) kit (Qiagen, Hilden, Germany). RNA from bone marrow was amplified by MessageAmp II aRNAkit (Ambion, Austin, TX). 1,6μg of RNA was used for cDNA preparation with random hexamers and SuperScript II Reverse Transcriptase kit (Invitrogen). qRT-PCR was performed as described[17]. Primers are listed in Table S-IV. In case of mouse erythroblasts we used β-tubulin as control; in case of human erythroblasts we used B2M selected from set of 32 endogenous controls (Applied Biosystems, Carlsbad, CA).

SDS-PAGE and Western blotting

Cell lysates, SDS-PAGE and Western blotting were performed as described[17]. Antibodies used were Rps19 (Abcam, ab40833), Rpl11 (Santa Cruz Biotechnology, sc-25931), Bag1 (Santa Cruz Biotechnology, sc-939), Csde1 (ProteinTech group, 13319-1-AP), Rpl8 (Abcam, ab55952), actin (Santa Cruz Biotechnology, sc-1616) and Erk1/2 (Santa Cruz Biotechnology, sc-94). Fluorescently-labeled secondary antibodies were used and blots scanned on Odyssey infrared scanner (LI-COR, Lincoln, NE).

Mice, flow cytometry and colony assays

The generation of Bag1 knockout mice was described[21]. All were housed in the central animal facilities of the University of Würzburg. The animal care and ethic committee approved all described procedures and experiments. The fetal liver cells from E12.5 embryos were resuspended

in PBS containing 2% BSA and 0.5 ug/ml 7-AAD and stained with antibodies against Ter119 and CD71 (BD, Franklin Lakes, NJ). For BFU-E colony assay, fetal liver cells were cultured in Methocult 3231 (StemCell Technologies, Vancouver, BC) supplemented with murine stem cell factor (SCF; 50 ng/mL), murine IL-3 (100 ng/mL, purified from supernatant of WEHI cells) and human erythropoietin (4 U/mL). For GM-CSF-induced colony formation, cells were cultured in Methocult 3231 supplemented with murine granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL). Cytokines were purchased from PeproTech (Rocky Hill, NJ), if not otherwise mentioned. Colonies were visualized with an inverted bright-field microscope and were assigned scores on day 5 (BFU-E) or day 10 (CFU-GM). All colony assays were done in triplicate with seeding 2*10^4 cells per plate.

RNA-protein pulldown

100*10^6 MEL cells were used for RNA-protein pulldown protocol as described [22]. For pulldowns we used streptavidin conjugated Dynabeads M-270 (Invitrogen). The NT2 washing buffer was supplied with 0.5% DOCH. RNA was isolated using Trizol (Invitrogen) and amplified with MessageAmp II amplification kit (Ambion).

DNA microarrays and data processing

Complementary RNA was generated from pooled subRNA and pbRNA fractions as described[17] and hybridized to Affymetrix MOE 430A2.0 mouse expression arrays according to manufacturer's protocols. Each sample had two paired hybridizations for polyribosomal and subpolyribosomal fraction. The raw data was processed using Affymetrix Microarray Suite 5 (MAS5) to target intensity values of 100. Probe sets, which had polyribosomal intensity lower than 100, were excluded to prevent systematic variation between experimental conditions. Subsequently, the intensity values were normalized per probe set for each sample within the associated class to quantify the mRNA transcript association with polyribosomes. These are computed by taking the polyribosomal intensity value per probe set of each sample with respect to the 'polysomal plus subpolysomal' intensity value within the sample. This resulted into arbitrary ratios that range between 0 and 1. Data files were uploaded to GEO with accession number GSE22903.

Bioinformatical analyses were performed using Matlab R2009b (MathWorks, Natick, MA). To determine transcripts with differential ratios of polysome recruitment we performed a multivariate analysis using ANOVA (analysis of variance) using the translational ratios. The multiple testing was controlled by employing a Benjamini Hochberg correction.

Statistical analysis

Results are depicted as means ± s.e.m, except when stated otherwise. We performed statistical analysis with Student's t-test using GraphPad Prism (v4, GraphPad, La Jolla, CA).

RESULTS

Loss of Rps19 and Rpl11 impairs expansion and differentiation of erythroblasts

To investigate how haploinsufficiency of ribosomal proteins affects mRNA translation in erythroblasts, we reduced the expression of Rps19 and Rpl11 in mouse p53-deficient I/11 erythroblasts using 2 distinct shRNA sequences for each ribosomal protein (Figure 1A). Single ribosomal subunits, monosomes and polysomes were separated on a 7-46% sucrose gradient (Supplemental Figure S1A). At day 3 knock down of Rps19 reduced the 40S subunit content, and loss of Rpl11 reduced 60S subunits (Figure 1B). At this stage, cell numbers and cell morphology

Figure 1

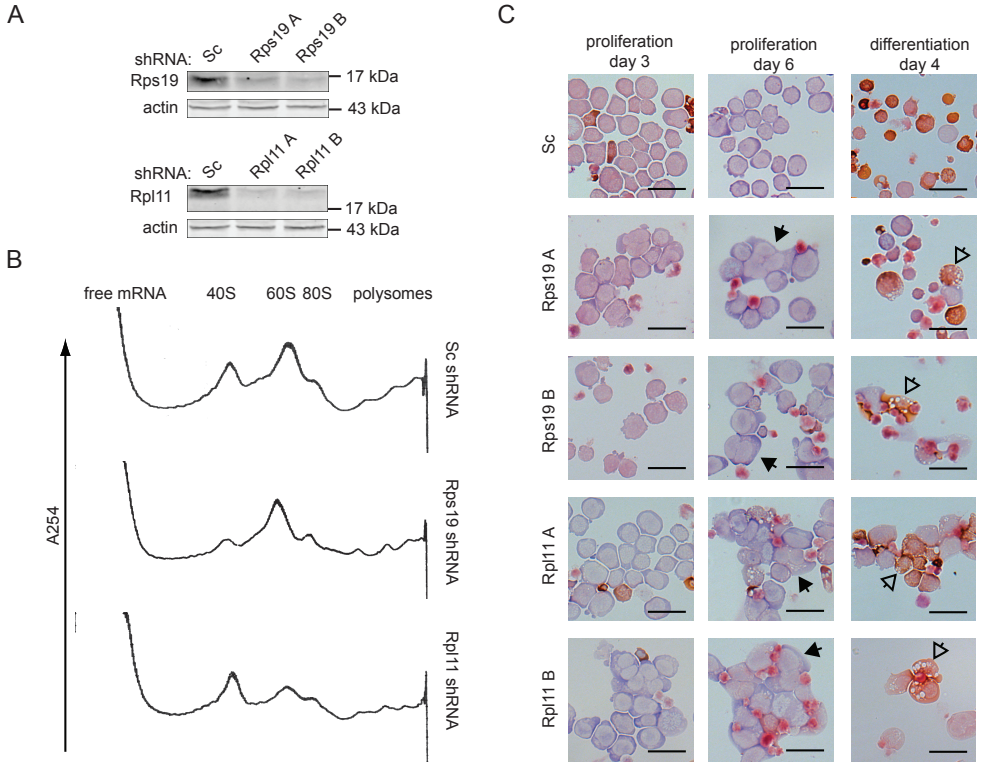

Figure 1: Phenotype of Rps19 and Rpl11 deficient erythroblasts. (***A***) Rps19 and Rpl11 protein levels in I/11 erythroblasts 3 days post transduction with lentiviral vectors expressing scrambled (Sc) shRNA or 2 distinct shRNA (A and B) specific for Rps19 or Rpl11, respectively. (***B***) RNA profiles measured by absorbance at 254 nm in a sucrose gradient loaded with cytoplasmic extract of shRNA treated erythroblasts 3 days post-transduction. The position of the 40S and 60S ribosomal subunits, the 80S monosome and polysomes is indicated (for peak identification see supplemental Fig. S1). (***C***) I/11 erythroblasts were transduced with lentiviral shRNA constructs as indicated. Cells were cultured for 3 (left panel) and 6 days (middle panel) in proliferation conditions or switched to differentiation conditions 3 days after transduction and differentiated for 4 days (right panel). Cells were stained for hemoglobin (brown color) and histological dyes. Full arrowheads point to multinucleated cells, empty arrowheads point to aberrantly differentiated cells. Bar represent 25 μm.

between cultures transduced with control shRNA or shRNAs directed against *Rps19* or *Rpl11* were still comparable (Figure 1C, Supplemental Figure S1B). Thereafter, reduced expression of Rps19 and Rpl11 inhibited proliferation of I/11 erythroblasts and resulted in aberrant cell morphology, characterized by occurrence of bi-nucleated and poly-nucleated cells, dependent on the shRNA used (Figure 1C, Supplemental Figure S1B,C). Rps19 and Rpl11 deficient erythroblasts failed to undergo differentiation divisions, accumulated less hemoglobin, and displayed aberrant vacuolised morphology (Figure 1C). In summary, the knockdown of Rps19 and Rpl11 resulted in functional reduction of corresponding ribosomal subunits followed by phenotypical changes in erythroblasts during proliferation and differentiation.

Figure 2

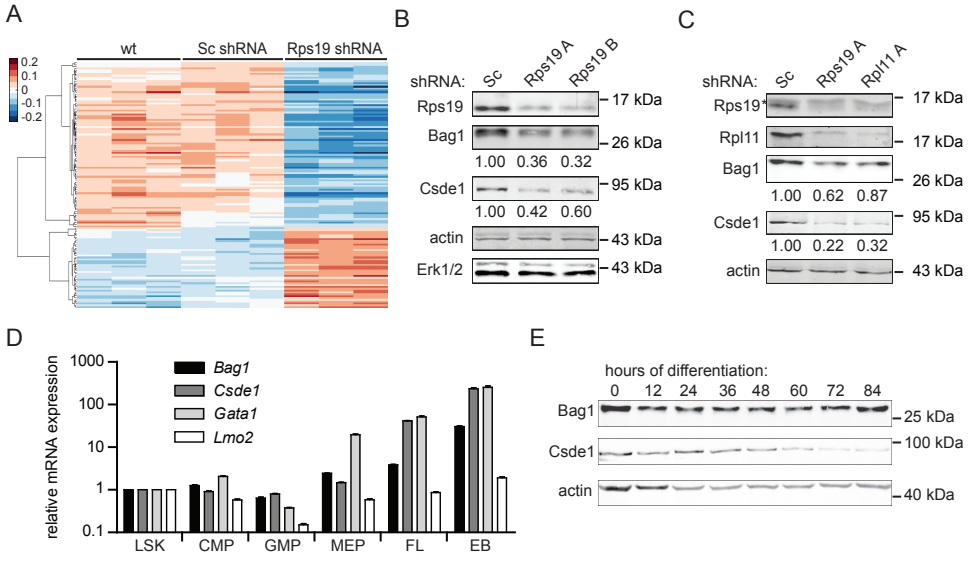

Figure 2: Polysome association of mRNAs in erythroblasts deficient for Rps19 and Rpl11. (***A***) Polysomal- and subpolysomal-derived cRNA generated from parental I/11 erythroblasts (wt), and cells transduced with Sc shRNA or Rps19 shRNA A was hybridized to expression arrays. Following normalization arbitrary ratios of polyribosomal recruitment were calculated (see Materials and methods). Transcripts that are differentially expressed between control cells (wt and Sc shRNA) and Rps19 deficient cells were identified by ANOVA (FDR<0.05) and hierarchically clustered. Three columns for each class represent independent biological replicates. (***B***) Protein levels of Bag1 and Csde1 in I/11 erythroblasts 3 days following transduction with Sc and Rps19 shRNA. Actin and Erk1/2 serve as loading controls. Quantified expression, corrected for actin, is indicated below the blots. (***C***) Protein levels of Rps19, Rpl11, Bag1 and Csde1 in primary erythroblasts obtained from E12.5 fetal liver cells 3 days after transduction with Sc, Rps19A and Rpl11A shRNA. Actin serves as control. Numbers indicate blot quantifications corrected to actin. * indicates a non-specific band in the Rps19 panel. (***D***) qRT-PCR on RNA obtained from sorted mouse bone marrow fractions (LSK, Lin-, Sca1+,c-Kit+; CMP, common myeloid progenitor, lin-, c-kit+, CD34+, CD16/CD32low; GMP, granulocyte-monocytic progenitor lin-, c-kit+, CD34+, CD16/CD32high; MEP, megakaryocytic-erythroid progenitor, lin-, c-kit+, CD34-, CD16/CD32low), day E13.5 mouse fetal liver (FL) and 5 days *in vitro* erythroid culture of the fetal liver cells (EB, erythroblasts). Expression is shown relative to LSK levels on a logarithmic scale. Error bars represent ± s.e.m. (n=4). (***E***) Mouse erythroblasts (I/11) were induced to differentiate. Every 12 hours protein samples were prepared to be analysed for expression of Bag1 and Csde1. Actin serves as a loading control. The position of size markers is indicated.

Reduced Rps19 expression impairs translation of a specific set of transcripts

To identify transcripts whose translation may be specifically affected upon loss of Rps19, we hybridized oligonucleotide arrays with cRNA derived from polyribosomal (pbRNA) and subpolyribosomal RNA (subRNA) of non-transduced wt cells (wt) and cells transduced with lentiviral vectors expressing control shRNA (Sc), or shRNA directed to *Rps19* or *Rpl11*. Arrays hybridized to polysomal or subpolysomal RNA were normalized separately and arbitrary translation ratios were computed for each paired sample to quantify mRNA ribosome loading ([polysomal signal]/[polysomal plus subpolysomal signal]; see Materials and methods). Multivariate analysis (FDR<0.05) identified 134 probe sets, corresponding to 130 genes that were differentially recruited to polysomes upon Rps19 knockdown compared to control samples (Figure 2A, Supplemental Table S-I). Most of these transcripts were regulated similarly upon Rps19 or Rpl11 knock down (Supplemental Figure S2A). Transcripts that were selectively lost from polyribosomes encoded among others mitochondrial proteins, translation initiation factors and mRNA processing factors. We measured the actual percentage of polysome recruitment by qRT-PCR for which we selected transcripts whose expression is increased in erythroid progenitors compared to other hematopoietic lineages[23], or for which a predicted RNA structure in the 5'UTR may explain regulated polysome association[19,24]. Upon Rps19 knock down, polysome recruitment of *Bag1 (Bcl2-associated athanogene 1)* mRNA dropped from 70 to 40%, *Csde1 (cold shock domain containing E1)* from 50 to 20%, *Fxc1 (fracture callus 1)* from 75 to 20%, and *Siva1* from 70 to 30%. A control transcript (*Hdhd2*) retained 65% polyribosome association. Loss of Rpl11 reduced polyribosome recruitment of *Bag1*, *Csde1*, *Fxc1* and *Siva* to a lesser extent than loss of Rps19, whereas the presence or absence of growth factor signaling (Epo and SCF) did not alter polyribosome recruitment (Supplemental Figure S2B). Protein levels of Bag1 (the predominant p36 or Bag-1S isoform) and Csde1 correlated with polyribosome recruitment in p53-deficient I/11 cells transduced with control or Rps19 siRNA (Figure 2B; Fxc1 and Siva could not be detected on Western blot with available antibodies). These effects are not specific for Rps19; reduced Rpl11 expression also decreased Bag1 and Csde1 protein expression (Supplemental Figure S2C). Knock down of Rps19 and Rpl11 expression in primary mouse fetal liver erythroblasts confirmed reduced Bag1 and Csde1 protein levels in p53 proficient cells, while RNA expression remained constant (Figure 2C, Supplemental Figure S2D). Thus, loss of Rps19 and Rpl11 negatively affects translation of *Bag1* and *Csde1* transcripts, independent of p53.

Because DBA is mainly manifested in the erythroid lineage, we compared expression of *Bag1* and *Csde1* in various hematopoietic lineages. Expression of *Bag1* and *Csde1* was low in stem cells and myeloid progenitors, but increased in megakaryocytic-erythroid progenitors, E13.5 fetal liver cells (90% Ter119$^+$ cells) and reached expression levels in cultured erythroblasts that were 30- and 250- fold higher, respectively, as compared to levels detected in stem cells (Figure 2D). Notably, the increase in *Csde1* expression was similar to the increase of the erythroid specific transcription factor *Gata1* that is abundantly expressed in erythroid progenitors. During terminal erythroid differentiation, the expression of Bag1 remained constant throughout differentiation. Csde1 expression was downregulated 60 hrs after induction of differentiation which corresponds

Figure 3

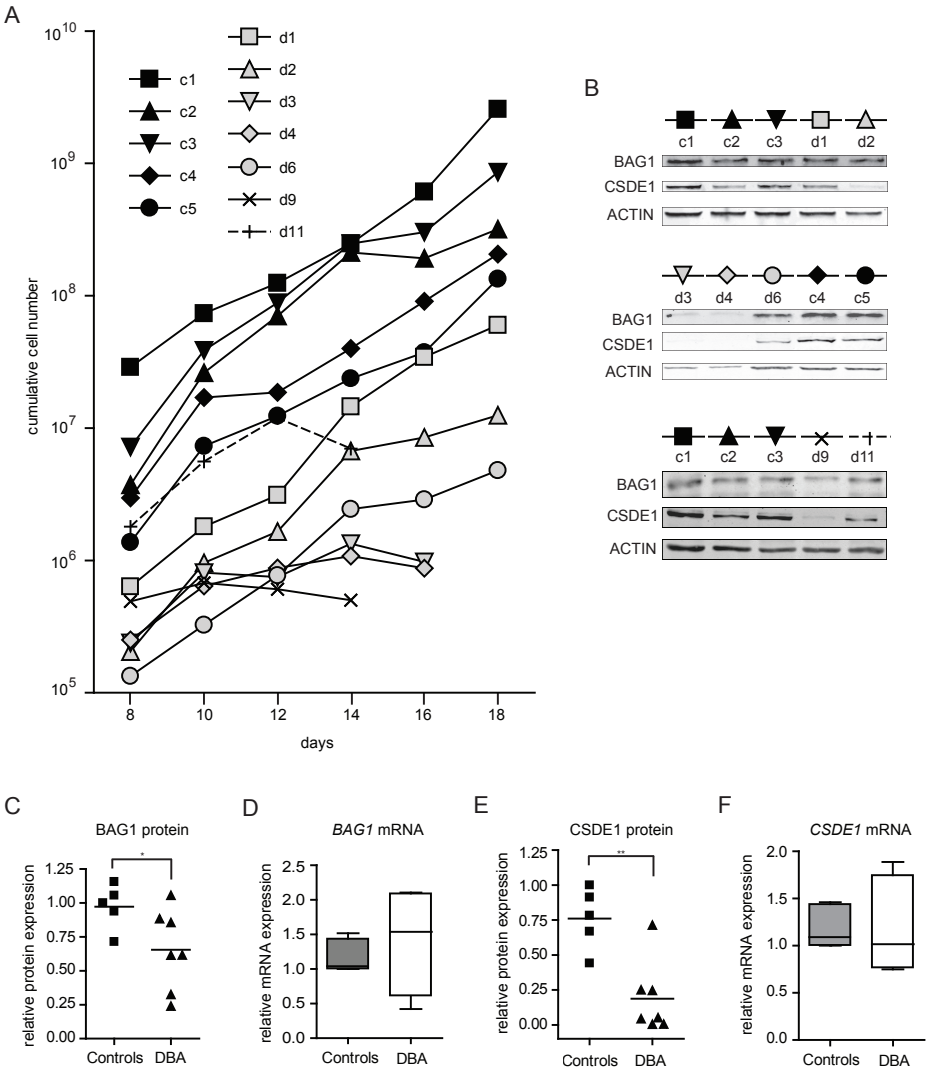

Figure 3: BAG1 and CSDE1 protein expression is decreased in DBA erythroblasts. (***A***) Proliferation of erythroblasts derived from peripheral mononuclear blood of DBA patients and healthy controls. Cumulative cell numbers were calculated; days after *in vitro* culture initiation are indicated. Samples for Western blotting and RNA isolation were harvested on day 16. Black symbols represent healthy controls (c1-c5); grey or open symbols represent DBA patients (d1-d11). Erythroblasts of 5 healthy controls and 7 DBA patients were harvested on day 16 of *in vitro* culture, only samples d9 and d11 were harvested on day 14. (***B***) Protein expression of BAG1 and CSDE1 was analysed by Western blot, using actin as a loading control. The symbols above the Western blot correspond to symbols used in (*A*). Patient characteristics are given in supplemental Table S-II. BAG1 (***C***) and CSDE1 (***E***) protein expression was quantified and corrected to actin expression. *BAG1* (***D***) and *CSDE1* (***F***) transcript levels were quantified by qRT-PCR (except patient d9, n=4 per sample). (C-F) Data are also given in supplemental Table S-III, lines indicate mean, (D, F) boxes indicate standard error of mean, error bars indicate maximum and minimum values. **P*=0.059, ***P*=0.0023

to the start of enucleation[15] (Figure 2E). The, increased expression of Bag1 and Csde1 throughout late stages of erythropoiesis suggests an erythroid specific function.

Expression of BAG1 and CSDE1 is reduced in DBA patient-derived erythroblasts

To examine whether the identified genes are target genes in DBA independent of the underlying mutation, we assessed the expression of BAG1 and CSDE1 in erythroblasts cultured from mononuclear cells isolated from 30 ml peripheral blood of DBA patients with various mutations, and of age- and sex-matched controls (Supplemental Table S-II). From 7 out of 11 patient samples we could expand erythroblasts, albeit with a total yield that was reduced compared to healthy controls (Figure 3A). The low quantity of patient-derived erythroblasts precluded the isolation of polysomes from sucrose gradients. Therefore, we harvested protein and RNA samples from the cultures to examine protein expression on Western blots, which we related to the mRNA expression measured by qRT-PCR (for quantitative data see Supplemental Table S-III). BAG1 protein levels were reduced in DBA samples, as compared to controls (Figure 3B,C), while *BAG1* mRNA levels remained constant or were even increased (Figure 3D). Expression of CSDE1 protein was on average 3-fold lower in DBA erythroblasts than in control erythroblasts (Figure 3B, E), whereas *CSDE1* mRNA levels were similar between DBA and controls (Figure 3F). To exclude that CSDE1 levels differences result from the differentiation status of the cultures, we stained the cells at the day of protein and RNA harvest (Supplemental figure S3). These results indicate that defective translation of specific targets occurs in DBA patient-derived erythroblasts as well, similar to the mouse *in vitro* cultures. Interestingly, reduced proliferation rates of DBA erythroblast cultures seem to correlate with low Bag1 and CSDE1 protein levels (compare Figures 3A and 3B).

Bag1 deficiency impairs erythroid differentiation

To study the function of *Bag1* in erythroid differentiation, different lentiviral shRNA expressing constructs were used to reduce Bag1 expression in I/11 erythroblasts to 50 and 25% of normal levels (Figure 4A). Reduced Bag1 expression did not affect expansion of erythroblast cultures (Figure 4B). However, upon induction of differentiation, characterized by size reduction and hemoglobin accumulation, a considerable subset of Bag1 depleted erythroblasts retained a blast size and morphology (Figure 4C,D). Inhibition of differentiation inversely correlated to the expression of Bag1 at the beginning of differentiation. Not all cells were retained in an immature state, and cells able to enter the differentiation program accumulated hemoglobin comparable to control cells (Figure 4D).

Bag1$^{-/-}$ mouse embryos die at day E13.5 with severe defects in brain development and small, anemic livers[21]. To investigate the hematopoietic defect in *Bag1*$^{-/-}$ livers, we examined fetal liver cells from E12.5 *Bag1*$^{-/-}$ embryos and *Bag1*$^{+/+}$ and *Bag1*$^{+/-}$ littermates. *Bag1*$^{-/-}$ fetal livers contained half the number of cells as compared to *Bag1*$^{+/+}$ and *Bag1*$^{+/-}$ fetal livers (Figure 5A). In colony-forming assays 10^4 *Bag1*$^{-/-}$ fetal liver cells yielded 4 times less BFU-E colonies as compared to *Bag1*$^{+/+}$ and *Bag1*$^{+/-}$ fetal liver cells, while the number of CFU-GM colonies was not significantly different between the genotypes (Figure 5B). To analyze maturation of *Bag1*$^{-/-}$ fetal liver

cells we measured expression of the erythroid markers CD71 and Ter119 on the fetal liver cells. The *Bag1*$^{-/-}$ fetal livers contain a TER119-negative, CD71-low population of immature cells that is hardly present in wt fetal livers. Within the CD71-high erythroid population, less *Bag1*$^{-/-}$ fetal liver cells co-expressed TER119 indicating delayed differentiation (Figure 5C, D). Together the data suggest that complete loss of Bag1 expression strongly impairs erythropoiesis. Less BFU-E were present in the fetal liver at day E12,5, and the erythroid progenitors that are generated are delayed in their maturation to TER119 positive cells. When Bag1 expression is reduced in established erythroblasts they are less prone to enter terminal differentiation, but part of the culture matures to hemoglobinised cells. In conclusion, Bag1 seems required for erythroblasts to enter a terminal differentiation program.

