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Sjögren, Sara

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On new and current treatments for Diamond-Blackfan anemia

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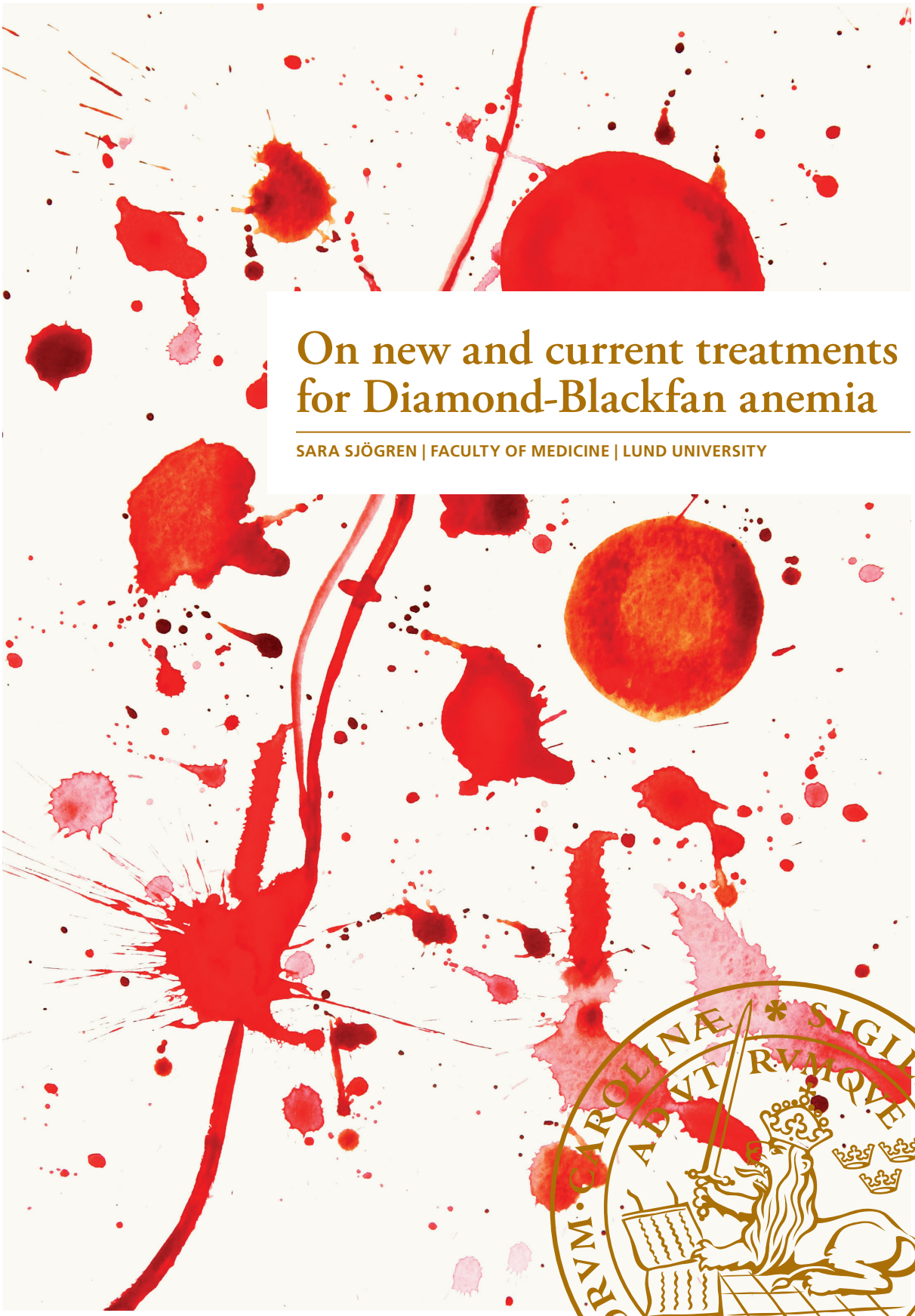

On new and current treatments for Diamond-Blackfan anemia

Sara Sjögren

DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden.
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FACULTY OPPONENT

Associate Professor Marieke von Lindern
Sanquin Research and Landsteiner Laboratory, Amsterdam UMC,
University of Amsterdam,
Amsterdam, The Netherlands