Figure 4

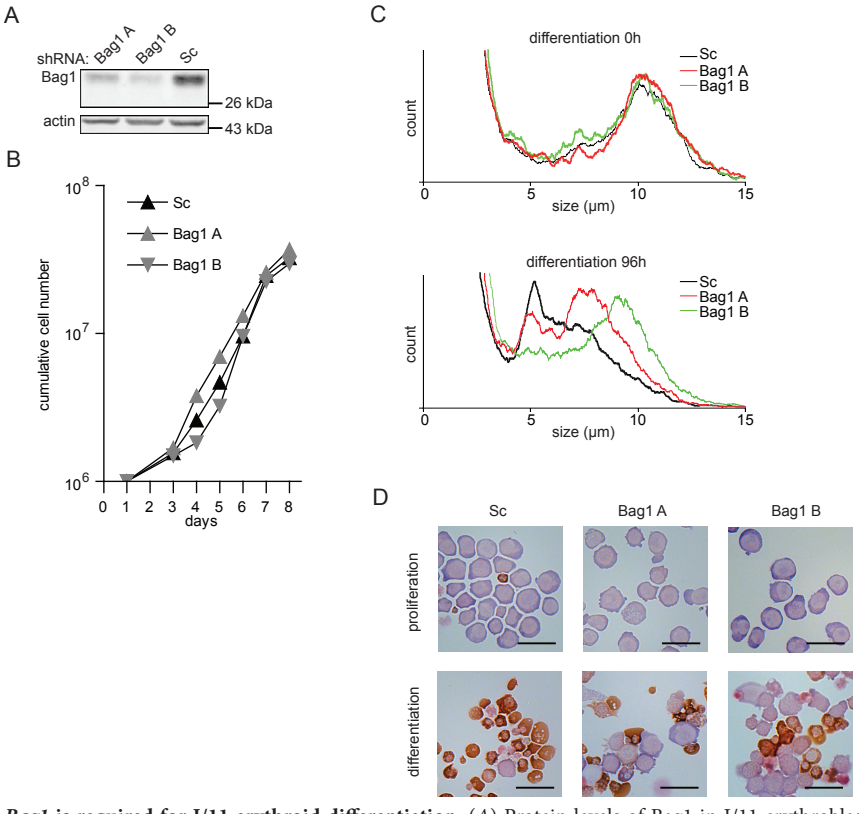

Figure 4: *Bag1* is required for I/11 erythroid differentiation. (*A*) Protein levels of Bag1 in I/11 erythroblasts 3 days post-transduction with Sc shRNA lentivirus and 2 distinct shRNA lentiviruses complementary to Bag1. (*B*) Cumulative cell numbers of I/11 erythroblast cultures following transduction with control shRNA (▲) and shRNA against Bag1 (A ▲ and B ▼). Cumulative cell numbers are calculated from 3 independent experiments. (*C*) Cell size profiles, measured by a cell counter, from differentiating Bag1 deficient I/11 erythroblasts shown on the day of Epo induction (0h) and after 96h. Peaks at 10µm size indicate erythroblasts, peaks at 5µm represent enucleated reticulocytes. (*D*) I/11 erythroblasts were transduced with lentiviral shRNA constructs as indicated. Cells were cultured for 3 days in proliferation conditions (upper panels), followed by 4 days differentiation conditions (lower panels) before being harvested for cytospins and stained for hemoglobin (brown color) and histological dyes. Bar represent 25 µm.

Figure 5

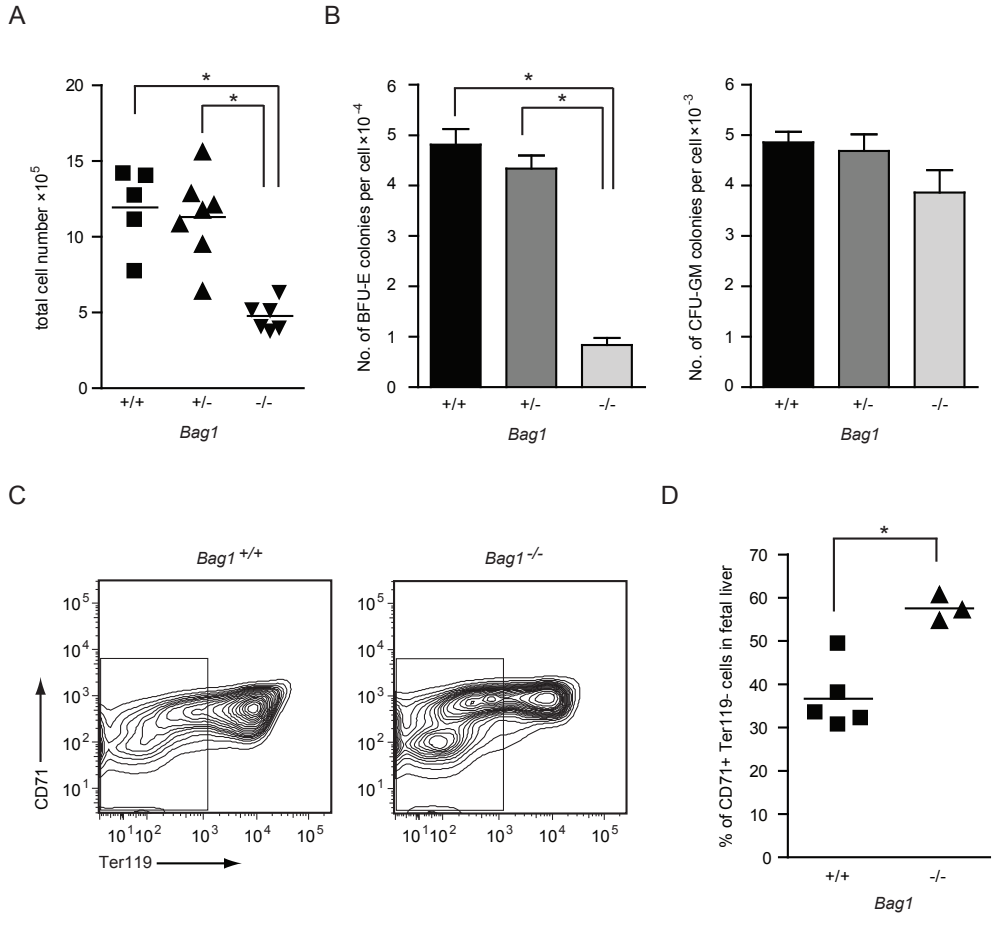

Figure 5: Bag1 deficiency inhibits maturation of erythroblasts *in vivo*. (*A*) Total number of nucleated cells in E12.5 fetal livers from Bag1$^{+/+}$, Bag1$^{+/-}$ and Bag1$^{-/-}$ embryos. *p<0.001 (*B*) Number of colonies in BFU-E and CFU-GM assays represented as colonies per cell from E12.5 fetal livers as in *A*. *p<0.001 (*C, D*) FACS analysis of the 7AAD$^-$ cells from fetal livers as above. The gated area represents the Ter119$^-$ cells, which are quantified as % of total in the graph *D*. *p<0.005

Loss of Csde1, an IRES-binding protein, inhibits erythroid proliferation and differentiation

Csde1 contains five copies of the RNA-binding cold shock domain (CSD), and is highly homologous with Drosophila u*pstream of* N-r*as* (*unr*) involved in mRNA translation[25]. We suppressed Csde1 expression in I/11 erythroblasts using lentiviral vectors expressing two distinct shRNAs. Expression was reduced to 10% (Csde1 shRNA-A) or 20% (Csde1 shRNA-B) of normal protein levels (Figure 6A), which impaired both erythroid proliferation and differentiation (Figure 6B, C). The cells with reduced Csde1 expression became mainly pycnotic and failed to mature to enucleated erythrocytes (Figure 6C).

Figure 6

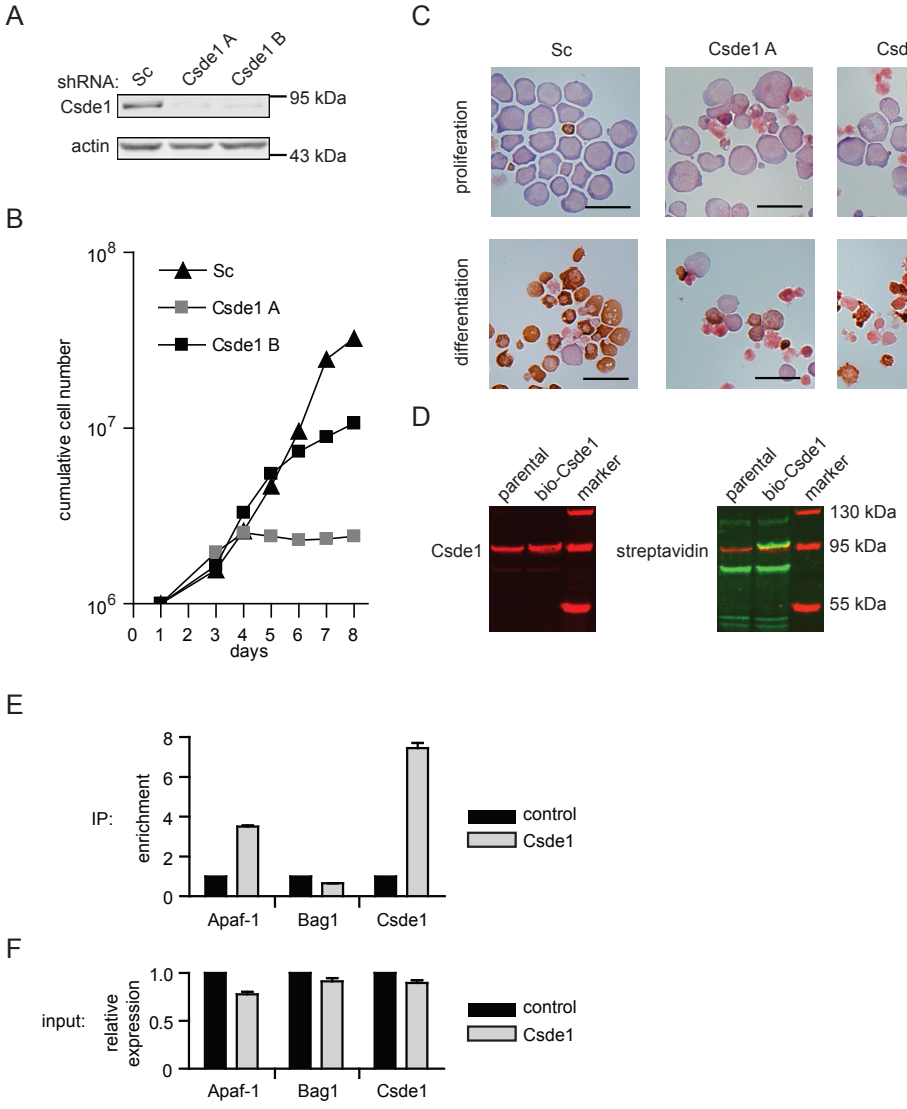

Figure 6: Csde1 is required for proliferation and differentiation of I/11 erythroblasts. (A) (*A*) Protein levels of Csde1 in I/11 erythroblasts 3 days post-transduction with Sc shRNA lentivirus and 2 distinct shRNA lentiviruses complementary to Csde1. (*B*) Cumulative cell numbers of I/11 erythroblast cultures following transduction with control shRNA (▲) and shRNA against Csde1 (A □and B ■). Numbers are calculated from 3 independent experiments. (*C*) I/11 erythroblasts were transduced with lentiviral shRNA as indicated. Cells were cultured for 3 days in proliferation conditions (upper panels), followed by 4 days differentiation conditions (lower panels) before being harvested for cytospins and stained for hemoglobin (brown color) and histological dyes. Bar represent 25 µm. (*D*) Western blots containing lysates from parental MEL-birA cells and cells expressing bio-tagged Csde1 were stained for Csde1 (red). The tagged band runs above the endogenous protein (left image) and was stained with fluorophore conjugated streptavidin indicating biotinylation (yellow-green band, right image). (*E*) The enrichment of *Apaf-1*, *Bag1* and *Csde1* in RNA isolated from Csde1 pulldowns with strepatavidin beads from MEL cells expressing biotagged-Csde11 compared to pulldowns from parental MEL cells (set at 1; n=4). (*F*) Realtime PCR on total RNA used as input in RNA-IP (n=4).

Translation of both *Csde1* and *Bag1* was shown to start from an IRES[26,27]. Interestingly, Csde1 protein binds to an IRES sequence in the 5'UTR of specific transcripts such as *Apaf-1* and *Cdk11A,* or *Rhinovirus* RNA[25]. It also binds to the IRES in the 5'UTR of its own transcript upon which it regulates its own translation in an autoregulatory loop[28]. Therefore, we investigated whether Csde1 protein binds its own IRES and the IRES of *Bag1* in erythroblasts. Mouse *Csde1* was fused to the recognition site of the prokaryotic biotin ligase BirA at its N-terminus, and the biotagged Csde1 protein was expressed in murine erythroleukemia (MEL) cells that stably expressed the biotin ligase[29]. Biotagged Csde1 was efficiently biotinylated (Figure 6D). Next we used streptavidin coated beads to pull down Csde1 and associated RNA. RNA was converted to cDNA and tested for the presence of *Csde1* nucleotide sequences (primers in 3'UTR) by real time PCR. *Csde1* was 8-fold increased in pull downs from MEL cells co-expressing BirA and biotagged Csde1 when compared to MEL cells expressing BirA only. The known target, *Apaf-1* was also enriched in Csde1 immunoprecipitations, but *Bag1* was not enriched (Figure 6E), while input mRNA levels before pulldown were comparable between control and Csde1 MEL cells (Figure 6F). This suggests that Csde1 is not involved in IRES-mediated translation of *Bag1* in erythroid cells.

Figure 7

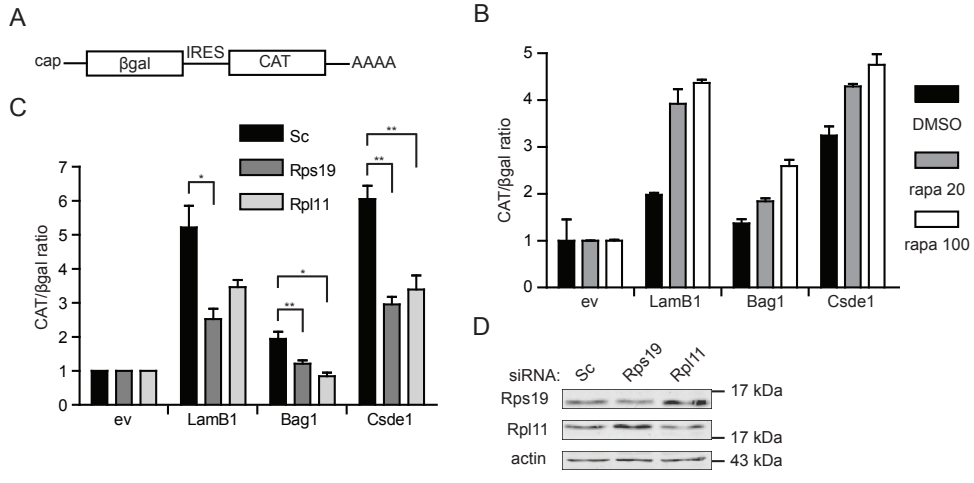

Figure 7: IRES activity is inhibited under ribosomal protein deficiency. (*A*) Schematic representation of the transcript produced by the bicistronic reporter plasmid with cap-dependent βgal and IRES-dependent CAT expression. (*B*) Hek293T cells were pretreated with 20 and 100 nM rapamycine, grey and white bars respectively, or with DMSO (black bars) for 12 hours. Cells were transfected with βgal-CAT bicistronic reporter plasmid lacking IRES sequence (ev) or harboring the 5'UTR of *LamB1*, *Bag1* and *Csde1* between the βgal and CAT ORF. Cells were harvested 48 hours post transfection and βgal and CAT were measured using ELISA asays. All βgal-CAT ratios were normalized to the ratio in lysate containing the bicistronic construct without IRES, which was set to 1 in each condition. Error bars shown are s.e.m. (n=4) (*C*) Hek293T cells were treated with Scrambled (Sc), Rps19 and Rpl11 siRNA and transfected after 12 hours with the same bicistronic plasmids. IRES activity is represented by CAT/βgal ratio. Data shown are mean ± s.e.m. (n=4) *P<0.05, **P<0.01 (*D*) Western blot showing knockdown of RPS19 and RPL11 by siRNA in Hek293T cells 3 days after transfections.

Finally, we investigated whether knockdown of Rps19 and Rpl11 can affect IRES-mediated translation initiation in general. For this we used a pβgal/CAT bicistronic reporter plasmid[30], in which we inserted the 5'UTR of *Bag1* and *Csde1* between the open reading frames of ß-galactosidase (βgal) and chloramphenicol acetyl transferase (CAT) (Figure 7A). The *LamB1* 5'UTR was used as positive control for eukaryotic IRES activity[31]. Cap independent translation from the IRES of *LamB1*, *Bag1* and *Csde1* was confirmed by an increased CAT/βgal ratio in transfected Hek239T cells upon treatment with rapamycine (Figure 7B). Notably, the CAT/βgal ratio activity of all constructs was decreased in cells transfected with Rps19 or Rpl11 siRNA compared to cells transfected with control siRNA, indicating that loss of Rps19 and Rpl11 decreased IRES-driven translation (Figure 7C). Knockdown of Rps19 and Rpl11 in Hek293T cells was confirmed by Western blot (Figure 7D). These data indicate that IRES-mediated translation is sensitive to deficiencies Rps19 or Rpl11 to a larger extent than cap-dependent translation. As the IRES activity of the positive control was affected as well, we suspect that these phenomena may be a general feature of translation driven by a cellular IRES.

DISCUSSION

Since the discovery that mutations in ribosomal proteins cause DBA it has been puzzling why such a general cellular defect predominantly causes profound anemia. Our previous findings that selective mRNA translation is crucial in the expansion of the erythroid compartment prompted us to investigate whether loss of ribosomal proteins impairs the translation of specific transcripts with an essential role in the proliferation or differentiation of erythroblasts. We identified transcripts that were selectively lost from polysomes upon knock down of Rps19 or Rpl11 compared to control erythroblasts. Among those are *Bag1* and *Csde1,* transcripts that are strongly upregulated in erythroid cells compared to other hematopoietic cells and that appeared essential for erythropoiesis. Both Bag1 and Csde1 are translated from an IRES and, interestingly, Csde1 is an RNA binding factor that controls IRES-mediated translation. However, Bag1 mRNA is not a target of Csde1 regulation. Instead, reduced expression of Rps19 or Rpl11 suppressed general IRES-mediated translation more profoundly than cap-dependent translation.

Ribosomal proteins are functional constituents of the ribosome, for instance to shape the mRNA exit channel[32]. Ribosomal proteins also function in maturation of ribosomal subunits, and knockdown of Rps19 was shown to affect the maturation of 21S to 18SE pre-rRNA[33]. The scattered location of DBA-associated ribosomal proteins on the 40S ribosomal subunit supports the suggested role in ribogenesis, rather than a specific functional role in translation[34].

Cap-dependent and IRES-mediated translation compete for translation factors, a process in which cap-dependent translation is favored under optimal conditions. Inhibition of cap-dependent translation by cell starvation, viral infection, or apoptosis induction increases IRES-dependent translation[35]. A shortage of ribosomes as it occurs in DBA may affect the less competitive mechanism of IRES-mediated translation much more compared to cap-dependent translation. A more quantitative approach should reveal whether all IRES-mediated translation is affected, or whether some IRES-containing transcripts are affected more than others.

We did not only identify transcripts that specifically failed translation upon knock down of Rps19 and Rpl11, some transcripts seemed to be preferentially translated. Among those were transcripts encoding ribosomal proteins (*Rps8, Rps25, Rpl8, Rpl36a*) and other transcripts with a 5'-TOP (terminal oligopyrimidine tract) sequence in their 5'UTR such as proteasome subunits *Psmb1* and *Psmb4*. Moreover, we found preferential translation of transcripts that depend on SCF-induced PI3K activation (*Use1, Nap1l1*)[17]. The translation of these transcripts is under control of the mTOR pathway and mTOR activity also enhances the translation of 5'-TOP mRNAs[36]. Following Rps19 and Rpl11 knockdown, we harvested polysomal RNA from cells cultured in presence of SCF. Under these conditions, the translation of transcripts with a structured 5'UTR is actively supported. The most likely explanation for the preferential translation of these transcripts is that their translation is still sustained by SCF-dependent mechanisms when the overall translation is reduced due to loss of ribosomes.

Mouse embryos lacking *Bag1* die at E13.5 and suffer from a major defect in brain development and lack of erythropoiesis. Embryo's lacking *Epo* or the *EpoR* also die at day 13.5 from a lack

of erythrocytes, which indicates that mouse embryo becomes dependent on definitive erythropoiesis at day E13.5[13]. Notably, the fetal liver of *Bag1*$^{-/-}$ embryos was devoid of late erythroid cells, suggesting that the embryos die from impaired erythropoiesis. Surprisingly, we were able to expand erythroblasts following knock down of *Bag1*, but they were severely delayed in their differentiation (Fig. 4C, D). Bag1 functions as nucleotide exchange factor for HSC70/HSP70, a chaperone which was reported to protect GATA1 from caspase-3-mediated cleavage during terminal erythroid differentiation[37]. Furthermore, Bag1 links the protein surveillance chaperone system with the proteasome degradation[38], and lack of Bag1 may enhance the unfolded protein response, which will suppress mRNA translation. This may be beneficial in a situation where ribosome synthesis is restricted.

Mouse embryos lacking *Csde1* die around post gestation day E10.5 pointing at a function in tissue development[25]. Csde1 is an RNA binding protein also known as Unr (Upstream of N-ras). In *Drosophila*, Unr is recruited by sex lethal (SXL) protein to the 3'UTR of male-specific lethal 2 (msl-2) to inhibit translation[39]. In mammalian cells Csde1 was shown to bind the IRES of p58^{PITSLRE} kinase and to enhance its translation[40]. p58^{PITSLRE} kinase is important for G2-M transition of the cell cycle and crucial for the completion of cytokinesis[41]. Haploinsufficiency of Rpl24, a ribosomal protein that was not shown to be mutated in DBA, also impairs IRES-mediated translation and particularly the expression of p58^{PITSLRE} [42]. It will be interesting to know whether this involves regulation of Csde1 expression, or whether haploinsufficiency of ribosomal proteins affects translation of p58^{PITSLRE} directly. Of note, the increase in multinuclear cells induced by loss of Rps19 or Rpl11 (Fig. S1C) indicates that mitosis, and more specifically cytokinesis of erythroblasts, is affected, similar to what was observed in a DBA mouse model expressing dominant negative *Rps19*[43]. Possibly, mitosis in such rapidly cycling cells relies more on proper IRES-driven translation of certain transcripts than in other cell types.

Although the knock down and knock out models clearly demonstrate that Bag1 and Csde1 are required for erythropoiesis, the moderately reduced expression levels in DBA samples may not be sufficient for any single gene to cause anemia. Similarly, the brain defect observed in Bag1-deficient mice is not typical for DBA and, for the same reason, heterozygous mice do not have a phenotype[21,44]. Bag1 and Csde1 are only two examples from the list of translationally regulated genes. We propose that reduced translation of a set of transcripts, among which are Bag1 and Csde1, that share a common regulatory element contributes to a compound effect resulting in the severe anemia of DBA.

Our patient cohort is too small and heterogeneous to draw conclusions about a relation between impaired translation of Bag1 or Csde1 and the severity of the disease or the mutated ribosomal protein, although translation seems to be less affected in the three cultures that expanded relatively well. Moreover, the expression data in cultured DBA-derived erythroblast are biased because we could only expand erythroblasts from 7 out of 11 patients.

It has also been suggested that the severe anemia associated with DBA is caused by a p53-dependent mechanism, and that erythroblasts are extremely sensitive to activation of p53[9]. A disbalance in ribogenesis enables quenching of Mdm2 by free Rpl5 or Rpl11, which reduces

p53 degradation and activates p53-dependent pathways[45]. Our data indicate that certain transcripts are specifically lost from polyribosomes in erythroblasts of DBA patients independent of p53 activation. However, we also show that Siva is lost from polysomes upon RPs19 knock down (from 70 to 30% polyribosome loading). Siva is a p53 target gene involved in negative feedback, and reduced expression may enhance the activity of p53[46]. Thus, it seems likely that the overall DBA phenotype is caused by a combination of defective mRNA translation and p53 activation. This is supported by experiments in zebrafish that also show p53-dependent and -independent mechanisms cooperate in the overall DBA phenotype caused by Rps19 knock down[11,12].

The recent finding that loss of Rps14 contributes to the phenotype of a subtype of myelodysplastic syndrome (MDS) characterized by loss of chromosome arm 5q[47] raised the question whether BAG1 and CSDE1 are aberrantly expressed in 5q- MDS. Due to the predominantly erythroid expression, however, BAG1 and CSDE1 protein expression was not detected in unfractionated BM, and also not in the blasts present in MDS, whether with or without a 5q aberration (data not shown). Therefore it is difficult to envision how loss of RPS14 and potential downstream targets such as BAG1 and CSDE1 contributes to the clonal expansion of blasts in MDS. Nevertheless, both DBA and 5q- MDS have relatively low risk of leukemic transformation. Loss of RPS14 is likely to exert the specific erythroid phenotype of 5q- MDS and at the same time it may protect from leukemic transformation, similar to the mechanism by which haploinsufficiency of Rpl24 protects mice from myc-induced cancer[42].

ACKNOWLEDGEMENTS

This work was supported by grants from the European Community (Marie Curie RTN EUrythron, grant. no. MRTN-CT-2004-005499), the Ministry of Education, Czech Republic (MSM 6198959205), the Ministry of Health, Czech Republic (NT11059) and the German Research Foundation (DFG, SFB 581, TP B1).

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SUPPLEMENTAL FIGURES LEGENDS:

Figure S1

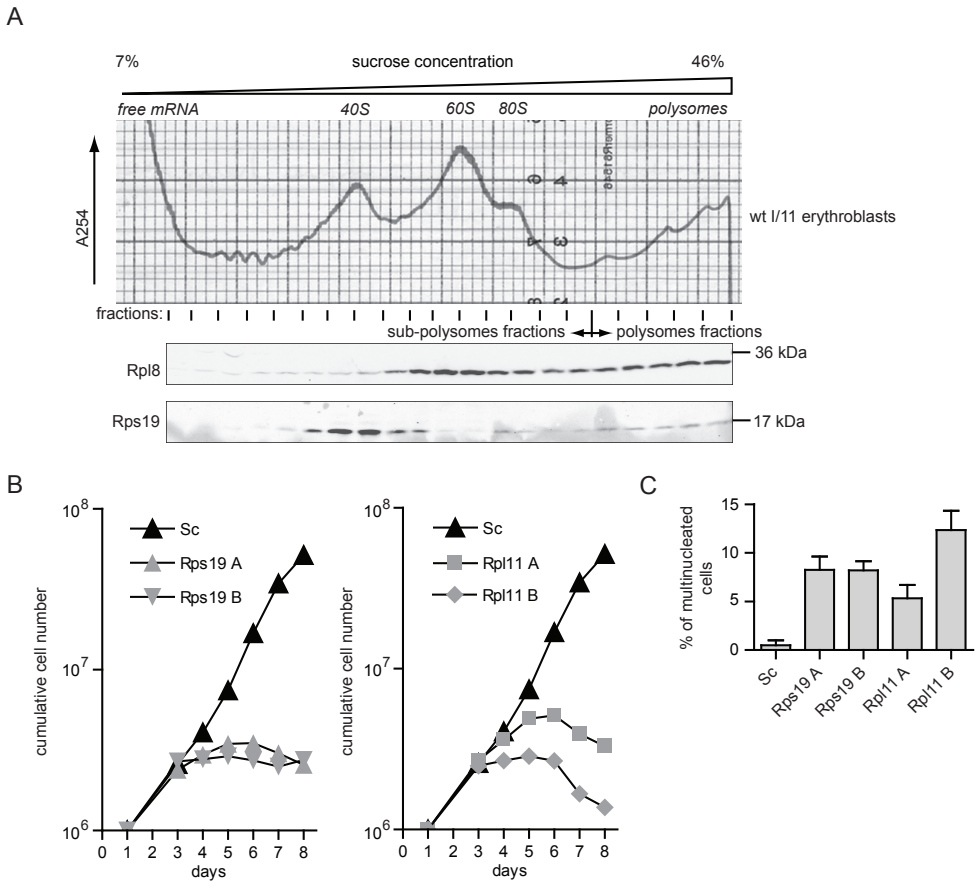


Figure S1: Identification of peaks on sucrose gradient profile. (*A*)Cell lysate was centrifuged on a 7–46% sucrose gradient and the distribution of RNA was measured by absorption at 254 nm. Fractions were collected as indicated. For the isolation of subpolysomal- and polysome-associated RNA the fractions were pooled as indicated by arrows. Proteins from the fractions were isolated with trichloroacetic acid. Western blot containing these samples was stained with Rpl8 (indicating 60S subunits) and Rps19 (indicating 40S subunits) antibody. (*B*) Proliferation of I/11 erythroblasts in presence of Epo, SCF and dexamethasone following transduction with control shRNA (Sc ▲) and 2 distinct shRNA against Rps19 (A ▲and B ▼) or Rpl11 (A ■ and B ♦), respectively. Cumulative cell numbers are calculated from 3 independent experiments. (*C*) Quantification of multinucleated cells six days after transduction with indicated shRNA. Numbers of bi- and multinucleated cells are shown as percentages of the total cell numbers At least 400 cells were counted, error bars represent ± s.e.m.

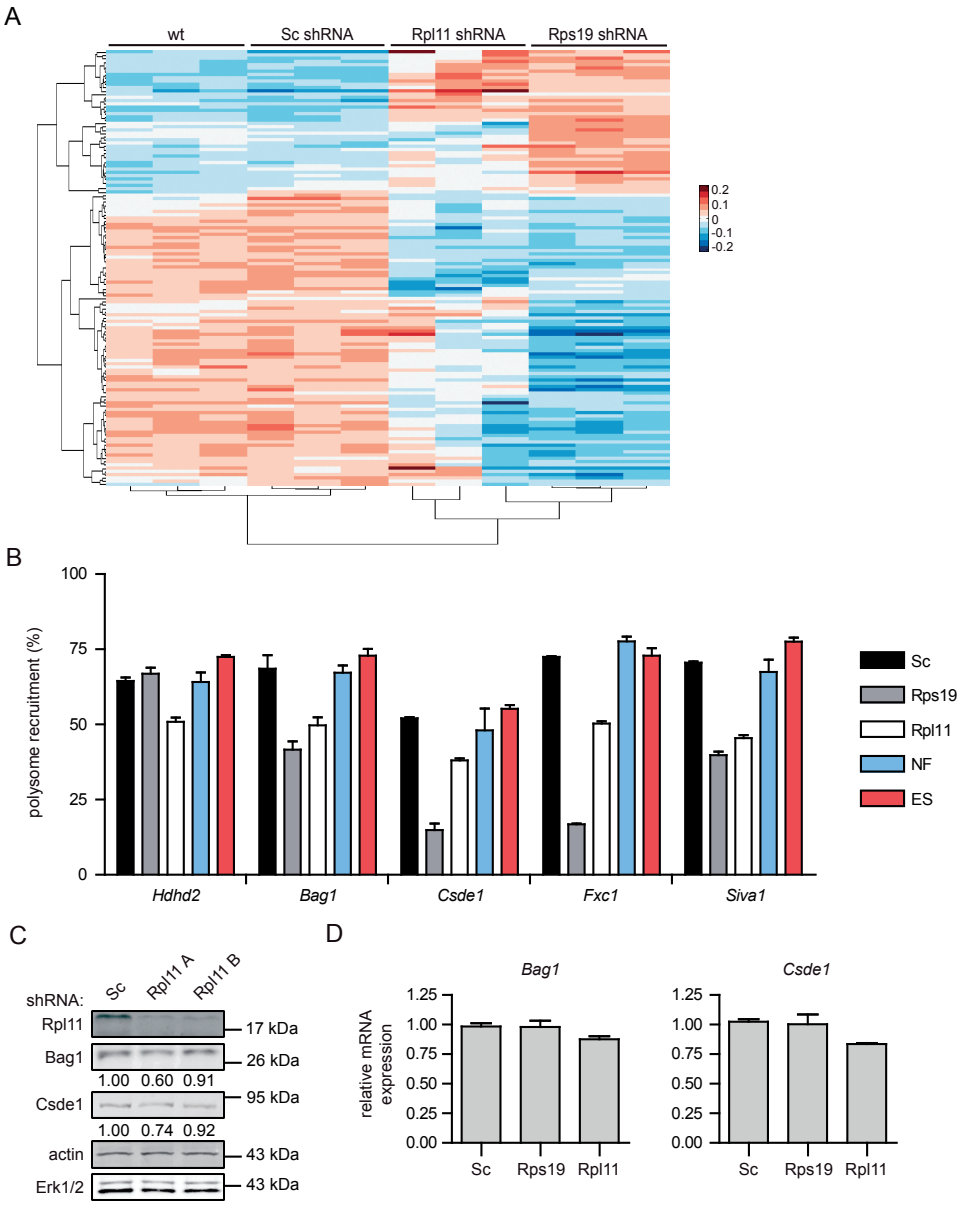


Figure S2: Identification of mRNA targets with reduced translation upon ribosomal deficiency (*A*) 134 probes with differential ratios between wt, Sc shRNA and Rps19 shRNA were hierarchically clustered together with the corresponding ratios in the Rpl11 shRNA dataset. (*B*) Polysome recruitment of control transcript (*Hdhd2*) and selected mRNAs (*Bag1, Csde1, Fxc1, Siva1*) in I/11 erythroblasts treated either with Sc shRNA, Rps19 A shRNA and Rpl11 shRNA A or factor deprived for 4 hours (NF) and re-stimulated with 5U/ml Epo and 200 ng/ml SCF for 2 hours (ES). Error bars represent ± s.e.m. (n=3). (*C*) Protein levels of Bag1 and Csde1 in I/11 erythroblasts 3 days following transduction with Sc and Rpl11 shRNA. Actin and Erk1/2 serve as loading controls. Quantified expression, corrected for actin, is indicated below the blots. (*D*) *Bag1* and *Csde1* mRNA levels relative to tubulin in total RNA in primary fetal liver erythroblasts 3 days after transduction with Sc, Rps19 and Rpl11 shRNA as measured by qRT-PCR. Error bars represent ± s.e.m. (n=4).

Figure S3

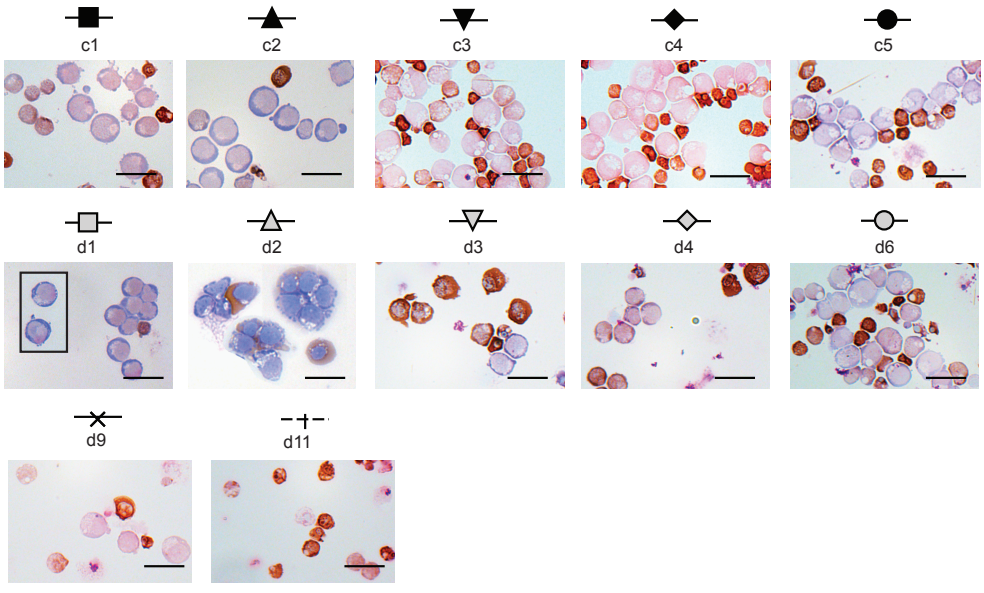


Figure S3: Analysis of DBA erythroblasts. (*A*) Cells from DBA patients and controls were harvested on day16 for cytospins and stained for hemoglobin (brown color) and histological dyes. Bar represent 25 µm. (*B*) Protein levels of BAG1 and CSDE1 in erythroblasts from 7 DBA patients and 5 controls are shown on 3 different blots. Actin is used as a loading control.

Table S-I

#	Affy ID	Change	*p*-value ANOVA	*p*-value FDR	gene name
1	1416798_a_at	down	5,55E-07	0,0103	*Nme4*
2	1451205_at	up	3,57E-06	0,0315	*Psmb4*
3	1429007_at	down	5,11E-06	0,0315	*Slc35b2*
4	1418235_at	down	8,17E-06	0,0330	*Atg5*
5	1448286_at	up	1,03E-05	0,0330	*Hsd17b10*
6	1426307_at	down	1,14E-05	0,0330	*Cyb5r4*
7	1448887_x_at	down	1,54E-05	0,0330	*Fxc1*
8	1449643_s_at	up	1,68E-05	0,0330	*Btf3*
9	1436416_x_at	down	2,02E-05	0,0330	*Fxc1*
10	1433554_at	down	2,36E-05	0,0330	*AU022870*
11	1417075_at	down	2,52E-05	0,0330	*2010309E21Rik*
12	1426581_at	down	2,57E-05	0,0330	*Ptpmt1*
13	1419298_at	up	2,91E-05	0,0330	*Pon3*
14	1448166_a_at	up	2,97E-05	0,0330	*Psmb1*
15	1429568_x_at	down	3,54E-05	0,0330	*Ube2f*
16	1423833_a_at	down	3,57E-05	0,0330	*Brp44*
17	1452191_at	up	3,64E-05	0,0330	*Prcp*
18	1423997_at	down	3,78E-05	0,0330	***Csde1***
19	1454607_s_at	up	3,82E-05	0,0330	*Psat1*
20	1423809_at	down	4,23E-05	0,0330	*Tcf19*
21	1418377_a_at	down	4,40E-05	0,0330	*Siva1*
22	1415852_at	up	4,43E-05	0,0330	*Impdh2*
23	1428186_at	down	4,60E-05	0,0330	*Kctd6*
24	1452621_at	down	4,67E-05	0,0330	*Pcbd2*
25	1455319_x_at	up	4,90E-05	0,0330	*Rps8*
26	1448122_at	up	4,91E-05	0,0330	*Tcp1*
27	1448543_at	down	4,93E-05	0,0330	*Slmo2*
28	1424382_at	up	5,09E-05	0,0330	*Rcn3*
29	1425668_a_at	down	5,34E-05	0,0330	*St3gal4*
30	1460707_at	down	5,36E-05	0,0330	*Ptp4a2*
31	1416458_at	up	5,57E-05	0,0332	*Arf2*
32	1435325_at	down	5,89E-05	0,0339	*Usp46*
33	1423242_at	down	6,24E-05	0,0339	*Mrps36*
34	1416807_at	up	6,29E-05	0,0339	Rpl36al
35	1416628_at	down	6,42E-05	0,0339	*0610006I08Rik*

#	Affy ID	Change	*p*-value ANOVA	*p*-value FDR	gene name
36	1454064_a_at	down	6,81E-05	0,0349	*Rnf138*
37	1417874_at	down	7,14E-05	0,0353	*Tmem9b*
38	1460711_at	down	7,38E-05	0,0353	*Dnajc21*
39	1449048_s_at	down	7,67E-05	0,0353	*Rab4a*
40	1433984_a_at	up	7,76E-05	0,0353	*Mdh2*
41	1452020_a_at	down	8,03E-05	0,0353	*Siva1*
42	1455625_at	down	8,38E-05	0,0353	---
43	1424316_at	down	8,63E-05	0,0353	*Slc25a19*
44	1420476_a_at	up	9,23E-05	0,0353	*Nap1l1*
45	1426456_a_at	down	9,23E-05	0,0353	*Pias2*
46	1450865_s_at	down	9,48E-05	0,0353	*Mrps24*
47	1449289_a_at	up	1,02E-04	0,0353	*B2m*
48	1452584_at	down	1,03E-04	0,0353	*1500032L24Rik*
49	1428272_at	down	1,04E-04	0,0353	*Eif1b*
50	1416651_at	down	1,06E-04	0,0353	*Znhit2*
51	1423241_a_at	down	1,08E-04	0,0353	*Tfdp1*
52	1416056_a_at	up	1,08E-04	0,0353	*Ndufb11*
53	1449710_s_at	up	1,08E-04	0,0353	*Atp5a1*
54	1428706_at	down	1,10E-04	0,0353	*Prr6*
55	1423880_at	up	1,11E-04	0,0353	*D10Wsu52e*
56	1438009_at	up	1,12E-04	0,0353	*RP23-480B19.10*
57	1455874_at	down	1,13E-04	0,0353	*Tmem179b*
58	1435488_at	down	1,14E-04	0,0353	*1110019K23Rik*
59	1419027_s_at	down	1,18E-04	0,0353	*Gltp*
60	1436803_a_at	down	1,19E-04	0,0353	*Ndufb9*
61	1438256_at	down	1,25E-04	0,0353	*Eif5a2*
62	1415961_at	up	1,27E-04	0,0353	*Itm2c*
63	1428489_at	down	1,29E-04	0,0353	*Zcrb1*
64	1436509_at	down	1,29E-04	0,0353	*2410014A08Rik*
65	1448685_at	down	1,31E-04	0,0353	*2900010M23Rik*
66	1455558_at	down	1,32E-04	0,0353	*Gm114*
67	1417341_a_at	down	1,33E-04	0,0353	*Ppp1r2*
68	1415714_a_at	down	1,33E-04	0,0353	*2610209M04Rik*
69	1416439_at	down	1,34E-04	0,0353	*2410015N17Rik*
70	1452307_at	down	1,37E-04	0,0353	*Cables2*
71	1435764_a_at	down	1,38E-04	0,0353	*Gemin7*

#	Affy ID	Change	*p*-value ANOVA	*p*-value FDR	gene name
72	1422480_at	down	1,38E-04	0,0353	*Snx3*
73	1417713_at	up	1,41E-04	0,0353	*Eif2s2*
74	1428863_at	down	1,42E-04	0,0353	*Ankrd39*
75	1426742_at	up	1,43E-04	0,0353	*Atp5f1*
76	1415781_a_at	up	1,49E-04	0,0361	*Sumo2*
77	1460644_at	down	1,52E-04	0,0365	*Bckdk*
78	1451291_at	down	1,56E-04	0,0370	*Obfc2b*
79	1451570_a_at	down	1,63E-04	0,0375	*6720467C03Rik*
80	1425023_at	up	1,64E-04	0,0375	*Usp3*
81	1460695_a_at	down	1,65E-04	0,0375	*2010111I01Rik*
82	1427879_at	up	1,67E-04	0,0375	*1810031K17Rik*
83	1429059_s_at	down	1,68E-04	0,0375	*Tmem107*
84	1428437_at	down	1,71E-04	0,0376	*Lsm14a*
85	1460428_at	down	1,74E-04	0,0377	*Ankrd13a*
86	1457069_at	up	1,81E-04	0,0389	*Ascc3*
87	1417762_a_at	up	1,97E-04	0,0416	*Rpl8*
88	1423817_s_at	up	2,00E-04	0,0416	*Use1*
89	1417287_at	up	2,00E-04	0,0416	*H13*
90	1424948_x_at	up	2,06E-04	0,0416	*H2-D1*
91	1418371_at	down	2,07E-04	0,0416	*Dynll2*
92	1416394_at	down	2,08E-04	0,0416	***Bag1***
93	1417594_at	down	2,09E-04	0,0416	*Gkap1*
94	1417916_a_at	down	2,14E-04	0,0420	*Fxc1*
95	1422998_a_at	down	2,19E-04	0,0425	*Glrx2*
96	1421963_a_at	down	2,22E-04	0,0425	*Cdc25b*
97	1460199_a_at	up	2,23E-04	0,0425	*Pafah1b1*
98	1436510_a_at	down	2,32E-04	0,0436	*Lrrfip2*
99	1439454_x_at	down	2,36E-04	0,0441	*Tm2d2*
100	1426323_x_at	down	2,39E-04	0,0441	*Siva1*
101	1448430_a_at	up	2,41E-04	0,0441	*Naca*
102	1428224_at	up	2,44E-04	0,0441	*Hnrpdl*
103	1451096_at	up	2,53E-04	0,0453	*Ndufs2*
104	1448405_a_at	up	2,64E-04	0,0466	*Eid1*
105	1419289_a_at	down	2,65E-04	0,0466	*Syngr1*
106	1423873_at	down	2,69E-04	0,0469	*Lsm1*
107	1451791_at	up	2,72E-04	0,0470	*Tfpi*

#	Affy ID	Change	*p*-value ANOVA	*p*-value FDR	gene name
108	1417964_at	up	2,84E-04	0,0478	*Ap3d1*
109	1436760_a_at	up	2,84E-04	0,0478	*Rps8*
110	1435446_a_at	down	2,85E-04	0,0478	*Chpt1*
111	1422747_at	down	3,04E-04	0,0484	*Chek2*
112	1424344_s_at	down	3,07E-04	0,0484	*Eif1a*
113	1460704_at	down	3,09E-04	0,0484	*Rfng*
114	1423956_at	down	3,10E-04	0,0484	*Smap1*
115	1417624_at	down	3,11E-04	0,0484	*Nab1*
116	1449404_at	down	3,12E-04	0,0484	*Pip4k2a*
117	1449523_at	down	3,14E-04	0,0484	*Bcl7c*
118	1428677_at	down	3,14E-04	0,0484	*Wdr73*
119	1451068_s_at	up	3,15E-04	0,0484	*Rps25*
120	1436038_a_at	down	3,16E-04	0,0484	*Pigp*
121	1423772_x_at	up	3,17E-04	0,0484	*Slc25a5*
122	1449059_a_at	up	3,26E-04	0,0494	*Oxct1*
123	1452674_a_at	up	3,30E-04	0,0494	*Eif3k*
124	1460169_a_at	down	3,32E-04	0,0494	*Pctk1*
125	1415792_at	down	3,35E-04	0,0495	*Rbck1*
126	1447703_x_at	up	3,39E-04	0,0496	*Zfp593*
127	1423976_at	down	3,46E-04	0,0498	*4930453N24Rik*
128	1450866_a_at	down	3,50E-04	0,0498	*Mrpl17*
129	1423831_at	down	3,53E-04	0,0498	*Prkag2*
130	1426374_at	down	3,55E-04	0,0498	*2410166I05Rik*
131	1435881_at	down	3,55E-04	0,0498	*Pcbp2*
132	1417728_at	down	3,57E-04	0,0498	*Mbd3*
133	1426277_at	down	3,60E-04	0,0498	*C730025P13Rik*
134	1436421_s_at	down	3,62E-04	0,0498	*Arpc5l*

Table S-II

Patient	Czech DBA registry ID	Sex	Age	Mutation	Status	Hb (g/l)	Current treatment	erythroblast yield *10^6	WB and qRT-PCR analysis performed
d1	CZ02	M	34	*RPS17 c.2T>G*	mild anemia	99	prednisone, leucine	59	yes
d2	CZ09	F	21	*RPS19 c196_206del*	severe anemia	72	steroids, transfusion dependent	12	yes
d3	CZ31	F	45	*RPL5 c.74-1G>A*	mild anemia	105	none	1	yes
d4	CZ12	M	22	*RPL5 c.74-1G>A*	mild anemia	101	steroids discontinued, transfusions	0,9	yes
d5	CZ21	M	11	*RPS26 c.1A>C*	severe anemia	72	transfusion dependent	0,2	no
d6	CZ19	M	15	*RPS26 c.3 G>A*	anemia; PB19[#]	86	steroids, transfusion dependent	5	yes
d7	CZ29	M	5	*RPL5 c.854C>T*	severe anemia	73	transfusion dependent, leucine	0,2	no
d8	CZ32	M	21	*RPL11 c.59T>A*	anemia	95	transfusion dependent	0,2	no
d9	CZ10	M	24	*N.A.*	anemia	N.A.	N.A.	0,5	only WB
d10	CZ39	F	17	*N.A.*	severe anemia	N.A.	transfusion depndent	0,2	no
d11	CZ01	F	39	*N.A.*	anemia	N.A.	steroids	7	yes

[#] parvovirus infection

Table S-III

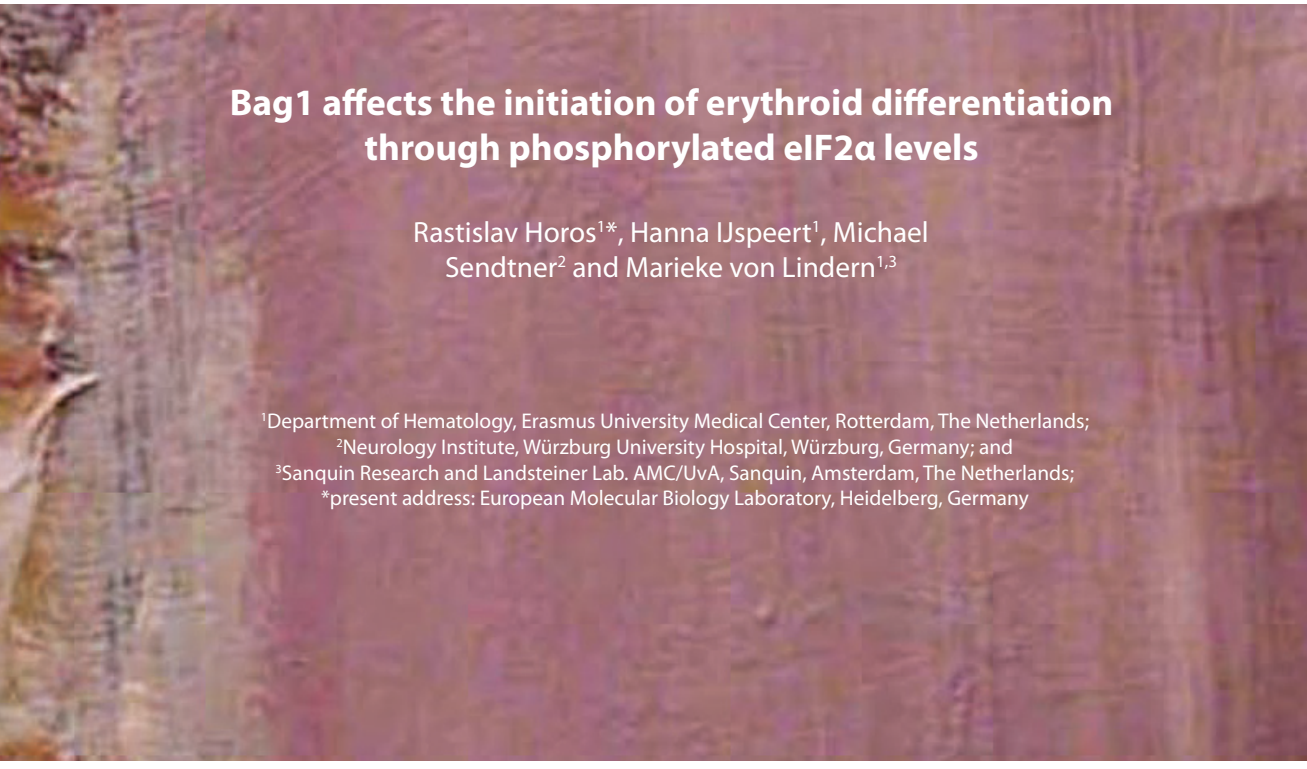
The expression levels of candidate genes in erythroblasts from controls and DBA patients				
Subject	relative protein levels (a.u.)		relative mRNA levels (a.u.) - mean	
	BAG1	CSDE1	BAG1	CSDE1
c1	1,000	1,000	1,000	1,000
c2	0,714	0,441	1,516	1,461
c3	0,937	0,913	1,039	1,420
c4	1,055	0,782	1,000	1,000
c5	1,157	0,667	1,357	1,090
d1	0,855	0,713	0,811	1,605
d2	1,056	0,051	1,915	1,888
d3	0,614	0,004	2,082	0,866
d4	0,324	0,005	2,107	0,777
d6	0,615	0,246	1,171	1,165
d9	0,238	0,043	N.A.	N.A.
d11	0,883	0,249	0,432	0,749