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Abstract

Diamond-Blackfan anemia (DBA) is a rare congenital disease where the patients suffer from macrocytic anemia due to reduced numbers of erythroid precursors in the bone marrow. Most patients carry mutations in ribosomal proteins, such as ribosomal protein S19 *(RPS19)*, which causes deficient ribosome biogenesis and affects protein translation. Ribosomal protein mutations also cause increased apoptosis in erythroid precursors due to upregulation of the tumor suppressor p53 and reduced translation of certain mRNAs important for erythropoiesis. This leads to a reduction of the pool of cells able to differentiate and give rise to red blood cells. DBA patients receive glucocorticoid treatment or blood transfusions to alleviate the anemia, but both treatments have severe side effects which negatively affects the quality of life for the patients. For this reason, it is important to gain deeper understanding of the mechanisms underlying DBA and utilize this knowledge to develop more disease-specific treatments for DBA. Studying mechanisms behind current treatments could provide knowledge needed to develop new therapies. About 40% of all DBA patients receive glucocorticoid treatment, but the disease specific mechanisms behind glucocorticoid treatment in DBA are not fully elucidated. Work presented here identifies that glucocorticoid treatment reduces p53 upregulation and increases the survival and delays differentiation of *Rps19* deficient erythroid precursors. In the search for new disease mechanisms behind DBA, we discovered increased levels of unbound intracellular heme in purified *Rps19* deficient erythroblasts. To identify of genes able to modify the disease phenotype we performed a custom shRNA based screen for 750 genes hypothesized to affect the DBA phenotype. The screen identified that knock down of the heme-sensing regulator of eukaryotic initiation factor 2a mediated translation (*HRI*) improved erythroid precursor proliferation in *Rps19* deficient mice and reduced both elevated heme levels and decreased the increased p53 activity. A small drug screen identified a1-microglobulin (A1M) to increase proliferation of both *Rps19* deficient murine erythroid precursors as well as erythroid percursors from DBA patients by reducing heme levels. Interestingly, A1M treatment did not affect either heme synthesis or p53 activity. Together, these studies show that glucocorticoid treatment decreases p53 activity in *Rsp19* deficient erythroid precursors and that elevated heme levels are seen in a mouse model for DBA. Work presented here propose that targeting elevated heme levels in DBA can be used as a new therapeutic strategy.

Key words: Diamond-Blackfan anemia, eryhtropoiesis, ribosome biogenesis, heme synthesis, glucocorticoids, p53, globin translation, HRI, α1-microglobulin

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On new and current treatments for Diamond-Blackfan anemia

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To anyone affected by this disease

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PREFACE

Thirst was made for water; inquiry for truth
C.S. Lewis (1898-1963)

The world around us is a beautiful thing. Most of us experience that through breathtaking experiences in nature and perhaps by beautiful art and music. Mankind is creative and at the same time we are able to enjoy things we have not ourselves made. For me, singing great masterpieces of music together with other choir members and a symphony orchestra have been overwhelming experiences of beauty and belonging. Even if - and probably because - I am not the composer, it has been truly wonderful to read someone else's score and interpret it in the best way possible. I look at science the very same way. I am not the originator of anything science set out to investigate; yet I get to read the scores, walk in someone's footsteps, re-search what is already there. This re-search should be done with great diligence and analytical sharpness. The world around us is predictable and logical and we are able to make sense of it; to prove and dismiss hypotheses, to verify previous assumptions. Even more, we are able to critically evaluate our own search for facts and dismiss and replace paradigms once proven wrong. The astronomer Johannes Kepler (1571-1630) once wrote: "*Those laws* [of nature] *are within the grasp of the human mind; God wanted us to recognize them by creating us after his own image so that we could share in his own thoughts*". To do science is to explore the world by putting our feet down where no one has gone before. That is, no man. Someone else has always been there before us; constructing the laws of the universe, enabling living organisms, laying foundations for cellular processes such as the differentiation and final maturation of erythroid precursors to form red blood cells. Walking in the footsteps of a true mastermind has been frustrating, challenging and one of the most exciting things I have done.