Table S-IV

List of primers	
Name	**Sequence 5' to 3'**
m_Bag1_F	ACCCCACAGCAAGGTAACAG
m_Bag1_R	TTTGCATTCCAAGTGCTGAC
m_Csde1_F	AATTGGAACGAGCAACCAAC
m_Csde1_R	TTTCTTTGGGCAGAGAGCAT
m_Fxc1_F	ACAGCTGTGCTGGGAAACTC
m_Fxc1_R	GGCGAGTCTCTGGTCTGTTC
m_Siva1_F	GATTGTTCCGTGAACCACCT
m_Siva1_R	CTCATGCACGATGAACAAGC
m_Gata1_F	CGGCCTCTATTTCAAGCTCC
m_Gata1_R	TTCCTCGTCTGGATTCCATC
m_Lmo2_F	TGCCATCCTGATAGGCTTCC
m_Lmo2_R	TGTACCTCCATGCAGTTTCCC
h_B2M_F	TGAGTATGCCTGCCGTGTGA
h_B2M_R	TCTTCAAACCTCCATGATGCT
h_BAG1_F	AACACCGTTGTCAGCACTTG
h_BAG1_R	GAGCTTCAGCTTGCAAATCC
h_CSDE1_F	GGCAACAAAGTCAGTGCAGA
h_CSDE1_R	ACCTTTCATATCGCCCTCCT
m_Bag1_IRES_F	AAGCTAGCTGTGAACAAACTCGGCCTGG
m_Bag1_IRES_R	AACTCGAGATGTTGTCGGTCTGGGTCACC
m_Csde1_IRES_F	AAGCTAGCGCGAGATTTATTCCTACGTACC
m_Csde1_IRES_R	AACTCGAGTCGCAGTGATACTCAAATATTGC
m_Csde1_3UTR_F	AATGAAGTGTGCCAGCAGTG
m_Csde1_3UTR_R	ATTGAACAGGGCACTGGAAC
m_Apaf1_F	GACAGCGAGAGGAAAACCAG
m_Apaf1_R	CCTCAGAGTTGCTCCCAGTC

List of shRNA plasmids clone ID	
Scrambled control	**SHC002**
Rps19 A	TRCN0000104260
Rps19 B	TRCN0000104264
Rpl11 A	TRCN0000104477
Rpl11 B	TRCN0000104479
Bag1 A	TRCN0000012369
Bag1 B	TRCN0000012372
Csde1 A	TRCN0000181609
Csde1 B	TRCN0000198576


CHAPTER
3



Bag1 affects the initiation of erythroid differentiation through phosphorylated eIF2α levels

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work in progress

ABSTRACT

Rationale: We previously observed reduced translation of *Bag1* (Bcl2 associated athanogene 1) mRNA in an in vitro model of Diamond Blackfan Anemia. *Bag1*$^{-/-}$ embryos die at embryonal day 13.5 with a complete lack of mature fetal erythrocytes. The aim of our study is to understand the role of Bag1 in erythropoiesis. Objectives: We examined the expression of Bag1 isoforms during differentiation of mouse erythroblasts and analysed how reduced expression or complete absence of Bag1 affects proliferation and differentiation of primary mouse erythroblasts. Because Bag1 is a cochaperone of the Hsc70/Hsp70 complex belonging to the ER protein surveillance machinery, we examined components of the ER stress pathway in Bag1-deficient erythroblasts. Findings: The p32 Bag1 isoform, expressed by IRES-mediated translation, is the most prominent isoform expressed in erythroblasts. Knockdown of *Bag1* suppressed terminal differentiation and sustained the growth of primary mouse erythroblasts. Similar effects were observed when erythroblasts were expanded from *Bag1*$^{-/-}$ fetal liver. Bag1 deficient cells exhibit increased levels of phosphorylated eIF2α, decreased basal levels of BiP and increased levels of CHOP. Conclusions: Bag1 deficient erythroblasts showed delayed entry into terminal differentiation coupled to increased eIF2α phosphorylation. The feedback mechanisms triggered upon eIF2α phosphorylation render the cells less sensitive to further stress and may contribute to sustained *in vitro* expansion. The results underline the importance of the eIF2 stress response pathway in erythroid development.

INTRODUCTION

Diamond Blackfan Anemia is characterized by congenital mutations in ribosomal proteins that result in haploinsufficiency and defective maturation of ribosomal subunits. We recently showed that IRES dependent translation of *Bag1* (*Bcl2-associated athanogene 1*) is reduced upon knock down of ribosomal protein S19 in mouse erythroblasts, a model system for Diamond Blackfan Anemia (DBA), and in erythroblasts cultured from DBA patients[1]. Bag1 was initially identified as a Bcl2 binding protein that protects cells from apoptosis [2]. Subsequently it appeared to be involved in various cellular processes such as proliferation and activity of nuclear hormone receptors[3,4]. Bag1 belongs to a 6-member family of BAG domain containing proteins that modulate the activity of Hsp70 molecular chaperones[5].

Whereas *Bag1*$^{+/-}$ embryos are born without obvious abnormalities, *Bag1* knockout mice die at embryonal day 13.5 with abnormalities in forebrain and fetal liver. *Bag1*$^{-/-}$ embryos were severely anemic, had smaller livers and showed increased apoptosis[6]. Analysis of hematopoiesis in *Bag1*$^{-/-}$ fetal livers showed that the number of early GM-CSF dependent colony forming units was unaffected, while BFU-E (Burst forming units-erythroid) were almost absent from Bag1-deficient fetal livers. Knock down of Bag1 expression in the erythroid cell line I/11 indicated that Bag1 is mainly required for erythroid differentiation and less for expansion[1].

The *Bag1* mRNA encodes distinct Bag1 protein isoforms[7]. The longer p50 isoform (BAG-1L in human) is translated from a non-canonical CUG initiation codon, and contains a nuclear localization signal and a number of acidic amino acid repeats of unknown function. The shorter p32 isoform (p36 or BAG-1M in human) is translated via an IRES element just upstream of the p32 start codon[8], which enables Bag1 translation when cap-dependent translation is inhibited. Both isoforms contain the central ULD (ubiquitin-like) and C-terminal BAG domain. The ULD domain is required for the association of Bag1 with the 26S proteasome[9], whereas the BAG domain interacts with Hsc70/Hsp70[10]. Bag1 functions as a cochaperone, which facilitates the exchange of ADP for ATP on Hsc70/Hsp70 and subsequent release of folded substrate from Hsc70/Hsp70 chaperone system[11].

Expression of Hsp70/Hsc70 is increased during late steps of erythroid differentiation, which is required for the increased pressure on protein quality control due to the increased hemoglobin production[12]. Unfolded proteins activate the unfolded protein response (UPR), which involves 3 downstream pathways[13]. The activation of PERK (PRKR-like endoplasmic reticulum kinase, or Eif2ak3) results in phosphorylation of the translation initiation factor 2 subunit α (eIF2α). During translation initiation eIF2 carries the methionine-bound initiator tRNA ($tRNA_{i}^{met}$) to the preinitiation scanning complex. The eIF2 is a GTPase and the exchange of GTP to GDP drives release of methionine at an AUG start codon to the P-site of the ribosome[14]. After release of $tRNA_i$ and methionine, the eIF2:GDP must be recharged to eIF2:GTP, which is inhibited by its phosphorylation on Ser51. Phosphorylation of eIF2α reduces general translation initiation rates, but particularly affects selection of AUG start codons following an upstream open reading frame (uORF) because this requires recruitment of a new eIF:GTP-$tRNA_i^{met}$ ternary complex while

the preinitiation complex continues scanning of the 5'UTR[15]. Phosphorylation of eIF2α leads to translation of *ATF4* (*Activating Transcription Factor 4)*, which in turn induce the expression of feedback proteins *Gadd34* (*Growth Arrest and DNA Damage-inducible protein 34*; also known as *Ppp1r15a*)[16] and *Chop* (*CCAAT/enhancer-binding protein homologous protein*; also known as *Ddit3*)[17]. Gadd34 is an inducible subunit of protein phosphatase 1 (Pp1) that enhances dephosphorylation of p-eIF2α[18]. Bag1 was shown to interact with Gadd34 and the binding of Bag1 decreased the dephosphorylation activity of Gadd34-PP1 complex *in vitro*[19]. Unfolded proteins also bind and activate IRE1 (inositol-requiring 1, also known as Ern2), which enhances splicing of *Xbp1* (*X-box binding protein 1*)[20]. Xbp_{short} is an active transcription factor that induces expression of additional ER chaperones such as Bip (*Binding immunoglobulin protein*; also known as *Hspa5*)[21]. The third leg of UPR is formed by activation and cleavage of transcription factor ATF6, which enhances expression of Xbp1 and further induces chaperones such as Bip[22].

Here we investigated how Bag1 deficiency affects erythroid differentiation. Primary erythroblasts lacking Bag1 initially proliferated slowly but they demonstrated a striking capacity to adapt. As a result, Bag1-deficient cultures had an extended proliferation capacity, which was at least partly due to a delay in differentiation. Lack of Bag1 initially increased the phosphorylation of eIF2α, which was reduced to intermediate levels upon prolonged proliferation of Bag1-deficient erythroblasts. This intermediate eIF2α phosphorylation could not be increased upon activation of UPR, which suggests active feedback control. This was supported by reduced levels of the UPR sensor target gene Bip. Given the important role of eIF2α phosphorylation in balancing iron availability with globin synthesis during erythroid maturation, the impaired regulation of eIF2α activity in Bag1-deficient cells may contribute to delayed differentiation.

DESIGN AND METHODS

Cell culture

I/11 erythroblasts, derived from p53-deficient mouse fetal livers, or primary fetal liver-derived erythroblasts were expanded in StemPro-34 medium (Invitrogen, Breda, The Netherlands) supplemented with 0.5 U/mL Epo (gift from Jansen-Cilag, Tilburg, The Netherlands), 100ng/mL of SCF (supernatant of CHO producer cells) and 10^{-6} M dexamethasone (Sigma-Aldrich, St Louis, MO)[23]. For differentiation, medium was supplemented with 5 U/mL Epo and 0.5 mg/mL iron-loaded transferrin (Scipac, Sittingbourne, UK). Cell number and size were determined with an electronic cell counter (CASY-1; Innovatis, Reutlingen, Germany). Hek293T cells were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) fetal calf serum (PAA, Pasching, Austria). For protein stability assays, cells were treated with 0.1mg/mL cycloheximide, harvested every 2 hours and washed with PBS before lysis. For induction of the p-eIF2α response cells were treated with 2.5 μg/mL tunicamycin.

Hemoglobin content and cell morphology

Cell morphology was assessed by cytospins stained with histological dyes and neutral benzidine[24], using 40x magnification (Leica, Wetzlar, Germany). Images were processed in Adobe Photoshop CS3, version 10.0 (Adobe, San Jose, CA).

Lentivirus production, titration and transductions

293HekT cells were transfected with pLKO.1-puro lentiviral construct containing shRNA sequences (MISSION® TRC-Mm 1.0 shRNA library; Sigma) against Bag1 (TRCN0000012369, TRCN0000012372) or Scrambled control (SHC002) togehter with packaging plasmids[25] using LT-1 transfection reagent (Mirus, Madison, WI). Medium was concentrated by ultracentrifugation at 60 000×g for 2h at 4°C. Lentivirus titer was assessed by colony assays in 293HekT cells. Erythroblasts were transduced with a multiplicity of infection of 3-5 and addition of 8 μg/mL polybrene (Sigma). 24 hours after the transduction cells were kept with 1μg/mL puromycine for the rest of the experiment.

Sucrose gradients

35×10^6 cells were incubated with 0.1 mg/mL cycloheximide (CHX) for 10 min at 37°C, washed with cold PBS containing 0.1 mg/mL CHX and lysed as described[24]. Polysomal RNA was separated on 10.5mL 7-46% linear sucrose gradients containing 10mM Tris-HCl, 12mM $MgCl_2$, 140mM NaCl and centrifuged at 190,000 g for 3 hours. Measurement of absorbance at 254 nm, visualization and fractionation were done using an Econo system (Biorad, Hercules, CA).

RNA isolation, cDNA synthesis, qRT-PCR

RNA was isolated from gradient fractions as described[24] and further purified with LiCl[26]. Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany). RNA from RNA-IPs was amplified by MessageAmp II aRNAkit (Ambion, Austin, TX). 1,6μg of RNA was used for cDNA

preparation with random hexamers and SuperScript II Reverse Transcriptase kit (Invitrogen). qRT-PCR was performed as described[24], data represented are relative to the levels of *α-tubulin* mRNA. Primers used are Bag1_F (5'-ACCCCACAGCAAGGTAACAG-3'), Bag1_R (5'-TTT-GCATTCC AAGTGCTGAC-3'), tub_F (5'-GGCACCGGCTCTG GCTTCACCTCTC-3'), tub_R (5'-GCCGTGGACACTTGGGGGGCTGG-3'), p53_F (5'-CCATCCTGGCTGTAG-GTAGC-3'), p53_R (5'-AATGTCTCCTGGCTCAGAGG-3'), p21_F (CAGGAGCAAAGTGTG CCGTTGTCTCTTC), p21_R (CAGAAGACCAAT CTGCGCTTGGAGTGAT), Bbc3_F (5'-CCAGAAATGGAGCCCAACTA-3'), Bbc3_R (5'-AAGGCTGGCAGTCCAGTATG-3'), Bip_F (5'- AGTGGTGGCCACTAATGGAG-3'), Bip-R (CAATCCTTGCTTGA TGCTGA), Chop_F (5'-GCATGAAGGAGAAGGA GCA G-3') and Chop_R (5'-CTTCCGGAGAGACA-GACAGG-3').

SDS-PAGE and Western blotting

Cell lysates, SDS-PAGE and Western blotting were performed as described[24]. Antibodies used were Rpl11 (Santa Cruz Biotechnology, sc-25931), Bag1 (Santa Cruz Biotechnology, sc-939) β-actin (Santa Cruz Biotechnology, sc-1616) and p-eIF2α Ser51 (Cell Signalling, 9721). Fluorescently-labeled secondary antibodies were used and blots scanned on an Odyssey infrared scanner (LI-COR, Lincoln, NE).*Mice*

The generation of Bag1 knockout mice was described[6]. All were housed in the central animal facilities of the University of Würzburg. The animal care and ethics committee approved all procedures and experiments.

RESULTS

Bag1 is expressed in the erythroid differentiation

We identified *Bag1* mRNA in a screen for transcripts with reduced polyribosome recruitment in mouse erythroblasts deficient for ribosomal proteins. We also showed that IRES-mediated translation was reduced in this model[1]. To investigate the potential function of Bag1 in erythroblasts we first analyzed its expression. In cultured primary erythroblasts and in the I/11 erythroblast cell line we detected both the p32 and p50 Bag1 protein isoform. p32 was the most prominent isoform, particularly in I/11 cells (Figure 1A). During terminal differentiation of erythroblasts to enucleated reticulocytes, which is characterized by cell size reduction, enucleation and concentration of hemoglobin in the cytoplasm (Figure 1B), the Bag p32 protein level remained stable while p50 slightly decreased when compared with β-actin as loading control (Figure 1C). We previously showed pronounced upregulation of Bag1 during differentiation of hematopoietic multipotent progenitors to erythroblasts[1]. During differentiation of erythroblasts to reticulocytes *Bag1* mRNA remains relatively constant similar to its protein levels (Figure 1D). Polyribosomal recruitment of *Bag1* mRNA, however, steadily decreased during the course of terminal differentiation (Figure 1E). This suggested increased stability of Bag1 p32 protein in erythroid cells,

Figure 1

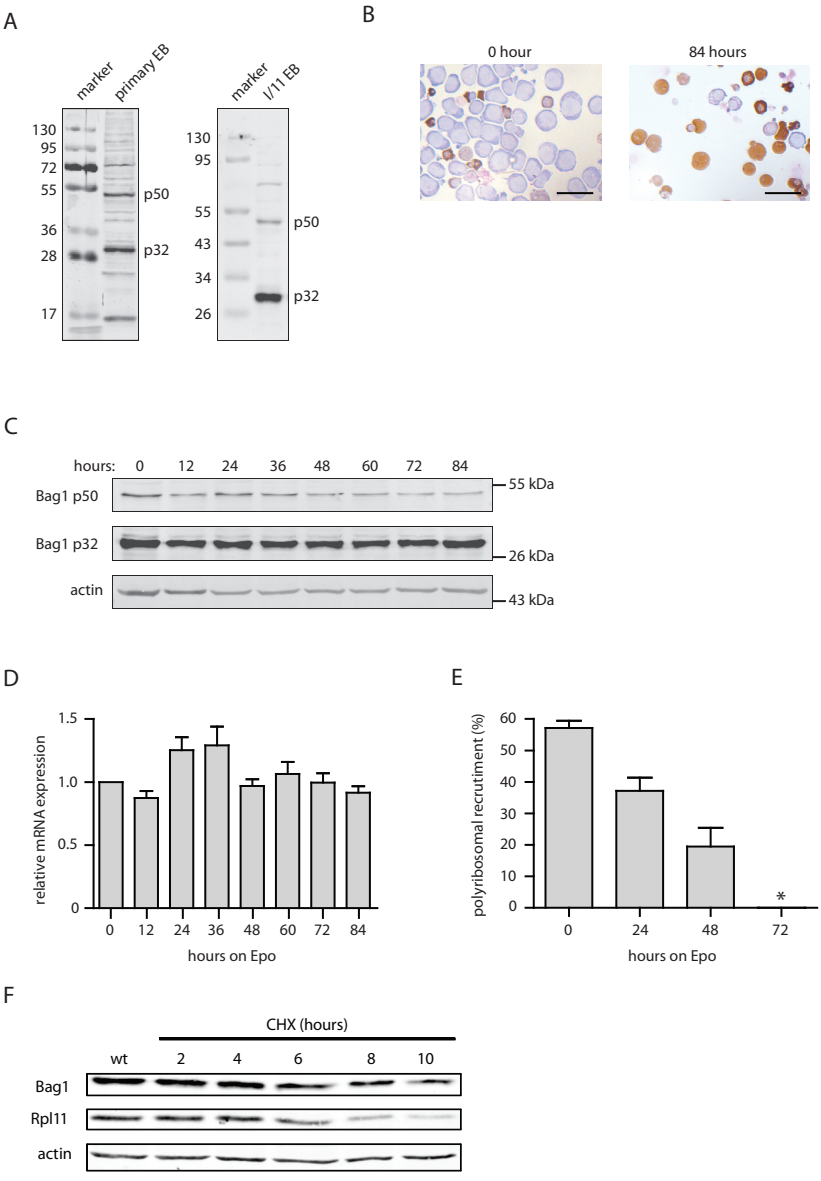

Figure 1. Expression of Bag1 isoforms in erythropoiesis. (A) Western blot showing protein levels of Bag1 isoforms in the primary erythroblasts isolated from E12.5 mouse fetal liver and I/11 cells. (B) The cytospins of I/11 cells showing cell morphology at 0h and 84h of treatment with Epo only. Cells were stained for hemoglobin (brown) and histological dyes. Scale bars represent 25 μm. (C) The protein levels of Bag1 p50 and p32 isoforms in the course of terminal erythroid differentiation. β-actin was used as loading control. (D) The *Bag1* mRNA levels were measured with qRT-PCR on the samples from differentiating I/11 erythroblasts every 12 hours. Data shown are mean ± s.e.m. (n=4) (E) The polyribosomal association of *Bag1* mRNA was measured with qRT-PCR on the cDNA prepared from polyribosomal and subpolyribosomal fractions of sucrose gradient. * indicates non-detectable *Bag1* mRNA signal in polyribosomal fractions. Data shown are mean ± s.e.m. (n=4) (F) Western blot showing protein levels of Bag1, ribosomal protein L11 and β-actin in I/11 cells treated with cycloheximide. The samples were harvested every 2 hours.

which we further supported by stability assays. The Bag1 protein stability in I/11 erythroblasts treated with CHX was pronounced when compared to e.g. Rpl11 protein and exceeded a $t_{1/2}$ of 8 hours (Figure 1F). Thus, the elevated expression of *Bag1* mRNA and protein in erythroblasts and the stability of the p32 isoform suggest a function in terminal erythropoiesis.

Loss of Bag1 promotes expansion of primary mouse fetal liver erythroblasts

Primary mouse erythroblasts can be expanded from E12.5 mouse fetal liver in serum free media supplemented with Epo, SCF and glucocorticoids *in vitro*[23]. These cultures extinguish after approximately 2 weeks due to an increasing rate of spontaneous differentiation to reticulocytes. Previously, we showed that lentiviral mediated knockdown of Bag1 protein had no effect on proliferation of p53-deficient I/11 erythroblasts but inhibited their differentiation[1]. To study the effect of Bag1 on erythroid maturation in primary, p53 proficient erythroblasts, we used two different *Bag1* shRNA lentiviruses (A and B), which reduced the Bag1 levels to 70% and 20%, respectively, when compared to cells infected with control shRNA (Sc) (Figure 2A). The Bag1 shRNA A slightly reduced the cumulative primary erythroblast number when compared to Sc treated cells, whereas the Bag1 shRNA B increased the expansion potential of primary erythroblasts by 10-fold at day 14 (Figure 2B). Size profiling indicated expansion of blast cells in Bag1 B shRNA cells relative to the fraction of enucleated (<5μm) erythrocytes towards the end of the experiment (Figure 2C). At the same time, the expression of Bag1 almost completely vanished (Figure 2D, left panel). We were able to culture the primary erythroblasts transduced with Bag1 B shRNA well beyond the usual 2-weeks proliferative time span and could repeatedly obtain "cell lines" with almost no Bag1 expression (Figure 2D, right panel). To exclude the possibility that Bag1 shRNAs may interfere with p53 expression or response and thus cause a growth advantage, we measured the mRNA levels of *Bag1*, *Trp53*, *p21* and *Bbc3* (Puma) in primary erythroblasts infected with Sc, Bag1 A and Bag1 B shRNA three days after lentiviral infection. Figure 2E shows that shRNA Bag1 B did not reduce the expression of p53 or its targets p21 and Bbc3. Therefore it is likely that the growth advantage of primary erythroblasts is caused by loss of Bag1 function.