Lund, November 2018

ORIGINAL PAPERS

Papers included in this thesis

Paper I **Sara E Sjögren**, Kavitha Siva, Shamit Soneij, Amee J George, Marcus Winkler, Pekka Jaako, Marcin Wlodarski, Stefan Karlsson, Ross D Hannan and Johan Flygare. *Glucocorticoids improve erythroid progenitor maintenance and dampen Trp53 response in a mouse model of Diamond-Blackfan anaemia*. Br J Haematol, 2015

Paper II **Sara E Sjögren**, Jun Chen, Magnus Gram, John G Doench, Jane-Jane Chen and Johan Flygare. *Heme-regulated eIF2α kinase can be targeted to normalize intracellular heme toxicity in Diamond-Blackfan anemia*. Manuscript

Paper III **Sara E Sjögren**, Abdul Ghani Alattar, Jun Chen, Helena Karlsson, Magnus Gram and Johan Flygare. *Human α1-microglobulin reduces excess intracellular heme and improves proliferation in Diamond-Blackfan anemia.* Manuscript

Paper not included in this thesis

Sara E Sjögren and Johan Flygare. *Progress towards mechanism-based treatment for Diamond-Blackfan anemia*. ScientificWorldJournal, 2012

POPULÄRVETENSKAPLIG SAMMANFATTNING

Diamond-Blackfans anemi (DBA) är en ovanlig blodbristsjukdom där nybildningen av röda blodkroppar i benmärgen är försämrad. Processen som bildar röda blodkroppar kallas erytropoes och hos patienter med DBA fungerar den inte som den ska. Utan behandling är DBA ofta en dödlig sjukdom eftersom man inte överlever med för få röda blodkroppar under en längre tid. För att öka mängden röda blodkroppar behandlas patienterna antingen med steroidhormon eller med tillförsel av blod från en donator, s.k. blodtransfusion. Båda behandlingarna är livslånga och ger svåra biverkningar, vilket sänker patienternas livskvalité. För att kunna utveckla nya och bättre behandlingar för DBA skulle man behöva veta mer om vad som ligger bakom sjukdomen. Det finns alltså ett stort behov av behandlingar som är mer specifika för DBA, eftersom de då skulle kunna ha färre biverkningar.

Varför blir man sjuk?

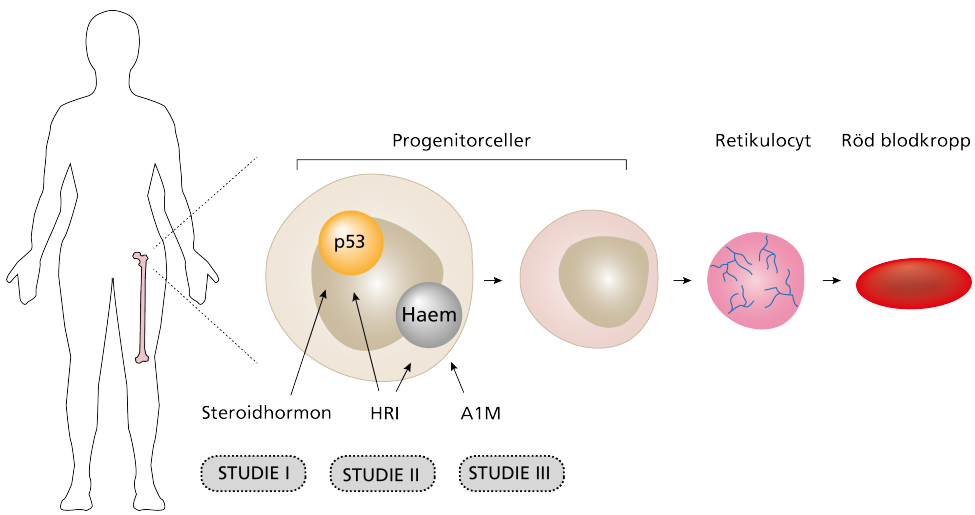
I en frisk människa bildas 2 miljoner nya röda blodkroppar – varje sekund. En sådan process bygger på att de progenitorceller som bildar röda blodkroppar har förmågan att föröka sig i snabb takt. I patienter med DBA slutar dessa progenitorceller att föröka sig och självdör på grund av att det bildas för mycket av ett protein som heter p53. I vanliga fall fyller p53 en viktig funktion för att förhindra att vanliga celler börjar ge upphov till tumörer, men hos patienter med DBA orsakar denna skyddsmekanism istället att progenitorcellerna som bildar röda blodkroppar dör.