Bag1$^{-/-}$ erythroblasts show reduced proliferation and differentiation capacity

Bag1$^{-/-}$ mouse embryos do not survive embryonic day E13.5 and have small, anemic livers[6]. We showed that *Bag1*$^{-/-}$ fetal liver cells have decreased erythroid potential and a decreased CD71^{+}/Ter119^{+} population [1]. To examine whether the decreased differentiation capacity of *Bag1*$^{-/-}$ erythroblasts also results in increased expansion in vitro, similar to what we observed upon knock down of *Bag1*, we initiated erythroblast cultures from *Bag1*$^{-/-}$ fetal livers together with control livers. Erythroblasts were kept under expanding conditions with Epo, SCF and dex and proliferation and morphology of the cells were monitored. Initially the proliferation of *Bag1*$^{-/-}$ erythroblasts was reduced, whereas *Bag1* heterozygote cells proliferated similarly as wt cells (Figure 3A). After a few days, however, the Bag1-/- erythroblasts started to proliferate with the same kinetics as wt controls. Similarly to shRNA-mediated knock down in primary cells, the lack of Bag1 maintained large, immature erythroblasts in the cultures, as shown on cell counter profiles (Figure 3B) and morphological staining (Figure 3C). The similar erythroid phenotype of

Figure 2

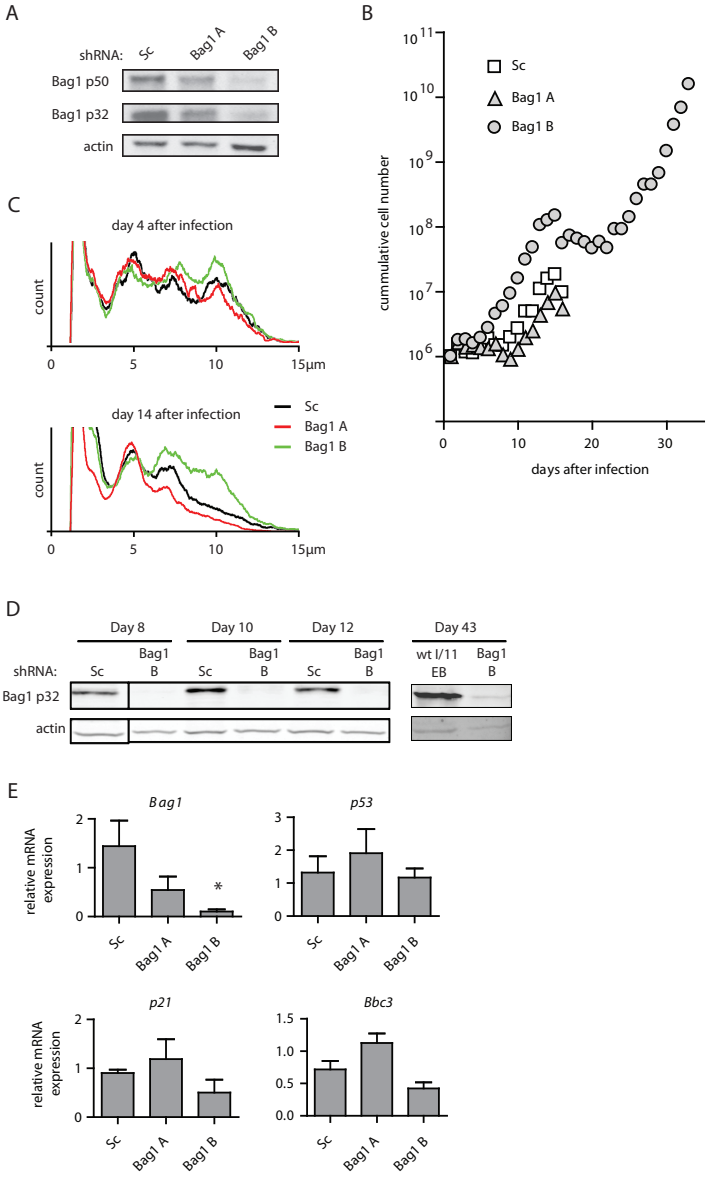

Figure 2. Bag1 knockdown inhibits erythroid differentiation and promotes erythroid expansion. Primary fetal liver erythroblasts were isolated, expanded in vitro for 3 days, infected with lentiviruses bearing Sc or Bag1 shRNAs and followed over time in culture. (A) After 2 days of puromycin selection, cells were harvested and lysates were analyzed on Western blot for expression of Bag1 and actin. (B) Growth curve of the infected cells; the cumulative number is shown on the days after infection. (C) Cell size profiles, measured by the Casy cell counter, shown on day 4 and 14 after infection. Peaks at 10µm size indicate erythroblasts; peaks at 5µm represent enucleated reticulocytes. (D) Western blot of cells infected with Sc and Bag1 B shRNA lentiviruses showing protein levels of Bag1 in time after infection. At the time point of 43 days, I/11 cells were used as a control. (E) mRNA levels of *Bag1* as well as genes involved in the p53 response were measured with qRT-PCR on samples from primary erythroblasts 3 days after infection. Data shown are mean ± s.e.m. (n=4) *p<0.01

Bag1$^{-/-}$ and Bag1 shRNA B erythroblasts suggests that the effects are caused by complete absence of Bag1 in both models.

Figure 3

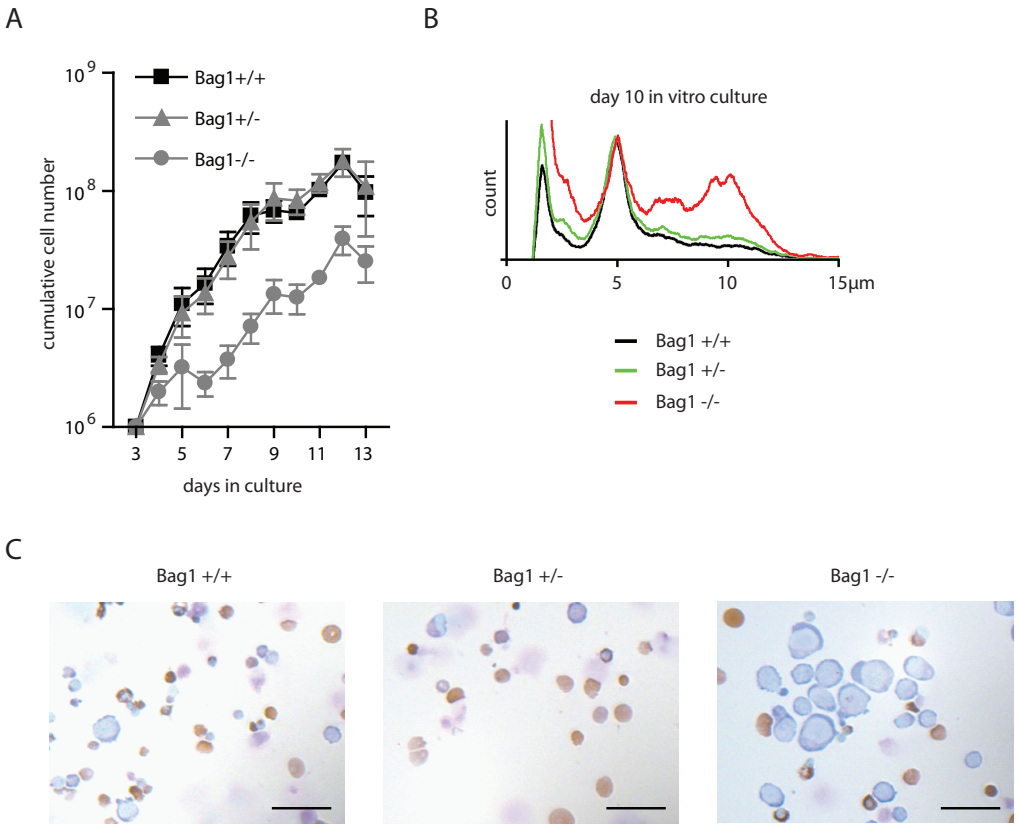

Figure 3. In vitro cultures of *Bag1*$^{-/-}$ fetal liver erythroblasts. Fetal liver cells were isolated from E12.5 mouse embryos and were cultured in medium supporting erythroblasts expansion. (A) Cumulative growth curve of erythroblasts. (B) Cell size profiles, measured by a cell counter, shown on day 10 of culture. Peaks at 10µm size indicate erythroblasts; peaks at 5µm represent enucleated reticulocytes. (C) Cytospins showing cell morphology at day 13 of culture. Cells were stained for hemoglobin (brown) and with histological dyes. Scale bars represent 25 µm.

Bag1 deficiency causes a sustained increase in p-eIF2α levels

Reduced expression of the cochaperone protein Bag1 may invoke an unfolded protein reponse (UPR) and eIF2 phosphorylation. Alternatively, Bag1 was reported to bind Gadd34 and decrease the dephosphorylation activity of Pp1[19], which would reduce eIF2 phosphorylation upon Bag1 deficiency. Therefore we investigated the levels of p-eIF2α in Bag1 deficient cells. *Bag1* knock-down in primary erythroblasts induced phosphorylation of eIF2α in short- (4-fold) as well as in long-term cultures (2-fold) (Figure 4A), which supports the hypothesis of UPR, but not the repression of Gadd34 activity. Phosphorylation of eIF2α can be induced in erythroblasts by

Figure 4

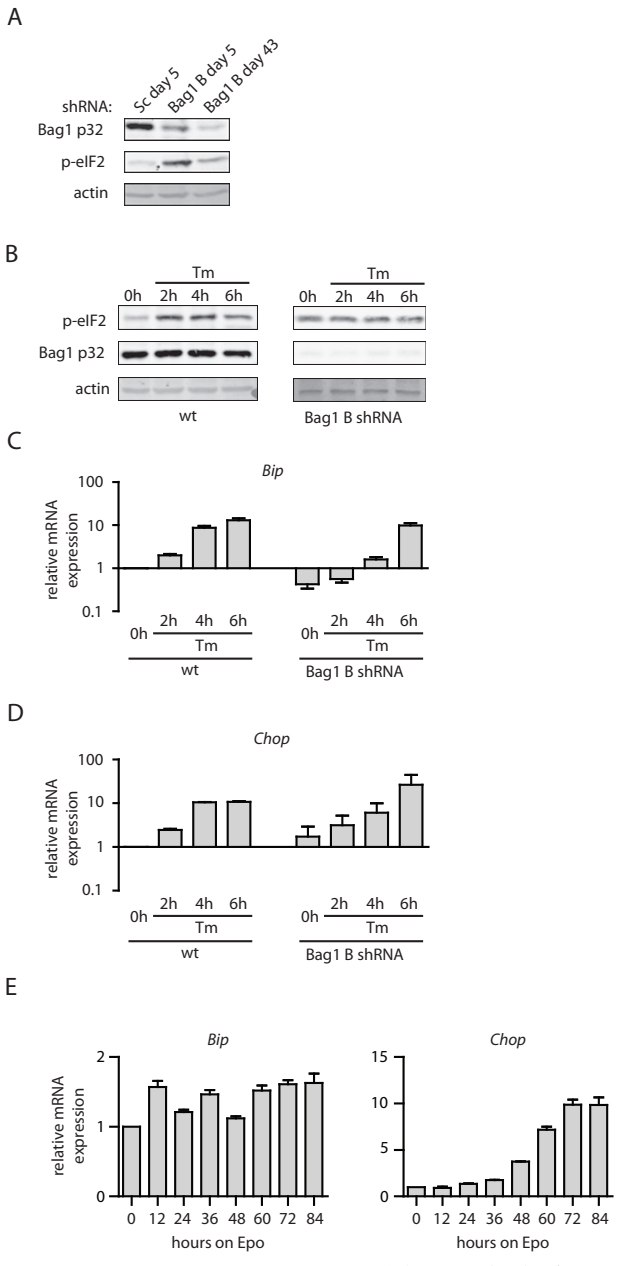

Figure 4. eIF2α phosphorylation pathway in Bag1 deficient cells. (A) Protein levels of Bag1 and p-eIF2α in primary fetal liver erythroblasts infected with Sc or Bag1 B shRNA lentiviruses on day 5 and 43 post infection. (B) eIF2 phosphorylation levels in wt I/11 cells and long term culture of Bag1 B shRNA cells after treatment with Tm. (C) mRNA levels of *Bip* measured by qRT-PCR in the cells from (B). (D) mRNA levels of *Chop* measured by qRT-PCR in the cells from (B). (E) *Bip* and *Chop* mRNA levels measured by qRT-PCR on the samples from differentiating I/11 erythroblasts every 12 hours as shown in Figure 1B. Data shown are mean ± s.e.m. (n=4)

tunicamycine (Tm, a glycosylation inhibitor in the Golgi apparatus), which resembles ER stress (Figure 4B, left panel). Phosphorylation of eIF2α did not affect the expression of Bag1. Because p-eIF2α levels were elevated in Bag1 deficient erythroblasts, we investigated if additional stress will further increase these levels. Treatment with Tm did not further increase the moderate eIF2α phosphorylation in long-term (>40 days) expanded Bag1 deficient erythroblasts (Figure 4B, right panel). Thus, Bag1 deficiency caused an increase in basal phosphorylation levels of eIF2α that rendered the cells insensitive to additional stress.

We next investigated Tm-induced expression of *Chop*, a target of eIF2α phosphorylation, and *Bip*, a sensor of unfolded proteins in the ER that is activated upon UPR through Ire1 and Atf6. In wt cells, the levels of *Bip* and *Chop* were increased after Tm treatment (Figure 4C,D). However the basal levels of *Bip* were lower in the long term growing Bag1 deficient primary cells and the response of *Bip* to the Tm treatment was delayed, when compared to the wt cells (Figure 4C). On the other hand, the basal levels of *Chop* were increased in Bag1 deficient cells (Figure 4D). Interestingly, during the course of normal terminal erythroid differentiation of wt I/11 erythroblasts, the mRNA levels of *Bip* were not changing, but those of *Chop* were increased by 10-fold (Figure 4E). Thus we conclude that misfolded protein sensing in Bag1 deficient cells is decreased, probably due to sustained feedback control caused by increased eIF2 phosphorylation and *Chop* levels.

DISCUSSION

Recently we identified *Bag1* as a transcript that is preferentially lost from polysomes in models for DBA, as well as in erythroblasts cultured from peripheral blood of DBA patients. Mice deficient for Bag1 die at E13,5 with a lack of mature erythrocytes. Their fetal liver contains early hematopoietic progenitors, but lacks erythroid specific colony forming cells. Together with a 20-fold increase of Bag1 mRNA expression in erythropoiesis, this points to an important role of Bag1 in erythropoiesis. In this manuscript we investigated the role of Bag1 in more detail. We show that the short p32 isoform of Bag1 which is translated through IRES mediated translation initiation is the major isoform expressed in erythroblasts. Primary erythroblast that lack Bag1 initially proliferated more slowly, but adapted and underwent increased expansion because they were largely protected from spontaneous differentiation. Slow expansion was associated with a 4-fold increase in eIF2α phosphorylation, which was reduced to a 2-fold increase when Bag1-deficient erythroblast cultures resumed at wt expansion rate. Strikingly, these Bag1-deficient erythroblasts became resistant to induction of eIF2 phsphorylation when UPR was induced by Tm, suggesting an active feedback control that formed a robust buffer against further eIF2α phosphorylation. Increased expression levels of the Atf4 target gene *Chop* and reduced induction of the unfolded protein sensing factor *Bip* in Bag1-deficient erythroblasts confirmed this hypothesis.

Although the Bag1-deficient embryos died with a complete lack of fetal erythrocytes, the erythroblasts taken in culture did differentiate: the cultures accumulated a cell population with a distict size of 5 μm, similar to the differentiated cells of control cultures. When Bag1 knockdown established cultures were shifted from renewal conditions (Epo, SCF, glucocorticoids) to differentiation conditions (Epo), these cells also differentiated (data not shown). In cytospins of cultured Bag1$^{-/-}$ erythroblasts, we also observed well hemoglobinised, enucleated erythrocytes (Figure 3C). Thus, it seems that the presence of Bag1 mainly impacts on the decision to mature, not on the maturation program itself. We hypothesize that the extended proliferation capacity is due to a combined effect of delayed entry of the differentiation program combined with the observed resistance to stress signals.

Bag1 has been shown to bind to Gadd34 and inhibit association of Gadd34 with the catalytic subunit of Pp1[19]. This would imply that loss of Bag1 increases Pp1 activity and lowers eIF2α phosphorylation, which is not what we observe. Alternatively, the role of Bag1 as a Hsp70/Hsc70 co-chaperone could imply that loss of Bag1 induces ER-stress and a general UPR. However, while lack of Bag1 increased eIF2α phosphorylation and Chop expression, it did not result in a general increase in expression of Bip, which is a target of UPR through activation of IRE1 and activation of the transcription factor Xbp1[27]. Thus, lack of Bag1 primarily affects eIF2α phosphorylation, possibly through PERK activation.

The importance of eIF2 phosphorylation in erythropoiesis is highlighted by functional studies in mice. The p-eIF2α dephosphorylation activity of Pp1 requires one of its two regulatory subunits: constitutively expressed Crep (Ppp1r15b) or Atf4-induced Gadd34 (Ppp1r15a). *Crep*$^{-/-}$ mouse die perinataly with severe anemia due to reduced number and aberrant shape of red blood

cells[28]. *Gadd34*$^{-/-}$ mice are born anemic with small and misshapen erythrocytes and display active stress erythropoiesis as their spleens are enlarged and contain an increased number of erythroid progenitors[29]. *Atf4*$^{-/-}$ fetal livers are pale, hypoplastic, and increased in nucleated erythroid cells, while the number of erythroid colony forming cells is reduced. Adult *Atf4*$^{-/-}$ mice are mildly anemic and erythroid colonies grown from bone marrow are reduced in size[30]. Thus, the inability to resolve eIF2 phosphorylation impairs erythroid differentiation similar to what is seen in Bag1-deficient mice, suggesting that the increased eIF2α phosphorylation observed in Bag1-deficient erythroblasts may directly be responsible for the delayed differentiation of these cells.

Phosphorylation of eIF2 by stress kinases HRI and PERK occurs on serine 51 of the alpha subunit of eIF2. The *eIF2*$^{S51A/S51A}$ knock-in mice, in which serine 51 is mutated to alanine, die perinataly due to hypoglycaemia, but they do not suffer from anemia[31]. Importantly, the S51A mutation in eIF2α restored erythrocyte size and number in *CReP*$^{-/-}$;*eIF2*$^{S51A/S51A}$ mice[28]. The final proof that increased eIF2α phosphorylation also causes delayed differentiation of Bag1-deficient cells could be obtained by crossing the *Bag1*$^{-/-}$ mice to the *eIF2*$^{S51A/S51A}$ mice.

However, eIF2 phosphorylation may also be beneficial to erythroblasts and a low level of eIF2 phosphorylation is always present in cultured erythroblasts. Selective inhibition of p-eIF2 dephosphorylation by salubrinal protects the cells from ER-stress[32], which resembles the resistence of Bag1-deficient cultures to Tm induced Bip expression.

We reproducibly established long-term erythroblast cultures following knock down of Bag1 in fetal liver-derived erythroblasts. Spontaneous differentiation of erythroblast cultures derived from primary cells is largely due to activation of p53 as a result of oxidative stress experienced in vitro when cells are exposed to 20% atmospheric oxygen. Our experience to date is that establishment of long-term cultures from E12,5 fetal livers requires loss of p53 expression. In the Bag1-deficient cultures, however, p53 itself and p53 target genes are expressed. Therefore we hypothesize that the Bag1-deficient erythroid cultures could be maintained due to their delayed differentiation and a reduced sensitivity to oxidative stress signals mediated by HRI.

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CHAPTER 4

Erythroid-upregulated RNA binding protein Csde1 binds mRNAs involved in cell cycle and signaling

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work in progress

ABSTRACT

Cold shock domain protein E1 (Csde1) is an evolutionary conserved RNA binding protein containing 5 cold shock RNA binding domains. Its expression is highly upregulated in erythropoiesis. *Csde1* mRNA translation is decreased in Diamond Blackfan Anemia, a congenital anemia characterized by haploinsufficiency of ribosomal proteins. This triggered us to investigate the role of Csde1 in erythropoiesis. Csde1 expression is high in (pro)erythroblasts and decreases upon induction of terminal erythroid differentiation. To investigate which mRNAs are bound by Csde1 protein, we expressed a bio-tagged version of Csde1 in MEL erythroblasts and identified its mRNA targets using RNA-protein immunoprecipitation coupled to microarray profiling (RIP-Chip). We found that Csde1 binds mRNAs involved in cell cycle and signaling. Analysis of the Csde1 binding site, followed by experiments that determine mRNA stability and translation, may give insight into the role of Csde1 in protein expression of bound transcripts.

INTRODUCTION

Cold shock domain contacting protein E1 (Csde1, known as unr in *Drosophila melanogaster*), is an RNA-binding protein whose name was originally derived from the finding that it is positioned immediately upstream of *N-ras*[1]. The transcription of both *Csde1*/*Unr* and *N-ras* genes is co-regulated, as deletion of the *Csde1* promoter by homologous recombination in mouse ES cells (mESC) led to an increase of *N-ras* mRNA in variety of tissues[2]H.</author><author>Dautry, F.</author></authors></contributors><auth-address>Genetique Moleculaire et Integration des Fonctions Cellulaires, CNRS UPR 9044, Institut de Recherches sur le Cancer, Villejuif, France.</auth-address><titles><title>Transcription of unr (upstream of N-ras, suggesting that the transcription of *Csde1* interferes with that of *N-ras*. *Csde1*$^{-/-}$ mESC showed decreased apoptosis and necrosis which could be restored by exogenous expression of wt Csde1 protein but not by mutant Csde1 protein in which the first cold shock domain (CSD1) was mutated such that it was deficient for RNA-binding[3,4]. Specifically, Csde1 renders mESC insensitive to γ-irradiation-induced DNA damage. This effect was not related to the increased repair activity or altered cell cycle response[3]. In mouse development, however, the ablation of *Csde1* expression was embryonically lethal at the gastrulation stage[2].

The molecular function of Csde1 is exerted through its RNA-binding properties. The Csde1 binding site is defined as a AAGUA/G or AACG sequence downstream of a purine stretch[5]. Csde1 contains five copies of the RNA-binding cold shock domain (CSD) and is highly homologous with Drosophila unr involved in mRNA translation[6]. Csde1 binds to its recognition sites in RNA and also single stranded DNA with high affinity whereas it does not bind double stranded nucleic acids[7].

In Drosophila, unr is required for the sex-dependent silencing of *msl-2* mRNA. In female flies, UNR forms a complex with sex lethal protein (SXL) and targets the 5'- and 3'-UTR of *msl-2* mRNA which leads to its translational repression[8]. UNR interacts directly with the translational activator poly-A binding protein (PABP), and this interaction is required for the efficient translational repression of *msl-2*[9]. Recent studies showed that UNR binds not only *msl-2* but also a large set of protein coding and non-coding RNAs in a sex specific manner[10]. Interestingly, UNR binds sequences in the 3'UTR that are differentially used in fly males and females due to alternative splicing, and it was suggested that the UNR protein RNA binding could be active already in the splicing events[10].

Previously, we showed reduced translation of *Csde1* and a distinct set of other transcripts in mouse erythroblasts expressing reduced levels of ribosomal protein L11 (Rpl11) or Rps19, and in erythroblasts of patients affected by Diamond Blackfan Anemia (DBA)[11], a congenital disease characterized by haploinsufficiency of ribosomal proteins. A common feature of these mRNAs is the presence of an IRES in their 5'UTR sequence, as is the case for Csde1[12]. Because Csde1 was described as an important ITAF for several viral and cellular IRESes[13-15], we speculated that the decrease in Csde1 protein levels may cause the general decrease in IRES mediated translation in DBA.

To begin to understand the role of Csde1 in erythropoiesis we identified Csde1-associated transcripts. Mouse erythroblasts express two Csde1 isoforms, both of which we cloned with a N-terminal biotag and tried to express constitutively in erythroblasts. Fetal liver-derived erythroblasts did not tolerate constitutive expression of Csde1, therefore we used murine erythroleukaemia cells (MEL) for expression studies. We performed RNA-protein pulldowns and identified mRNA targets of Csde1, which included transcripts involved in cell cycle progression and growth factor receptor signaling cascades.

MATERIAL AND METHODS

Cell culture

Fetal liver-derived erythroblasts and I/11-birA cells were expanded in StemPro-34 medium (Invitrogen, Breda, The Netherlands) supplemented with 0.5 U/ml Epo (gift from Jansen-Cilag, Tilburg, The Netherlands), 100ng/ml of SCF (supernatant of CHO producer cells) and 10^{-6} M dexamethasone (Sigma-Aldrich, St Louis, MO)[16]. Cell number and size were determined with electronic cell counter (CASY-1; Innovatis, Reutlingen, Germany). Hek293T and MEL-birA cells were cultured in DMEM (Invitrogen) supplemented with 10% (v/v) fetal calf serum (PAA, Pasching, Austria).

SDS-PAGE and Western blotting

Cell lysates, SDS-PAGE and Western blotting were performed as described[17]. Primary antibodies used were Csde1 (ProteinTech group, 13319-1-AP), anti-HA tag (Abcam, ab13834) and β-actin (Santa Cruz Biotechnology, sc-1616). Fluorescently-labeled biotin and secondary antibodies were used and blots scanned on Odyssey infrared scanner (LI-COR, Lincoln, NE). Images were processed in Adobe Photoshop CS3.

Lentivirus and retrovirus production, titration, transductions and transfections

For retroviral production, the pCR-Eco packaging plasmid (gift of E.Bindels, ErasmusMC) was cotransfected with pMSCV plasmid into HEK293T cells and the supernatant was harvested in media 48h after the transfections. Virus was first immobilized on the plates coated with retronectin (TaKaRa, Shigu, Japan), I/11-birA cells were then co-incubated on the coated plates for 48 hour, harvested, reseeded and selected in fresh media containing 2μg/ml of blasticidine.

Cloning

The Csde1 ORF was PCR-amplified from I/11-birA cDNA using primers pr_BamHI_ biotag_mCsde1_F 5'-AAGGATCCACCATGGGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAGT GGCACGAAAGCTTTGATCCAAACTTCTCC-3', pr_mCsde1_XhoI_R 5'-AACTCGAGTTAGTCAATG ACACC AGCTTGACGG-3' and subcloned into pCR2.1 using TAcloning kit (Invitrogen). The insert was sequence verified; the sequencing showed that both

murine Csde1 isoforms were cloned. We mutated the phenylalanine at the position 29 to the alanin using mutagenesis kit (Stratagene) with primers mut_mCsde1_F29A_F 5'-CCTCTTACGGAGCTATTCAGTGTTCAGAACG-3' and mut_mCsde1_F29A_R 5'-CGTTCTGAACACTGAATAGC TCCGTAAGAGG-3' and sequence verified the clones. The inserts were cloned into pMSCV(blast) using BamHI and XhoI sites for the fragments and BglII and XhoI for the vector. Similarly, we cloned the Csde1 is1 wt and F29A into pBud_neo using the BamHI and XhoI sites for the insert (BamHI site was blunted) and the KpnI and XhoI sites for pBud_neo vector (KpnI site was blunted). The pBud_Csde1_is1 was electroporated into MEL-birA[18] cells which were then selected with 1 μg/ml neomycin.

RNA immunoprecipitation

100x10[6] MEL cells were used for RNA immunoprecipitation protocol as described[19]Messenge r/*analysis/*isolation & purification</keyword><keyword>Ribonucleoproteins/*analysis/chemistry/isolation & purification</keyword></keywords><dates><year>2006</year></dates><isbn>1750-2799 (Electronic. For pulldowns we used streptavidin conjugated Dynabeads M-270 (Invitrogen). The NT2 washing buffer was supplied with 0.1% SDS or 0.5% DOCH as indicated. RNA from beads was isolated using Trizol (Invitrogen) and amplified with MessageAmp II amplification kit (Ambion, Austin, TX).

RNA isolation, cDNA synthesis, qRT-PCR

Total RNA was isolated using Trizol. 1,6μg of RNA was used for cDNA preparation with random hexamers and SuperScript II Reverse Transcriptase kit (Invitrogen). qRT-PCR was performed as described[17] using primers for Csde1 m_Csde1_3UTR_F 5'-AATGAAGTGTGCCAGCAGTG-3', m_Csde1_3UTR_R 5'-ATTGAACAGGGCACTGGAAC-3', eIF5a m_eIF5A_F 5'-ACTTCCAGCTGATTG GCATC-3',m_eIF5A_R 5'-ACAGCTGCCTCCTCTGTCAT-3' and β-tubulin m_Tubb4b_F 5'-AAGGCATGGATGAGATGGAG-3', m_Tubb4b_R 5'-TGGACATGGCTAGCAGACAG-3'.

DNA microarrays, data processing and analysis

Complementary RNA was hybridized to Affymetrix MOE 430A2.0 mouse expression arrays according to manufacturer's protocols. The raw data was processed using RMA normalization and log2 transformed. Bioinformatical analyses were performed using ArrayTools v3.7.2. To determine the transcripts with differential abundance from RIP-Chip in the Csde1 overexpressing cells, as compared to the parental cells, paired Student t-test was used. Clustering analysis was performed on the whole dataset using centered correlation and average linkage. The online search tool UTRdb was used to find Csde1 binding sites in mRNAs[20].

RESULTS AND DISCUSSION

Cloning of Csde1 and stable expression cells derivation

The RNA-binding protein Csde1 exerts its function through the regulation of (m)RNA stability and/or translation. To investigate which RNAs are bound by Csde1 we aimed to transiently express the Csde1 in erythroid cells and then identify bound RNAs by an RNA-protein pulldown method. Two isoforms of Csde1 are annotated in the mouse genome; a longer isoform 1 (798 AA) and a shorter isoform 2 (767 AA). Both isoforms contain 5 cold shock domains, distributed over the whole protein length. Isoform 2 lacks a 31 AA stretch between the first and second CSD (Figure 1A). To create stable cell lines expressing a tagged Csde1 we made use of the biotag that can be biotinylated in vivo by the prokaryotic biotin ligase BirA. Biotagged proteins can be efficiently purified by streptavidin coated beads[18] (Figure 1A). The biotag was fused to the N-terminus of both Csde1 isoforms and cloned in the retroviral vector pMSCV. Expression and biotinylation of the constructs was tested in HEK293T cells co-transfected with birA (Figure 1B). The tagged isoform 1, running slightly higher on the gel, was biotinylated more efficiently than the isoform 2. The HEK293T cells also express the human isoform which appeared comparable in size to mouse isoform 2.

Next, we mutated the sequence encoding a phenylalanine residue on position 29 of Csde1 to alanine (TTC to GCT). This residue is located in the putative binding site RNP-2 that is required for the RNA binding activity of the whole protein[21]/. Next we transduced the *Csde1* wt and F29A constructs in the $p53^{-/-}$ BirA expressing erythroblasts derived from fetal livers of $p53^{-/-;\ birA}$ embryos. These cells express BirA constitutively from the Rosa26 locus (Pourfarzad et al, submitted and[22]). We analysed expression in mass cultures (Figure 1C). Of note, we could only express biotinylated Csde1 isoform 1, wt or F29A, in erythroblasts but not Csde1 isoform 2. Compared to transiently transfected HEK293T cells, Csde1 expression in birA erythroblasts was low, although the biotinylation was similarly efficient. We prepared single cell derived clones for Csde1 isoform 1 (wt and F29A) by limiting dilution with the aim to isolate clones efficiently expressing biotinylated Csde1 (Figure 1D). We identified clones Csde1_is1 clone 5 and Csde1_is1_F29A clone 7 as expressing the highest levels of biotagged proteins. However, we did not consider the expression of biotynilated Csde1 sufficient for RNA-IP experiments. Therefore, we turned to the MEL (murine erythroleukemia cells) cell line that was constructed to constitutively express the birA ligase[23]. The wt form of Csde1 is1 was cloned in the pBud plasmid and transfected in MEL-birA cells. Unfortunately, the F29A subcloning into pBud_neo led to accumulation of mutations in the coding region, therefore it was not used for furher experiments. Single cell derived clones were screened for the expression of biotynilated wt Csde1 (Figure 1E). Clone number 9 was selected for further experiments. Finally, we compared the expression and biotinylation of Csde1 in I/11 and MEL-derived cells. The biotinylation and expression of Csde1 was considerably higher in MEL-birA cells compared to $p53^{-/-;birA}$ cells (Figure 1F), therefore we proceeded only with the MEL-birA cells.

Figure 1

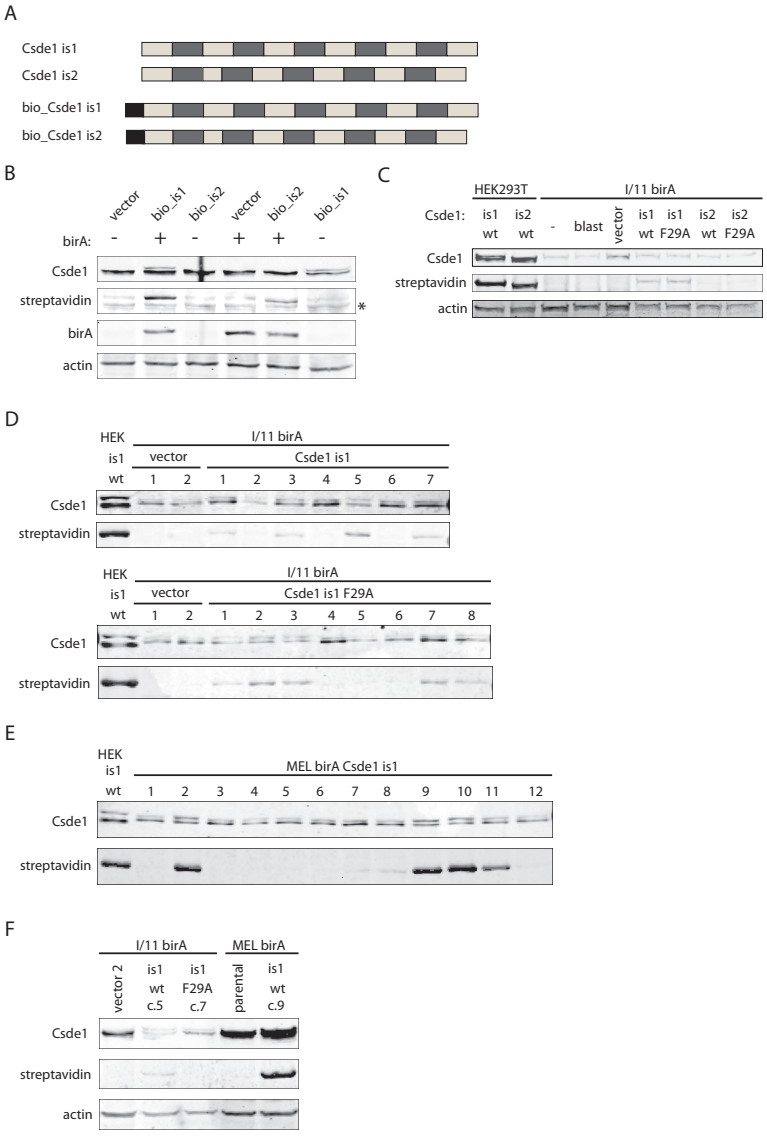

Figure 1: Establishment of a stably Csde1 overexpressing erythroid cell line. (*A*) **Schematic depiction of the Csde1 protein. Blue rectangles represent the CSD domains. Yellow rectangle represents the biotag.** (*B*) **HEK293T cells were transfected with pMSCV constructs bearing the biotagged Csde1 isoforms and co-transfected with the birA ligase. Csde1 antibody recognizes both isoforms. birA ligase was detected with anti-HA tag antibody. *indicates non-specific band.** (*C*) **I/11 birA cells were transduced with the pMSCV vectors bearing the bio-tagged Csde1 isoforms. Bulk cultured cells were selected for 3 days with blasticidine and harvested for WB lysates. As a control for biotagged Csde1 we used the lysates as in** (*B*)**.** (*D*) **Western blot on lysates from single cell derived cultures of I/11-birA cells transduced with wt or F29A mutant of Csde1. Cells were derived from the bulk cultured cells as in** (*C*)**. Similar control for biotinylated Csde1 was used.** (*E*) **Csde1 expression and biotinylation in the single cell derived clones of MEL-birA cells transuded with biotagged Csde1 is1 together with the control lysates from** (*B*)**.** (*F*) **Comparison of the Csde1 expression and biotinylation from the clones of I/11-birA cells and the MEL-birA cells.**

Previously, we showed that Csde1 mRNA expression increases during the differentiation stages from HSCs towards definitive erythropoiesis[11]. Interestingly, Csde1 was validated as a target for transcriptional upregulation by the short isoform of Gfi-1B, a transcription factor indispensable for erythroid maturation[24]. The expression profiles of Csde1 mRNA resembled those of Gfi-1B in the differentiating erythroid cells acquired from umbilical blood $CD34^+$ cell[24].

RNA immunoprecipitation

To identify the potential mRNA targets of Csde1 in mouse erythroid cells we performed RNA immunoprecipitation (RIP) of biotinylated Csde1_is1 from MEL-birA cells. As control, we used parental MEL-birA cells, in which some endogenous proteins are biotinylated (visible as several biotinylated bands) that were previously shown to be predominantly mitochondrial proteins[25] (Figure 2A). We followed the published protocol[19], used streptavidin conjugated magnetic beads and a washing buffer containing 0,1% SDS or 0,5% deoxycholate (DOC). The yield of RNA in µg isolated from the beads is summarized in Table 1. The yield of RNA after the first RIP was slightly higher in the Csde1_is1 expressing cells as compared to the parental cells. To increase the amount of RNA for cDNA synthesis and microarray hybridization we performed a RNA amplification using an oligo-d(T) primer. Remarkably, the amount of amplified RNA was 5-fold increased in the Csde1 RIP compared to the control RIP.

Next we employed the fact that Csde1 binds its own transcript to test the required stringency of washing buffers (Figure 2B). Primers that can amplify endogenous Csde1 mRNA were designed in the 3'UTR to ensure that the overexpressed mRNA cannot be recognized. Quantitative RT-PCR amplified Csde1 mRNA from pull downs of Csde1-expressing cells, not from parental cells. The signal was retained when the RIP was washed with DOC buffer, not in SDS buffer. As a control, eIF5 was amplified from pull downs of Csde1 expressing cells, but the signal disappeared upon washing with buffers containing either DOC or SDS. This analysis indicated that *i*) addition of SDS to the washing buffer was too stringent for the RIP conditions used, *ii*) unspecific binding of mRNAs occurs in similar amounts in control RIPs, and *iii*) addition of deoxycholate to the NT2 washing buffer efficiently prevents unspecific binding of mRNA to streptavidin beads or Csde1 complexes. Importantly, there was no difference in expression of endogenous mRNAs for Csde1 or eIF5a in the input samples (Figure 2C). Thus, we showed that the biotag-Csde1 expressed in the MEL cells was binding its expected target mRNA and that these samples could be used for the microarray experiment.

Array experiments design and data analysis

For an unbiased search for Csde1-bound mRNAs in erythroid cells, we used the mRNA extracted from the RIP performed with 0,5% DOC containing NT2 buffer for the mRNA expression analysis using mouse oligonucleotide arrays. We used RNA from 2 RIP experiments that included both parental and biotag_Csde1 expressing cells, and from one sample of RNA isolated from the initial input samples during the lysis step. Although normalization of arrays following RIP is debatable, we used the RMA algorithm to normalize the data and subsequently clustered the

Table 1. Obtained amounts of RNA in the RIP experiments in µg.

Cells	NT2 buffer	RIP 1		RIP 2	
		after IP	after amplification	after IP	after amplification
parental	no additive	0.145	0.56	-	-
	+ 0,1% SDS	0.162	0.135	-	-
	+ 0,5% deoxycholate	0.145	3.18	0.271	0.822
Csde1_is1	no additive	0.111	0.95	-	-
	+ 0,1% SDS	0.496	1.25	-	-
	+ 0,5% deoxycholate	0.334	23.87	0.063	27.622

samples. This clearly separated the total input RNA samples from the RIP samples and indicated that RIP samples of parental and Csde1 expressing cells are paired and closer to each other than to the input samples (Figure 2D). Next, we performed a t-test analysis to identify the mRNAs differentially enriched in the two RIP conditions. Using the cutoff of 1% ($p>0.01$), we identified 166 mRNAs, out of which 114 were enriched in the biotag-Csde1 cells RIP and 52 were enriched in the parental cells RIP. We list the mRNAs which appear with more than 1.5 fold enrichment in the biotag_Csde1 cells in Table 2. Among the transcripts enriched in the biotag-Csde1 expressing cells was *Csde1* mRNA itself, which was in agreement with the control experiment using qPCR detection and previous literature[26]. To exclude enrichment due to transcriptional upregulation as a result of Csde1 overexpression, we compared the expression levels of the identified transcripts in total RNA samples. Transcripts that were more than 1.5-fold enriched in biotag-Csde1 cells compared to parental cells were marked to indicate mRNAs that could be enriched due to higher expression (white labeled in the Table 2). Transcripts enriched in the parental RIPs samples were typically highly expressed mRNAs coding e.g. for membrane transporters or ribosomal proteins that are often contaminants in these types of assays (*Abce1, Rps13, Tmem144*).

Csde1 RIP enriched mRNAs involved in cell cycle and signaling

The Drosophila unr binding site motif was identified as a AAGUA/G sequence immediately downstream of a purine stretch, which is usually 11-14 nt long[5]. Several transcripts enriched in the Csde1 RIP contain this unr binding motif and some encode proteins involved in cell cycle progression. Xlr (X-linked lymphocyte regulated) has a role in meiosis and, interestingly, is expressed in lymphocytes[27]. Its mRNA contains a binding motif for unr in the coding-region. Mphosph9 (M-phase phosphoprotein 9) is a protein kinase localized in the Golgi Apparatus during the interphase, and in the cytoplasm during the M-phase of the cell cycle[28]. Its mRNA 3'UTR contains two typical unr binding motif that are conserved between man, mouse and rat. Smc3 (structural maintenance of chromosomes 3, also known as Cspg6) is required for proper sister chromatid cohesion during mitosis[29]. In addition, several mRNAs enriched in the Csde1 RIP dataset encode proteins involved in cell signaling. Pik3ca, the catalytic subunit of phosphoinositide-3-kinase, propagates signals from membrane bound receptors to the downstream signal machinery through phosphorylation of phosphatidylinositols (PtdIns)[30]. Ribosomal protein S6

kinase (Rps6kb1) phosphorylates Rps6 upon activation of mTOR[31], and contains 2 binding sites for Csde1. Finally, Socs2, an important negative regulator of cytokine signaling[32], appears twice in the list and contains a Csde1 binding site in its 3'UTR.

Previously, CSDE1/UNR was shown to affect cell cycle progression in HeLa cells[33]. The UNR IREs activity was enhanced during the G2M stage of cell cycle, which was caused by increased binding affinity of hnRNP C1/C2 proteins to the UNR IRES and displacement of PTB. Knockdown of UNR inhibited cell growth and delayed progression through G2M[33]. Interestingly, cell cycle progression is tightly controlled in the expanding and differentiating erythroid compartment[16,34]. Thus, it will be important to further validate binding of Csde1 to its mRNA targets during cell cycle progression and in response to growth factor availability.

Except for *Csde1* itself, we did not identify other IRES-containing transcripts that were previously shown to be bound by Csde1 such as Cdk11/Pitslre[33]. Possibly, the activity of IRES mRNAs depends on the conditions under which the cells are grown. An example is *Apaf-1*[15], encoding an adaptor protein that binds cytochrome c at the initial stage of apoptosis[35]. Its translation via an IRES was shown to be dependent on Csde1 and induction of apoptosis[36], which could explain why we did not enriche for *Apaf-1* mRNA in the RIP experiments.

Csde1 does not only binds mRNAs in the IRES sequences but also in the 3'UTR and coding regions. The Csde1 binding motif appears frequently in 5' and 3' UTR sequences in the human genome; 502 5'UTRs contain in total 591 binding sites, whereas 2433 3'UTRs contain 3622 binding sites for Csde1[20]. This suggests, that Csde1 may have a more general function in RNA metabolism, for instance mRNA stability and translation, which is further supported by the fact that *Drosophila* unr was shown to interact with PABP[9]. Alternatively, the specificity of Csde1 binding may be further exerted by additional binding partners that may bind mRNA as well as other proteins in a regulatory complex, such as shown for FOS mRNA[37]. Some of the identified targets from the Csde1 pulldown list have a predicted IRES sequence. It will be interesting to clone those 5'UTRs together with mRNAs that showed a Csde1 binding site in their 5'UTR into bi-cistronic reporters and test for their IRES activity[38].

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Table 2. List of enriched mRNAs in RIP-Chip experiments in the birA-Csde1 samples

#	Probeset	Gene name	*p*-value t-test	Fold change	Csde1 binding site/IRES
1	1438285_at	*2210015D19Rik*	0.00496	3.78036	
2	1427174_at	*3100002L24Rik*	0.003378	3.399426	
3	1418507_s_at	*Socs2*	0.001505	3.200581	1x 3'UTR
4	1418685_at	*Tirap*	0.001515	3.199801	
5	1420916_at	*Prpf40a*	0.009919	3.194717	
6	1436330_x_at	*LOC278757*	0.006694	3.065167	
7	1449972_s_at	*Zfp97*	0.006836	3.041685	
8	1456483_at	*Zfp9*	0.00167	3.040235	IRES
9	1440125_at	*A530054K11Rik*	0.000303	2.944235	
10	1440171_x_at	*1700049G17Rik*	0.005463	2.789421	IRES
11	1449109_at	*Socs2*	0.001838	2.771718	1x 3'UTR
12	1425095_at	*BC002059*	0.00064	2.711931	
13	1430212_at	*Zfp712*	0.000547	2.708384	IRES
14	1440992_at	*3110052M02Rik*	0.001894	2.690282	
15	1439093_at	*Hspa4l*	0.003926	2.685465	
16	1438230_at	*Pggt1b*	0.007325	2.653368	2x 3'UTR
17	1424872_at	*2310001H12Rik*	0.001408	2.615021	
18	1436401_at	*9330128J19Rik*	0.006035	2.532896	
19	1452056_s_at	*Ppp3ca*	0.004046	2.526767	
20	1460445_at	*Sfrs2ip*	0.000418	2.478684	
21	1440202_at	*LOC622175*	0.009769	2.421628	
22	1442982_at	*Ccdc66*	0.004181	2.415833	
23	1443992_at	*Nipbl*	0.007996	2.37117	
24	1436172_at	*9530028C05*	0.003993	2.285583	
25	1439065_x_at	*MGC117846*	0.001562	2.208571	
26	1455385_at	*Exoc6*	0.003347	2.153129	
27	1429106_at	*4921509J17Rik*	0.001383	2.149532	
28	1423997_at	*Csde1*	0.009842	2.124201	IRES
29	1428162_at	*4933421E11Rik*	0.008192	2.106142	
30	1453134_at	*Pik3ca*	0.000326	2.08974	
31	1439901_at	*2610208M17Rik*	0.008416	2.089692	
32	1418524_at	*Pcm1*	0.008803	2.085719	1x 3'UTR
33	1455451_at	*AI449310*	0.00569	2.080062	
34	1438063_at	*Mphosph9*	0.001189	2.06912	2x 3'UTR
35	1437926_at	*E430012M05Rik*	0.000819	2.055472	

#	Probeset	Gene name	*p*-value t-test	Fold change	Csde1 binding site/IRES
36	1448712_at	*Chm*	0.000272	2.053485	
37	1444169_at	*3110052M02Rik*	0.001148	2.042648	1x 5'UTR
38	1450900_at	*AW011752*	0.008633	2.030859	
39	1425773_s_at	*Nmnat1*	0.008523	1.976602	
40	1439075_at	*Polr3f*	0.000557	1.941905	1x 3'UTR
41	1455057_at	*Gmps*	0.001829	1.933626	IRES; 1x 3'UTR
42	1455434_a_at	*Ktn1*	0.00201	1.929962	
43	1426903_at	*Fndc3a*	0.006648	1.903471	IRES; 1x 3'UTR
44	1449589_x_at	*2610020O08Rik*	0.005824	1.867599	
45	1453078_at	*8430408J07Rik*	0.001658	1.846082	
46	1437774_at	*1700020I14Rik*	0.004704	1.840741	
47	1422011_s_at	*Xlr*	0.000323	1.803532	1x CDS; IRES
48	1442465_s_at	*Strbp*	0.007227	1.746262	
49	1455687_at	*Ick*	0.004087	1.717521	
50	1437395_at	*Zcchc11*	0.004804	1.704084	
51	1428849_at	*Rps6kb1*	0.004183	1.703352	2x 3'UTR
52	1434526_at	*Abhd7*	0.000664	1.690517	
53	1426367_at	*Cab39l*	9.93E-05	1.686505	IRES
54	1453230_at	*Zfp74*	9.25E-05	1.679551	
55	1450901_a_at	*AW011752*	0.004804	1.657114	
56	1452374_at	*Zfp322a*	0.001317	1.632807	1x 3'UTR
57	1423432_at	*Phip*	0.002531	1.626408	
58	1441198_at	*Zfp39*	0.001427	1.626133	
59	1418012_at	*Sh3glb1*	0.000707	1.607231	
60	1426945_at	*Ranbp5*	0.006413	1.606279	
61	1423620_at	*2610528M18Rik*	0.003279	1.581937	
62	1450951_at	*Cspg6*	0.009984	1.563673	
63	1437634_at	*Thoc2*	0.007371	1.561383	
64	1428654_at	*1200016B10Rik*	0.001823	1.546288	IRES
65	1428287_at	*Cul5*	0.001246	1.525363	IRES; 1x 3'UTR
66	1438786_a_at	*2610021A01Rik*	0.00909	1.519284	
67	1434148_at	*Tcf4*	0.000852	1.504697	


CHAPTER
5

Summary and General discussion

SUMMARY

Diamond Blackfan Anemia is a bone marrow failure syndrome in which patients lack erythroid precursor cells in the bone marrow. Therefore it is also designated as pure red cell aplasia, as other hematological indices are at normal levels. Around half of DBA patients bear an autosomal dominant mutation in one of the ribosomal protein genes which leads to decreased cellular ribosomal content. There are many different ribosomal genes, encoding proteins for both large and small subunit of ribosome, which can be mutated or deleted in DBA. This fact led to the description of DBA as a "ribosomopathy" together with a few other hematological disorders which also show defects in ribosomal biogenesis. However, it is as yet unknown how such a general deficiency in ribosomes could lead to an erythroid restricted phenotype. In this thesis we have explored the pathophysiology of Diamond Blackfan Anemia with special focus on the translation regulation of gene expression. Ribosomes are responsible for translation in every cell type and there are no differences in the quality of ribosomes for different tissues described. Therefore we postulated that the erythroid cells must possess a feature which makes them especially sensitive to disturbances in ribosome production or levels. As opposed to the proposed mechanisms involving p53 in pathogenesis of the disease, we speculated that defective translation regulation may be an important lead in the erythroid specific phenotype of the diseased cells.

In our study, we reduced the expression of ribosomal proteins in mouse erythroblasts to mimic the conditions in erythroblasts of DBA patients. The use of p53-deficient cells enables us to find p53-independent mechanisms. Knock down of Rps19 or Rpl11 decreased proliferation and impaired differentiation in the cultured cells. Erythroblasts with reduced expression of Rps19 or Rpl11 exhibited a poly-nucleated phenotype which suggested defects in the cell cycle and/or cytokinesis. Employing polyribosome fractionation coupled to oligonucleotide expression arrays we identified transcripts of which polyribosome loading is altered under DBA conditions (**Chapter 2**). Whereas knock down of Rps19 and Rpl11 generally reduced polysome recruitment, certain transcripts, including several containing 5'TOP signals, were retained in the polyribosomes. A second fraction of mRNAs, however, displays reduced translation initiation as compared to the other mRNAs. As a consequence protein synthesis from these mRNAs was decreased. We showed that many of these transcripts contain an IRES in their 5'UTR and that IRES-dependent translation initiation is generally reduced in response to the decreased ribosomal content in the cells. Importantly, we could culture erythroblasts from peripheral blood of DBA patients and detect decreased protein levels of the candidate genes despite normal mRNA levels. This analysis indicated that the translation of these transcripts is regulated in human erythroid cells in a similar fashion to the mouse cells.

Further on, we studied the function of candidate proteins in erythropoiesis and explored mechanisms by which they could contribute to the erythroid phenotype of DBA. We decided to study the role of Bag1, because Bag1-deficient mouse embryos die at embryonic day E13.5 with a lack of definitive erythrocytes. We showed (**Chapter 2** and **3**), that Bag1 expression increases during erythroid differentiation, and is stable during terminal erythroid differentiation.

Knockdown of Bag1 showed that it is important in differentiation and dispensable for erythroid expansion. We observed the same phenomena in fetal liver cells isolated from *Bag1*$^{-/-}$ embryos and showed that specifically the erythroid lineage was affected. Moreover, we repeatedly observed that defective differentiation with retained proliferation, which are hallmarks of depletion of Bag1, endowed erythroid cells with a growth advantage. This was not mediated by the loss of p53. Bag1 functions as a co-chaperone in ER. Therefore, we analyzed the effects of Bag1 deficiency on the stress pathway mediated by phosphorylated eIF2 and detected an activated unfolded protein response.

Another mRNA whose translation was affected by reduced ribosomal subunits in erythroblasts encoded Csde1 (**Chapter 2** and **4**). Csde1 expression is increased in the erythroblasts when compared to other blood lineages and decreased during terminal erythroid differentiation. Knockdown of Csde1 caused inhibition of proliferation and differentiation of erythroblasts and led to the disturbances of the cell cycle. Csde1 is a RNA-binding protein that binds mRNAs on distinct binding motifs and affects their translation efficiency. Both positive and negative regulation of mRNA translation by Csde1 has been reported. We established erythroid cells that expressed a tagged version of Csde1 for RNA-protein immunoprecipitation assays coupled to expression array (RIP-Chip). By this approach we identified putative cellular mRNA targets of Csde1. Several of those contain a Csde1 binding motif and were involved in progression through the G2M phase of the cell cycle. We also identified mRNAs for important factors in Epo/SCF-mediated cell signaling. Because cell cycle progression and signaling are tightly regulated in the differentiation program of erythroid cells, Csde1 may an important player that could orchestrate these events. Further experiments on the role of Csde1 will shed more light on its function in erythropoiesis.

In summary, we identified novel genes that are subject to selective translation and important for erythropoiesis. Reduced translation of the encoded transcripts may contribute to the pathophysiological mechanism by which mutations in ribosomal proteins in Diamond Blackfan Anemia patients cause the decrease in erythroid expansion and anemia.

GENERAL DISCUSSION

Translation regulation of SCF signaling dependent transcripts and 5'TOP mRNAs

Previously, we identified a set of mRNAs for which translation regulation was strictly dependent on Epo/SCF signaling through PI3K and mTOR and the availability of eIF4E for translation initiation[1,2]. Over-expression or knock-down of some of these mRNAs (e.g. *Igbp1*, *Use1*) affected the proliferation and differentiation of erythroblasts, which hinted to the importance of the Epo/SCF signaling cascade to regulate translation initiation of specific transcripts. Given these results, we hypothesized that translation initiation of these mRNAs could also be affected in cells with a ribosomal protein deficiency. As shown in Chapter 2 (Supplemental table 1), we identified transcripts whose polysomal association was affected upon the knockdown of Rps19 or Rpl11 as

Figure 1

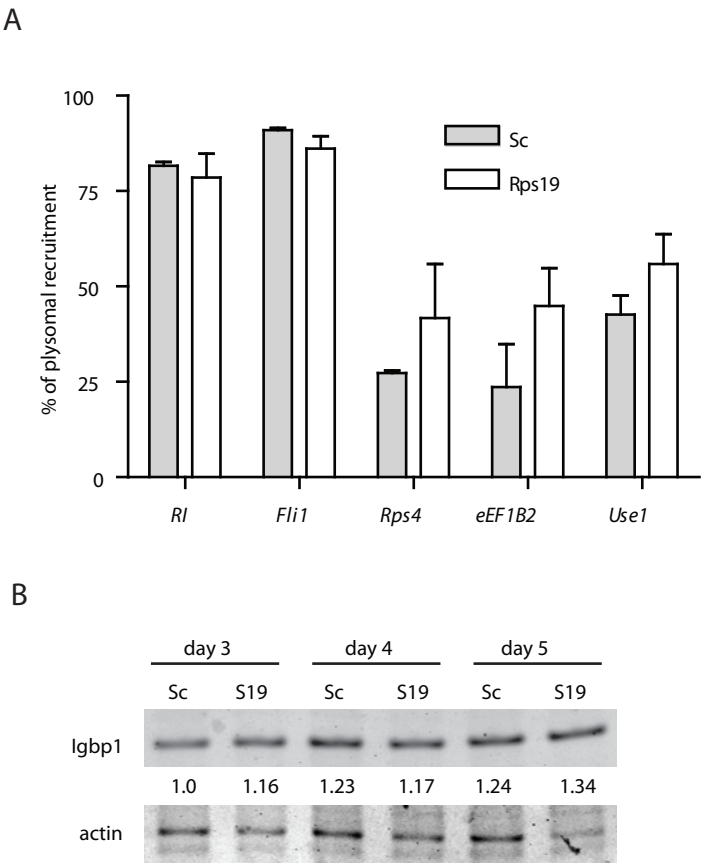

Figure 1. SCF-dependent transcript translation in cells deficient for Rps19. (A) Polysome recruitment of control transcripts (*RI, Fli1*) and selected mRNAs were tested from the polysomal profiles of cells treated either with Sc or Rps19 shRNA for 3 days. Error bars represent ± s.e.m. (n=3). (B) Protein levels of Igbp1 in cells as in (A) at days 3, 4 and 5. Quantified expression, corrected with actin protein levels, is indicated below the blot.

compared to controls. To our surprise, the transcripts that depend on SCF for translation (e.g. *Nap1l1*, *Use1*) showed increased polysomal association upon the decreased ribosomal content. To further test increase in polysomal association of these and other transcripts, we used qRT-PCR to measure the polysomal recruitment for control transcripts (*RI*, *Fli1*) and for transcripts subject to SCF-dependent translation (*Rps4*, *eEF1B2*, *Use1*) in the polyribosome samples of cells treated with Sc control and Rps19 shRNA (Fig. 1A). This confirmed the microarray results, and again indicated increased polysomal recruitment for the SCF-dependent transcripts. We also detected an increase in the protein levels of *Igbp1* upon knockdown of Rps19 compared to Scrambled control (Fig. 1B) at day 3 and 5 after the shRNA viruses transductions, which further justified the correlation between the mRNA polysomal loading and protein yield.

These results disproved our hypothesis that a reduced number of ribosomes may impair translation of the same transcripts that are hypersensitive to SCF signaling. The knockdown of

Rps19 and Rpl11 and polyribosome loading was tested in cells exposed to erythropoietin and SCF. The data suggest that SCF actively promotes the translation of certain transcripts and that the mechanism by which this is achieved renders translation of these transcripts less competitive in absence of SCF, but more competitive when e.g. the number of ribosomal subunits is reduced.

Translation regulation in cells depleted of *Sbds*

Schwachman Bodian Diamond Syndrom (SDS) belongs to the ribosomopathies and is caused by mutations in the *SBDS* gene which are found in more than 90% of patients[3]. The protein product of the *SBDS* gene is involved in late steps of maturation of 60S ribosomal subunit. SBDS functions to release the translation initiation factor eIF6 from the 60S subunit, which is required for the 60S subunit to become translation competent[4]. In yeast cells depleted for Sdo1, an ortholog of human SBDS, the 60S subunit bound to Tif6 is accumulating in the cytoplasm, decreasing the cytoplasmatic 40S:60S subunits ratio[5]. Although the hematopoietic phenotype of SDS initially presents as a neutropenia, whereas DBA presents as severe anemia, mutations in SBDS and for instance RPL11 both decrease availability of the 60S ribosomal subunit. Therefore we considered the lack of *Sbds* as a related mechanism involved in bone marrow failure due to deregulation of translation initiation. To investigate mRNA ribosome loading upon knock down of *Sbds*, we used a similar setup of experiments as described in Chapter 2: isolation of polysomal and subpolysomal RNA after knock down of *Sbds* in combination with expression profiling on microarrays.

Cells depleted for *Sbds* did not show signs of cell death by day 3 after transduction, when the pellets for polysomal profiles were harvested (Figure 2A). However, after 3 days the cells stopped proliferating and showed apoptosis. Upon induction of differentiation, the cells depleted for *Sbds* yielded low amounts of hemoglobinizated reticulocytes. As seen on the polysomal profile, the depletion of *Sbds* increased the 60S subunit in the cytoplasm, and decreased the polysome peaks as compared to the Sc shRNA control (Figure 2B). This was in concordance with published reports in yeast and mammalian cells. Next, we isolated polysome-bound and subpolysomal RNA and used it for microarray profiling. Surprisingly, the number of mRNAs regulated at the level of polyribosome loading in *Sbds* cells as compared to the wt and Sc control was very low, indicating no translation regulation mechanisms involved (data not shown).

These results suggested that the knockdown of *Sbds* in I/11 cells does not affect polyribosome loading of mRNAs, in contrast to the knockdown of DBA related ribosomal proteins genes *Rps19* and *Rpl11* (Chapter 2). This may be explained by the role of the SBDS protein in the maturation of the 60S subunit. Rps19 and Rpl11 are as structural ribosomal proteins required for formation of 40S and 60S subunits. We observed that knock down of Rps19 also affects the expression of Rpl11. Although knockdown of Rps19 primarily affected synthesis of 40S subunits, and knock down of Rpl11 primarily affected synthesis of the 60S subunit, there seems to be some crosstalk. In contrast, knockdown of Sbds only affects the availability of the 60S subunit. The 40S subunit is part of the 48S pre-initiation complex scanning the 5'UTR of mRNAs for the initiation codon, the 60S subunit only joins the 40S once the proper AUG is recognized. Therefore, in our

polysomal profiling assays, we may have only captured those mRNAs associated with polysomes which were already actively translated by already joined ribosomal subunits. In the *Sbds* knock-down erythroblasts, decreased levels of 60S subunits may not cause selection of the mRNAs for polysomal association, but may be a limiting factor for the general translation output.

Figure 2

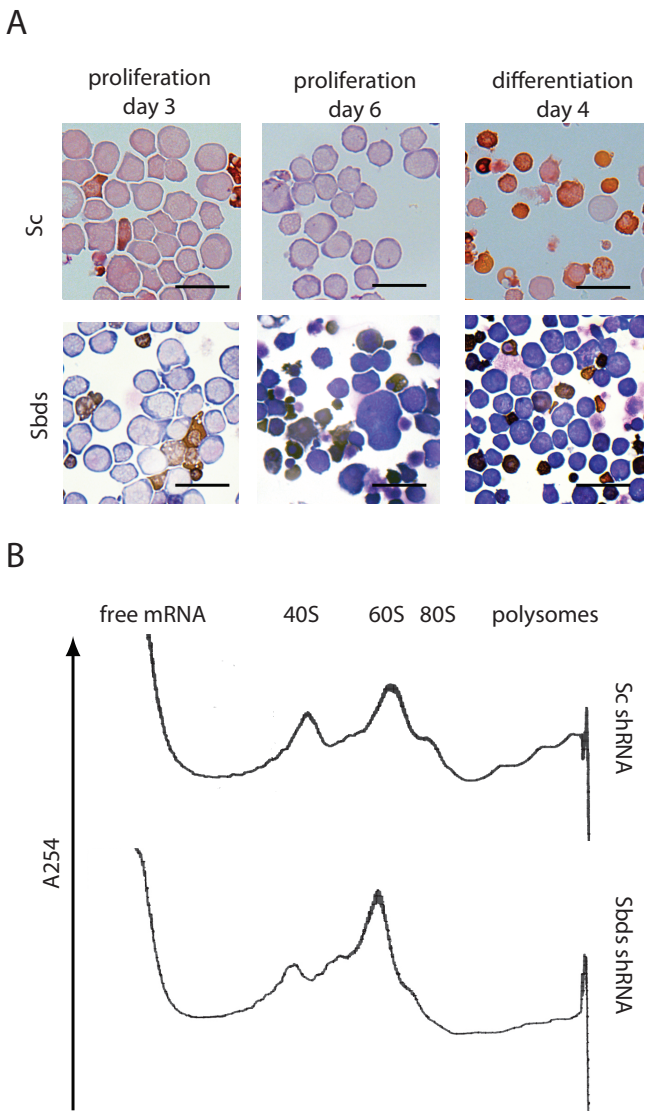

Figure 2. Knock down of Sbds in I/11 erythroblasts. (A) Cells were treated with Sc or Sbds shRNA lentiviruses and cultured in expansion media for 3 days. Afterwards, the cells were split for further expansion (day 6) or induced for terminal differentiation for additional 4 days. Scale bar represent 25 µm. (B) Cells were harvested for polysomal analysis on day 3 after the transduction with shRNA lentiviruses, similarly as was described in Chapter 2.

Translation regulation of IRESes in DBA and Csde1

Structurally and regarding the primary mRNA sequence, the internal ribosomal entry sites (IRESes) are poorly defined. They are mostly an Y-shaped RNA secondary structure with a stable stem region and loops that contain recognition sequences for IRES Trans-Acting Factors (ITAFs)[6]. Several widely expressed IRES binding proteins are known, such as polypyrimidine tract binding protein (PTB)[7], CSDE1[8] and some of the heterogeneous nuclear ribonucleoproteins (hnRNPs), such as hnRNPQ[9] or hnRNPA1[10]. These subsequently interact with more specific ITAFs and translation initiation factors to recruit the 40S ribosomal subunit. For numerous individual transcripts it becomes clear which ITAF bind the IRES to initiate translation. It also becomes clear that IRESes mainly serve to promote translation initiation in transcripts that need to be activated during mitosis and under stress conditions when transcription and cap-dependent translation initiation are largely abrogated[11]. To date, however, little is known about the interaction of ITAFs with the 40S subunit. Whether they bind specific ribosomal proteins or sequences in the rRNA is unknown. Therefore we do not know whether the ribosomal proteins mutated in DBA could have a direct role in recruitment of ribosomes to the IRES. Most likely, however, the reduced availability of ribosomal subunits further decreases the poor competitiveness of IRES-mediated translation initiation versus cap-dependent translation initiation. We observed that reduced expression of Rps19 and Rpl11 impairs IRES dependent translation independent of the IRES used in a bicistronic reporter assay, while cap-dependent translation was hardly affected (Chapter 2). We interpret from this observation that in the competitive process of cap-dependent recruitment of ribosomes the availability of eIF4E remains the rate limiting step, while less competitive IRES-mediated recruitment of ribosomes is generally impaired in our DBA model.

Importance of Csde1 (also called unr) in cellular differentiation is also highlighted by the fact that depletion of Csde1 resulted in differentiation of Csde1$^{-/-}$ mESC into the primitive endoderm lineage, which could be reverted by ectopic expression of unr[12]. The fact that we could not establish p53$^{-/-}$ erythroblasts with sufficient levels of Csde1 overexpression or the mutated form of Csde1 in any of the cell models, points to the importance of Csde1 protein levels in the cells. This is further supported by the fact that many of the bound mRNA targets codes for proteins which represent essential nodes in the growth factor dependent signaling cascades and the cell cycle.

Translation regulation in DBA patients' cells

In Chapter 2 we report on translation regulation in the erythroid cells derived from blood of DBA patients from the Czech National DBA Registry. In total, we have acquired blood samples of 11 patients with variable disease severity and glucocorticoids treatment response. We implemented an established protocol for in vitro erythroid culturing[13], which allowed us to expand erythroid progenitors from whole blood samples in the presence of Epo, SCF and dexamethasone. On FACS analysis, the majority of the cells in these cultures was positive for CD71 and Gly-A, indicating the acquisition of primary erythroid cells from DBA patients (data not shown). However the cell numbers were not sufficient to perform polysomal gradients coupled to microarray profiling as was done for mouse I/11 cells. Therefore, we independently measured the levels of BAG1 and

CSDE1 proteins and their respective mRNAs to indirectly assess the translation rates (Chapter 2). Protein expression levels are not only regulated by translation efficiency but also by protein stability. We therefore measured protein stability of Bag1 and Csde1, that both appeared to have a half-life of approximately 4 hours (Figure 3).

Figure 3

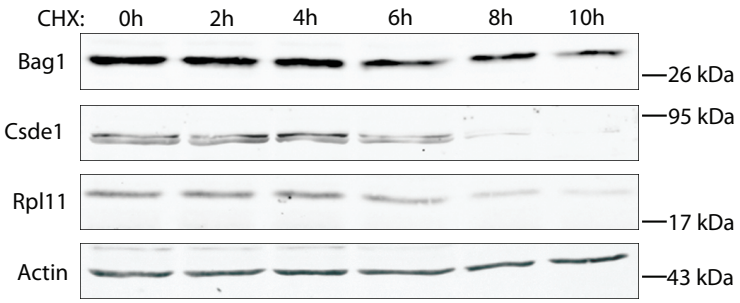

Figure 3. Stability of Bag1 and Csde1 in I/11 erythroblasts. I/11 cells were treated with cycloheximide and every 2 hours sample for WB was collected. Rpl11 serves for comparison and actin as a control for stable protein.

Led by the striking correlation between the levels of CSDE1 protein in diseased human erythroblasts and their in vitro growth rates, we speculated that there could be yet undiscovered mutations in the *CSDE1* gene in DBA patients who had no ribosomal protein gene mutations. This could result in decreased CSDE1 protein levels and hence to dysregulation of important mRNAs for erythropoiesis. To this end, we designed primer pairs spanning the exons of the human *CSDE1* gene and screened for mutations using a WAVE Nucleid Acid Fragment Analysis System, which analyzes the PCR fragments by denaturing high performance liquid chromatography (DHPLC). However, even by this sensitive method we did not uncover any mutations or polymorphisms in the *CSDE1* exons of three DBA patients tested (d9, d10, d11). This is in agreement with recent studies showing that not only ribosomal protein gene mutations, but also their deletions can occur[14,15], which cannot by detected by methods as DHPLC.

Strikingly, many but not all DBA patients respond to glucocorticoids with improved erythropoiesis. In our culture system, we could expand erythroblasts from some but not all patients. Whereas a single glucocorticoid receptor (GRα) is expressed in mouse cells, human cells express many GR variants due to the presence of (partly non-synonymous) SNPs and alternative splicing. The most common isoform, GRα, requires ligand binding for nuclear translocation, dimerization and activation of the transcription transactivating domain. In contrast the GRβ isoform is constitutively located in the nucleus, unable to bind ligand or to activate transcription. The GRα and GRβ can heterodimerise, which results in an inactive receptor[16,17]. A nucleotide polymorphism (SNP) at position A3669G is associated with expression of the GR-β isoform.

The expression of A3669G, predicting a GR-β, occurred mainly in the DBA samples from which we could not expand erythroblasts[18]. Polyribosome recruitment of Bag1 and Csde1 was however not affected by factor dependent signaling (Chapter 2), clearly separating these two events in the possible pathophysiological mechanisms in DBA.

Future perspectives

For identification of translationally deregulated transcripts in a DBA model, we used micro-array profiling of polysome bound mRNA, a protocol which is in use for more than two decades. This method proved to be a powerful tool to assess rapid polyribosome recruitment of specific mRNA in response to environmental factors, upon activation of T-cells, or activation of oncogenes[2,19,20]. Recent advances in high throughput sequencing techniques have shown that sequencing is more quantitative than micro-array profiling of RNA. In addition, the application of UV crosslinking and use of translational inhibitors enable global analysis of mRNA translation at several levels, and not only in polyribosome recruitment. Currently, also the translation start sites, the translation rate and the binding sites of RNA-binding proteins can be identified on complete transcriptomes[21].

If we would have to start again with the identification of mRNAs whose translation is hypersensitive to reduced expression of ribosomal proteins, we would use a new technique known as "ribosome profiling". The "ribosomal profiling" protocol was described in yeast[22] and in mouse ES cells[23]. In this approach, cycloheximide is used to "freeze" the ribosomes on mRNA, which then protect a 28-nt long RNA from partial RNAse digestion. The protected fragments can be ligated to barcode adapters and used for cDNA library preparation. Next generation sequencing will reveal sequences of mRNAs loaded with ribosomes in their coding regions. When the drug harringtonine is added to cells, the same procedure will reveal the relative efficiency with which translation start codons are used. Harringtonine binds the free 60S ribosomal subunit that can subsequently bind the 40S subunit to form a ribosome that is stabilized and "frozen" on the start codon. Already assembled ribosomes do not bind harringtonine and will run off the mRNA[24]. Thus, ribosome profiling can detect polysome recruitment, the efficiency of translation, and the use of distinct translation start sites. Because ribosome profiling yields additional information, particularly on the selection of translation start sites, it should be considered to reanalyze the effect of Rps19 and Rpl11 knockdown on mRNA translation using this technique.

In Chapter 3 we demonstrated that reduced expression of Bag1 resulted in phosphorylation of eIF2, which affects the selection of translation start sites following upstream open reading frames. It remains to be established whether deregulation of eIF2 phosphorylation causes the differentiation arrest of erythroblasts. Phosphorylation of eIF2 and expression of Atf4 and its target Chop is increased during erythroid differentiation. Downregulation of Bag1 increases eIF2 phosphorylation and subsequently induces a balance with moderate phosphorylation of eIF2, and activation of the negative feedback pathway of Atf4-induced expression of Gadd34 and activation of protein phosphatase 1. This activated feedback may inhibit differentiation. To investigate

how reduced Bag1 expression and eIF2 phosphorylation control erythroid differentiation we first need to investigate how eIF2 phosphorylation alters mRNA translation in erythroblasts. The above mentioned technique of ribosome profiling in presence of harringtonine is a powerful tool to examine the effect of eIF2 phosphorylation on the selection of mRNA translation start sites. We can next investigate whether Bag1 suppression alters translation initiation.

The other gene on which we focused was Csde1, an RNA binding protein shown to bind to specific RNA sequences in the context of an IRES in the 5'UTR such as present in *Cdk11* and in *Csde1* itself[7,25]. The *Drosophila* homologue of Csde1, unr, is known to bind the 3'UTR and regulate the translation msl-2 mRNA[26]aling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes</title><secondary-title>Mol Cell</secon/ as well as many other mRNAs and non-coding RNAs in gender-specific fashion[27]. We identified putative Csde1-bound mRNAs by performing a RIP-Chip, and found many mRNAs coding for proteins involved in the cell cycle and receptor signaling. Some of these bear Csde1 binding sites and/or predicted IRES sequence (Chapter 4, Table 2).

The original RIP-Chip protocol has been used extensively to identify mRNA targets of many RNA-binding proteins[28]. Its advantages are simplicity and robustness of the isolation procedure of bound mRNAs. Today, however, novel techniques are available. First, RNA sequencing surpasses micro-array technology in sensitivity and quantitative assessment. Second, the binding sites can be predicted more accurately by efficient pulldown and identification of protein-bound RNA using UV-crosslinking to create covalent bonds between the RNA and RNA-binding protein[29,30]. In the CLIP (crosslinking and immunoprecipitation) protocol, UV light at 254 nm induces the formation of free radicals from nucleotides that subsequently attack aromatic residues, positive groups and SH groups of amino acids[31]. The PAR-CLIP (photoactivatable ribnucleoside-CLIP) protocol uses analogs of ribonucleotides (e.g. 4-thiouridine) that are incorporated into nascent mRNAs. These bear additional SH group that, after UV irradiation at 356 nm, create free radicals, attacking similar groups in the amino acids side chains[30]. Importantly, UV-crosslinking improves the protocol by covalently stabilizing the RNA-protein complex rendering it resistant to the stringent washings conditions in subsequent IP. Moreover, the purified RNAs can be identified by next generation sequencing, which extends the identification of RNAs to pre-mRNAs, non-coding RNAs and others[32]. Thus, experiments using the MEL-birA Csde1 overexpressing cells, where the CLIP-seq methods could be applied, can yield significantly better insights into the nature of the RNAs bound by the Csde1 protein.

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ABBREVIATIONS

40S	small ribosomal subunit
60S	large ribosomal subunit
80S	complete eukaryotic ribosome
AML	acute myeloid leukaemia
BFU-E	burst forming unit-erythroid
CFU-E	colony forming unit-erythroid
CFU-GEMM	colony forming unit - granulocyte, erythroid, macrophage, megakaryocyte
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
DBA	Diamond Blackfan anemia
DC	Dyskeratosis congenita
dex	dexamethasone
DNA	deoxyribonucleid acid
EB	erythroblasts
Epo	erythropoietin
EpoR	erythropoietin receptor
ER	endoplasmatic reticulum
FA	Fanconi anemia
FL	fetal liver
GMP	granulocyte-monocytic progenitor
GR	gluccocorticoid receptor
HEP	human erythroid progenitor
HSC	haematopoietic stem cell
IBMFS	Inherited bone marrow failure syndromes
IP	immunoprecipitation
iPS	induced pluripotent cells
IRES	internal ribosomal entry site
MCV	mean corpuscular volume
MDS	myelodysplastic syndrome
MEP	megakaryocytic-erythroid progenitor
miRNA	micro RNA
MPP	multipotent progenitor
mRNA	messenger RNA
mTOR	mammalian target of rapamycine
ORF	open reading frame
p53	tumor suppressor protein 53
PCR	polymerase chain reaction

Pol	RNA polymerase
polyA	poly-adenin tail on mRNA
RNA	ribonucleid acid
RP	ribosomal protein
RPL	ribosomal protein large
RPS	ribosomal protein small
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor
SCN	Severe congenital neutropenia
SDS	Shwachman-Diamond syndrome
snoRNA	small nucleolar RNA
SNP	single nucleotide polymorphism
TCS	Treacher Collins syndrome
tRNA	transfer RNA
UPR	unfolded protein response
UTR	untranslated region

NEDERLANDSE SAMENVATTING

Diamond Blackfan Anemie (DBA), ook wel het syndroom van Diamond-Blackfan genoemd, is een aangeboren aandoening die vooral gekenmerkt wordt door een ernstige anemie die vaak al in het eerste levensjaar optreedt. Hoewel DBA wordt gezien als een erfelijke vorm van beenmerg falen, is er geen gebrek aan witte bloedcellen maar vooral een tekort aan erythrocyten (rode bloedcellen). Bij iets meer dan de helft van de patiënten wordt een mutatie gevonden in één van de genen die coderen voor ribosomale eiwitten. De mutaties zijn autosomaal dominant en reduceren het aantal ribosomen in de cel. Er zijn mutaties gevonden in genen die coderen voor ofwel eiwitten in de kleine ribosomale subunit, of eiwitten in de grote subunit. Deze eiwitten blijken vooral betrokken bij de aanmaak van ribosomen. Samen met enkele andere erfelijke aandoeningen waarbij de ribosoomaanmaak is verstoord wordt DBA wel aangemerkt als een "ribosomopatie".

In dit proefschrift beschrijf ik ons onderzoek naar de pathofysiologie van DBA waarbij we vooral de translatie van messenger RNA hebben bestudeerd.

Ribosomen zijn in iedere cel essentieel voor de synthese van eiwitten, en het is nog volstrekt onduidelijk waarom een gebrek aan ribosomen zo specifiek tot een gebrek aan rode bloedcellen kan leiden. Tot nu toe is er nooit vastgesteld dat ribosomen kunnen verschillen in afzonderlijke weefsels of cellen. Daarom denken we dat rode bloedcelvoorlopers een eigenschap hebben die deze cellen extra gevoelig maakt voor een tekort aan ribosomen. De gangbare mening op dit moment is dat voorlopercellen van de erythrocyte extreem gevoelig zijn voor het antitumor eiwit p53 dat geactiveerd wordt als er een disbalans is tussen ribosomaal RNA en ribosomale eiwitten. Wij zijn echter uitgegaan van de hypothese dat de translatie van erythroid specifieke mRNAs verstoord is in DBA, en dat de gevoeligheid van rode bloedcelvorming voor een tekort aan ribosomen wordt veroorzaakt door het specifieke transcriptome dat vertaald moet worden in eiwit. Door onze analyses uit te voeren in cellen die het p53 coderend gen missen, is p53 geen verstorende factor in ons model.

Verminderde expressie van Rps19 of Rpl11 in muis erythroblasten remde de groei en uitrijping naar erythrocyten. Erythroblast kweken waarin Rps19 expressie geremd was, vertoonden een groot aantal cellen met meerdere kernen wat duidde op een verstoorde regulering van de celdelingscyclus. Door poyribosomaal en subribosomaal RNA te profilen op oligonucleotide arrays, konden we transcripten identificeren die aanzienlijk minder associeerden met ribosomen bij verminderde Rps19 expressie (beschreven in **hoofdstuk 2**). Als voorbeeld hebben we in meer detail de translatie van *Bag1* en *Csde1* onderzocht. De eiwitten die gecodeerd worden door deze transcripten bleken ook verminderd tot expressie te komen in vergelijk tot controle eiwitten. Voor *Bag1*, *Csde1* en veel andere transcripten is voorspeld of aangetoond dat translatie initiatie niet vanaf de cap maar vanaf een IRES (interne ribosomale entry site) wordt gestuurd. We hebben een bi-cistronisch reporter plasmide gebruikt om aan te tonen dat na reductie van Rps19 of Rpl11 de IRES-afhankelijke translatie initiatie sterker geremd wordt dan de cap-afhankelijke translatie initiatie.

Vervolgens hebben we erythroblasten opgekweekt uit het bloed van DBA patienten en van gezonde vrijwilligers. In de cellen afkomstig van de patienten was de expressie van Bag1 en Csde1 eiwit verlaagd in vergelijk met controle cellen, terwijl er geen verschil was in de mRNA expressie. Dit maakt het heel waarschijnlijk dat de translatie initiatie van deze transcripten in patient cellen net zo verstoord is als in het model van muis erythroblasten.

Om te onderzoeken hoe de verstoorde translatie van specifieke transcripten de erythropoiese beinvloedt, hebben we de regulering en functie van Bag1 en Csde1 in erythroblasten onderzocht. We besloten de rol van Bag1 nader te onderzoeken omdat muis embryo's die geen Bag1 tot expressie kunnen brengen doodgaan op dag 13,5 na conceptie met een volledig gebrek aan erythropoiese in de foetale lever. We hebben laten zien dat de expressie van Bag1 ongeveer 30-voudig toeneemt tijdens de ontwikkeling van hematopoietische stamcellen tot erythroblasten (**hoofdstuk 2 en 3**). Reductie van de Bag1 expressie remde de differentiatie, maar had geen effect op de proliferatie van erythroblasten. Ook de erythroblasten van Bag1-deficiente muizen konden wel prolifereren, maar slecht differentieren. Als gevolg daarvan waren we in staat om van primaire cellen met verminderde Bag1 expressie cellijnen te genereren terwijl p53 expressie instand bleef. Omdat Bag1 een hsp70 co-chaperone eiwit is, hebben we onderzocht of gebrek aan Bag1 de "*unfolded protein response*" activeert, een stress reactie die phosphorylatie van de translatie initiatie factor eIF2 induceert. Op korte termijn bleek dit inderdaad het geval te zijn, terwijl op lange termijn de terugkoppeling van deze signaalroute ervoor zorgde dat er een stabiel evenwicht werd ingesteld dat niet meer reageerde op stress.

Daarnaast hebben we de rol van Csde1 bestudeert (**hoofdstuk 2 en 4**). De expressie van Csde1 was meer dan 100-voudig toegenomen tijdens de differentiatie van hematopoietische stamcellen naar erythroblasten, en vermindert weer tijdens terminale differentiatie naar erythrocyten. Reductie van de Csde1 vermindert zowel de proliferatie als ook de differentiatie van erythroblasten, en leidde tot verstoring van de celdelingscyclus. Net als bij reductie van Rps19 of RPl11 ontstonden er meerkernige cellen. Csde1 is een RNA-bindend eiwit dat aan specifieke sequenties bindt en zo de translatie van deze transcripten beinvloedt. Zowel positieve als negatieve regulatie is vermeld in de wetenschappelijke literatuur. Om uit te vinden welke transcripten door Csde1 gebonden worden hebben we een gelabeld Csde1 in erythroblasten tot expressie gebracht, om dat gelabeld Csde1 vervolgens met gebonden RNA weer te isoleren. Die RNAs zijn geidentificeerd op oligonucleotide arrays, en enkele van de gebonden RNAs bleken bekende Csde1 bings-plaatsen te bevatten. De functie van de gebonden RNAs suggereert dat Csde1 de signaaltransductie van erythropoietine en de celdelingscyclus van erythroblasten reguleert.

Samenvattend hebben we nieuwe genen gevonden die van groot belang zijn voor de erythropoiese, en waarvan de transcriptie sterk opgereguleerd wordt in erythroblasten terwijl de translatie afhankelijk is van voldoende ribosomen. De functie van deze genen suggereert dat deregulatie van de translatie bijdraagt aan de anemie die optreedt by DBA patienten.

SLOVENSKÉ ZHRNUTIE

Diamond Blackfan anémia (DBA) patrí k syndrómom zlyhaní kostnej drene, ktorá vedie k absencii prekurzorov červených krviniek v kostnej dreni. Ostatné bunkové parametre krvi nevykazujú zmeny, preto je DBA čistou apláziou červených krviniek. Približne polovica pacientov trpiacich na DBA nesie autozomálnu dominantnú mutáciu v jednom z génov pre ribozomálne proteíny, následkom čoho je zníženie obsahu ribozómov v bunkovej cytoplazme. DBA mutácie alebo delécie sa vyskytujú u viacerých génov pre ribozomálne proteíny, preto sa DBA označuje aj ako "ribozómopátia". Do rovnakej skupiny ochorení patrí aj niekoľko ďalších hematologických syndrómov, ktoré tiež vykazujú defekty v produkcii ribozómov. Ešte stále však nie je úplne objasnené, ako môže všeobecná bunková porucha ribozómov viesť k tak špecifickej poruche červených krviniek. Zároveň, viac ako polovica DBA pacientov nevykazuje žiadne iné tkanivové poruchy.

V tejto dizertačnej práci sme preskúmali patogenézu DBA s osobitným dôrazom na reguláciu mRNA translácie. Ribozómy sú zodpovedné za transláciu vo všetkých typoch buniek, pričom neexistujú kvalitatívne tkanivové rozdiely v ribozómoch. Z toho vyplynul náš predpoklad, že istá vlastnosť alebo mechanizmus v erytroidných bunkách je zodpovedný za to, že erytroidné bunky sú neobyčajne náchylné na poruchy v produkcii alebo množstve ribozómov. V protiklade ku často diskutovanému mechanizmu, ktorý zahŕňa p53 v patogenéze DBA, sme predpokladali, že chybná regulácia translácie môže byť dôležitým vodítkom k objasneniu podstaty patogenézy v postihnutých bunkách.

V našej práci sme znížili expresiu ribozomálnych proteínov v myšacích erytroblastoch, za účelom napodobniť podmienky nastávajúce v erytroblastoch DBA pacientov. Použitie buniek s p53-deficienciou nám umožnilo identifikovať nový mechanizmus, ktorý nie je závislý na prítomnosti p53. Vykonaný "knockdown" proteínov Rps19 alebo Rpl11 viedol k spomaleniu množenia erytroblastov a zastavil ich diferenciáciu. Erytroblasty so sníženou hladinou Rps19 alebo Rpl11 vykázali mnoho-jadrový fenotyp, čo naznačilo poruchu priebehu bunkového cyklu alebo cytokinézy. Použitím protokolu frakcionizácie polyribozómov spojeného s microarray sme identifikovali tie mRNA, ktoré majú zmenenú asociáciu s polyribozómami (**Kapitola 2**). Zatiaľ čo knockdown proteínov Rps19 a Rpl11 viedol ku všeobecne zníženej asociácii mRNA v polyribozómoch, určité mRNA (napr. obsahujúce 5'TOP sekvencie) boli v polyribozómoch zachované. Určitá skupina mRNA v porovnaní so zvyškom mRNA vykázala výrazne zníženú iniciáciu translácie, čo viedlo k zníženej syntéze proteínov. Taktiež sme poukázali na to, že mnoho mRNA z tejto skupiny obsahuje IRES sekvenciu v ich 5'UTR. Následne je translácia, ktorá závisí na týchto IRES sekvenciách, znížená, ako reakcia na nižšiu hladinu ribozómov v bunkách. Naviac sme dokázali kultivovať erytroblasty získané z periférnej krvi DBA pacientov, u ktorých sme detekovali zníženú produkciu proteínov identifikovaných v myšacom modeli, aj napriek normálnej hladine ich respektívnych mRNA. Z toho vyplýva, že translácia danej skupiny mRNA je v ľudských erytroblastoch regulovaná podobnými mechanizmami, aké prebiehajú v myšacích in vitro modeloch.

V ďalšom výskume sme sa zamerali na funkciu kandidátnych proteínov v erytropoéze a študovali mechanizmy, ktorými prispievajú k erytroidnému fenotypu vyskytujúcemu sa u DBA. Z dôvodu, že *Bag1*$^{-/-}$ myšacie embryá zahynú v embryonálnom dni 13.5 s jasným deficitom červených krviniek, sme sa rozhodli bližšie preskúmať práve proteín Bag1. Zistili sme, (**Kapitola 2** a **3**), že expresia Bag1 sa zvyšuje počas vývinu krvných buniek smerom k erytroblastom a je stabilná počas terminálnej erytroidnej diferenciácie. Bag1 proteín je doležitý pre diferenciáciu červených krviniek, ale nie je potrebný pre množenie erytroblastov. Rovnaký fenomén sme pozorovali v bunkách izolovaných z fetálnej pečene *Bag1*$^{-/-}$ embryí a preukázali sme, že u týchto myší je výhradne narušená červená rada krvotvorby. Navyše sme opakovane pozorovali chybnú diferenciáciu spojenú s neustálym množením erytroblastov, charakteristickú pre in vitro Bag1 knockdown model. Bag1 deficitné bunky vykazovali neustále množenie, ktoré nebolo spôsobené stratou p53 proteínu. Bag1 slúži ako šaperón v endoplazmatickom retikulu. Na základe toho sme analyzovali reakciu buniek na stratu Bag1 s osobitným dôrazom na stresové pochody sprostredkovávané fosforyláciou eIF2, výsledkom čoho bola detekcia fenoménu označeného ako "unfolded protein response".

Ďalšia mRNA, ktorej translácia bola ovplyvnená zníženým obsahom ribozomálnych podjednotiek, kóduje proteín Csde1 (**Kapitola 2** and **4**). Produkcia proteínu Csde1 je v porovnaní s ostatnými bunkovými radami krvi v erytroblastoch zvýšená, zatiaľ čo počas terminálnej diferenciácie červených krviniek je znížená. Knockdown proteínu Csde1 spôsobil inhibíciu množenia a diferenciácie erytroblastov, a viedol k chybnému bunkovému cyklu. Csde1 je RNA-viažuci proteín, ktorý sa viaže na špecifické motívy na mRNA, dôsledkom čoho reguluje ich transláciu. V literatúre bola popísaná pozitívna i negatívna regulácia translácie spôsobená proteínom Csde1. My sme pripravili erytroidné bunky, ktoré produkovali "označenú" formu proteínu Csde1 a použili sme ich na RNA-proteín imunoprecipitácie spojené s microarray (RIP-Chip). Týmto sme identifikovali pravdepodobné mRNA, ktoré sú viazané proteínom Csde1 v erytroidných bunkách. Viacero z týchto mRNA obsahujú motívy, na ktoré sa proteín Csde1 viaže, a ktoré spĺňajú rôzne funkcie pri G2M fáze bunkového cyklu. Taktiež sme identifikovali mRNA pre proteíny doležité pre prenos signálov z rastových receptorov. Kedže bunkový cyklus a rastové receptory podliehajú striktnej regulácii v erytroidných bunkách, Csde1 môže plniť dôležitú úlohu pri zoladovaní týchto procesov. Ďalšie experimenty by mohli vniesť viac svetla do funkcie Csde1 v erytropoéze.

V tejto práci sme identifikovali nové gény dôležité pre erytropoézu, ktorých expresia je silno ovplyvnená hladinou ribozómov v bunkách. Znížená translácia mRNA identifikovaných génov môže prispieť k patofyziologickým mechanizmom vedúcim k zníženej krvotvorbe a anémii.

WORDS OF THANKS

Finally, the long odyssey has come to its end... I would not imagine at the very beginning, in July 2006 when I started at the Department of Hematology in Rotterdam, that almost 7 years would come and go, until I finally can close this chapter of my life! But at the same time, this experience shaped my scientific thinking, opened many possibilities and, most of all, established friendships with many remarkable people, so it was definitely worth the time! More I will be happy to see many of them at my thesis defence. To them all I would like to thank in the following words.

First of all, I would like to devote my thoughts to Marieke, who was always so unbelievably enthusiastic, even when I thought I stare at a dead end. Marieke, I remember as it was only yesterday, when we met in Erasmus MC and had our first chat, how natural and opened you were to that "strange guy from East Europe". The more my astonishment was, when you sheltered me for the first visit, as it was the most normal thing in the world. Only with time I understood how extraordinary person, friend and scientist you are and what the word friendship really means. I wish you all the best at Sanquin, "hard hand" ruling the department and hope for collaboration in future! Naturally, I would also like here to thank Freek, who always warmly welcomed me in Leiden and made the stay enjoyable.

Dear Ivo, thank you for your everlasting support both at the department and during the time afterwards. I am indebted to you for being my promotor, particularly because you fulfilled this duty not only on the paper, but followed, criticized, and supported my development as scientist very closely throughout all these years.

I would like to express my gratitude to prof. Bob Löwenberg for his support, the possibility to stay at the department and enthusiasm expressed at my work discussions and international meetings. At the same time, I would like to thank Sjaak, Ruud and Paul Coffer for reading my thesis and their critical input. Members of my large thesis committee, dear Dagmar Pospisilova, Maarten Fornerod and Cor Calkhoven, thank you for your time and devotion for my thesis defence.

As to my fellow colleagues, first I want to mention Andrzej, without who all these years would turn quite boring! I enjoyed a lot your company in the lab, especially your good humour and everlasting smile. We had a lot of fun in these years and I gladly recall with joy our trips, parties and weddings! Dear Suming, in time we became good friends and I miss your right-to-the-point humour. Tanja, thank you for everything both in the lab and outside, you were always very helpful and kind. Hope all is going well and you're enjoying your return to home. Dear Annemarie, I enjoyed our tennis evenings where we formed "unbeatable" pair in doubles lasting till dark, though Karishma and Joost tried really hard...

Especially I would like to thank again Dagmar Pospisilova from University Olomouc, for your enthusiastic input in our common project and warm welcome in Olomouc for the OHD. Without your help we would hardly publish our paper so high! Special thanks and very nice memories I give to groupleaders at our and other departments, who supported me in various manners, ranging from helpful discussions, through facility sharing, to kindly providing assays and consumables. So thank you, Ruud, Peter V., Mojca, Tom, Sjaak, and Frank.

I greet all my fellow PhD students, postdocs and lab managers that left EMC before and after my time, namely Astrid, Albert, Eric, Fatima, Godfrey, Goran, Jurjen, Jurgen, JO, Mahban, Marshall, Menno, Merel, Saman, Sanne, Renée, and hope for forgiveness from those I forgot... I liked always our Boudewijn Fridays, dinners and cinema visits. For those that are still at Hematology, Simone, Lucilla, Roberto, Veronika, Noemi, Athina, Farshid, Hans, and others, good luck with your projects and success with publications! Dear Eric B., cheers for all your helpful discussions and funny weekends spent at bench...

I enjoyed sharing my office, first in the "kopkamer" with (sometimes too) many PhD fellows and later with especially great guys, Stefan ("Please stop hitting that keyboard like that!") and Arturo ("Calm down please, that is the only way I can export data from Excel to Prism..."). We had lot of fun and I am always happy to come back to our office.

Marijke, Paulette, Onno, Claudia, Marije and Judith G., it was a pleasure to work with you all these years. Though not directly working in common projects, your help has made my life at the department almost a walk through a rose garden. In particular I would like to mention "my" students who helped me to move on with my project. Hanna, I cross my fingers for your thesis and your family and hope to receive your theses soon! Chris, good luck with your future career and many many live concerts of your favourite singer! Fane, I hope you found yourself nice job which gives you lot of possibility to pursue your soccer career too! William, thank you for your devotion and superb-quality maxi preps which you mastered in no-time! And finally, though not directly "my" student, Marleen, all the best with your PhD project and many healed patients in future.

Ans, Monique, Jan, Sevilay and Thomasia, thank you for your administrative, computational and lab support and organization! Dear Egied, thank you for your editing work and dear Tim, I am grateful to you for being so kind and share your artwork for the cover.

My thanks belong naturally also to my current "boss" Matthias Hentze for his understanding and support, and to the whole lab at EMBL Heidelberg. I enjoy staying in this fantastic and inspiring work environment and hope to get most of it.

Finally, I give my thanks to Patrícia, to whom is this book also devoted. You always supported me in my decision to pursue scientific career and believed I could really achieve something. You stayed at my side even during the time when huge distance separated us, and later followed me to the Netherlands and Germany. You gave up your professional career for me, friends and family in Slovakia. The feelings I bear towards you can be hardly described by words, but at least I want to thank you this way for all you did for me and look forward to the things that await us...

CURRICULUM VITAE

The author of these theses was born in Košice, Czechoslovakia, on 31.8.1982. He studied veterinary medicine and gained the diploma of Doctor of Veterinary Medicine in 2006. During these studies he got interested in molecular genetics and immunology, where he would spend much time in the lab instead of stables. In 2005 he worked on his diploma thesis in Vila Real, Portugal in the field of cytogenetics. He then started his PhD in the group of Marieke von Lindern in Hematology Department, Erasmus Medical Center, in Rotterdam, the Netherlands, where he studied mRNA translation regulation in erythropoiesis. Since 2011 he works on post-doctoral project in the group of Matthias Hentze in European Molecular Biology Laboratory, in Heidelberg, Germany.

PUBLICATIONS

Castello A, **Horos R**, Strein C, Fischer B, Eichelbaum K, Steinmetz LM, Krijgsveld J, Hentze MW: System-wide identification of RNA-binding proteins by interactome capture. *Nature Protocols* in press

Horos R, von Lindern M: Molecular mechanisms of pathology and treatment in Diamond Blackfan Anaemia. *British Journal of Haematology* 2012 Dec;159(5):514-27

Castello A, Fischer B, Eichelbaum K, **Horos R**, Beckmann BM, Strein C, Davey NE, Humphreys DT, Preiss T, Steinmetz LM, Krijgsveld J, Hentze MW: Insight into RNA biology from an atlas of mammalian mRNA binding proteins. *Cell* 2012 Jun 8;149(6):1393-406.

Horos R, Ijspeert H, Pospisilova D, Sendtner R, Andrieu-Soler C, Taskesen E, Nieradka A, Cmejla R, Sendtner M, Touw IP, von Lindern M: Ribosomal deficiencies in Diamond-Blackfan-Anaemia impair translation of transcripts essential for differentiation of murine and human erythroblasts. *Blood* 2012; Jan 119(1):262-272

Esteghamat F, van Dijk TB, Braun H, Dekker S, van der Linden R, Hou J, Fanis P, Demmers J, van IJcken W, Ozgür Z, **Horos R**, Pourfarzad F, von Lindern M, Philipsen S: The DNA binding factor Hmg20b is a repressor of erythroid differentiation. *Haematologica* 2011 Sep;96(9):1252-60.

Rasmussen KD, Simmini S, Abreu-Goodger C, Bartonicek N, Di Giacomo M, Bilbao-Cortes D, **Horos R**, Von Lindern M, Enright AJ, O'Carroll D: The miR-144/451 locus is required for erythroid homeostasis. *Journal of Experimental Medicine* 2010 Jul 5;207(7):1351-8.

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: Rastislav Horos
Erasmus MC Department: Hematology
Research School: Molecular Medicine (MM)

PhD period: 3.7.2006-31.12.2010
Promotor: Prof.Dr. I.P.Touw
Supervisor: Dr. Marieke von Lindern

1. PHD TRAINING

	Year	ECTS
General courses		
• In vivo imaging – from cells to organisms	2007	1.8
• From development to disease (MGC)	2007	1.8
• Signal transduction and control of cellular proliferation (LUMC)	2007	1.8
• Basic and translational oncology	2005	1.8
Specific courses		
Laboratory Animal Science, FELASA C (Utrecht)	2008	3.0
Seminars and workshops		
• Basic data analysis on gene expression arrays	2008	0.6
• Matlab fundamentals and statistical methods	2009	1.0
• Photoshop CS3 Workshop	2010	0.3
• Erasmus Hematology Lectures	2006-2010	3.0
• Hematology department Workdiscussions	2006-2010	4.0
• Journal Club Presentations	2006-2010	3.0
• AIO/Postdoc meetings	2006-2010	1.6
Presentations		
Hematology Department workdiscussions (6x)	2006-2010	3.0
Journal Club Presentations (3x)	2006-2010	1.5
AIO/Postdoc meetings (2x)	2006-2010	1.0
(Inter)national conferences		
Oral presentations		
• 5th International Congress on Shwachman Diamond Syndrome	2009	1.0
• 51st American Society of Hematology Congress	2009	1.0
• 4th Dutch Hematology Congress	2010	1.0
• 11th DBA International Consensus Conference (Invited speaker)	2010	1.0
• 24th Olomouc Hematological Days (invited speaker)	2010	1.0
• Marie-Curie RTN Network meetings (4x)	2006-2010	1.2

Poster presentations

• EMBO conference on protein synthesis and translation control	2007	0.6
• EMBO Meeting	2009	0.6
• 15th Congress of the European Hematology Association	2010	0.6
• 17th Conference on Hemoglobin switching	2010	0.6

2. TEACHING

	Year	ECTS
• Supervising Master's theses		
• Master student Molecular Medicine (6 months)	2009	3.0
• Supervising bachelor theses		
• Bachelor student (4 months)	2008	3.0
• Supervising high school student		
• High school student (5 months)	2008	2.0
• High school student (5 months)	2010	2.0
Total		**47.8**