Röda blodkroppar har en livsuppehållande uppgift i att transportera syre runt i kroppen och ta med koldioxid tillbaka till lungorna. För att kunna transportera syre innehåller röda blodkroppar otroligt stora mängder hemoglobin. Vid bildandet av hemoglobin behöver varje globinmolekyl vara bunden till en haem-molekyl. Om det finns mer haem än globin kan cellen skadas, eftersom haem som inte är bundet till en annan molekyl är giftigt och gör att cellen dör.

Nya och nuvarande behandlingar för DBA

Ett bra sätt för att kunna utveckla nya behandlingsmetoder är att se vad som fungerar bra i nuvarande behandlingar och sedan utveckla det. Patienter med DBA har behandlats med steroidhormon sedan 1950-talet, men fortfarande är lite känt om varför det ökar antalet röda blodkroppar. I studie I fann vi att steroidhormon ökar mängden röda blodkroppar hos patienter med DBA för att de hindrar p53. I studie II kunde vi se att progenitorcellerna har förhöjda nivåer av haem, vilket är skadligt för progenitorcellerna och troligen bidrar till att de dör. Vi såg även att när vi tystade en gen som heter *HRI* så sjönk både nivåerna av haem och p53 och fler röda blodkroppar kunde bildas. I studie III fann vi att behandling med ett kroppseget protein som heter A1M gjorde att progenitorcellerna kunde föröka sig betydligt bättre eftersom behandlingen sänkte haemnivåerna.

Sammanfattningsvis presenterar den här avhandlingen tre studier och en av dem visar på mekanismen varför nuvarande behandling ökar mängden röda blodkroppar (Figur 1, studie I). Vi fann också att progenitorceller med DBA har förhöjda haemnivåer och att detta troligtvis bidrar till blodbristen hos patienterna. Med två olika strategier sänkte vi haemnivåerna i DBA-cellerna och ökade därmed deras överlevnad och förmåga att föröka sig (Figur I, studie II och III). Vår förhoppning är att dessa fynd ska kunna leda till bättre och mer sjukdomsspecifika behandlingar för patienter med DBA i framtiden.



Figur 1. Progenitorceller i benmärgen genomgår successivt förändringar för att till slut ge upphov till röda blodkroppar som kan transportera syre runt i kroppen. I tre olika studier har vi försökt öka mängden röda blodkroppar hos patienter med DBA.

ERYTHROPOIESIS

The circulating red blood cell is the cell type responsible for carrying oxygen (O_2) to tissues in the body, bringing back carbon dioxide (CO_2) for removal. Red blood cells are highly specialized cells able to cope with extreme mechanical force as it pushes through blood vessels and capillaries, reaching distant tissues. This life-sustaining differentiated cell is generated from a much less differentiated hematopoietic stem cell residing in the bone marrow. The gradual commitment of the stem cell towards finally becoming a committed red blood cell is a process called erythropoiesis. During mammalian development erythropoiesis occurs first in the yolk sac and later in the fetal liver, to finally relocate to the adult bone marrow (Palis 2014). Erythropoiesis is a highly dynamic process subjected to regulation at multiple levels to both ensure self-renewal and extensive proliferation of the earliest progenitors, accompanied by upregulation of lineage specific programs promoting terminal maturation. Terminal erythroid differentiation is a process involving cell cycle exit, extrusion of the nucleus and cellular rearrangements sufficient to generate the oxygen transporting red blood cell (Hattangadi *et al.* 2011).

The first committed erythroid precursor in the bone marrow are the burst forming unit erythroid (BFU-E), that further differentiate and form colony forming unit erythroid (CFU-E) progenitor cells. These early precursors are named after their ability to produce erythroid colonies after 14 to 7 days respectively (in mouse), when grown in semi-solid media (Gregory et al. 1977; Gregory et al. 1973; McLeod et al. 1974; Stephenson et al. 1971). While BFU-Es have a great potential to expand, CFU-E expansion is less dramatic as it enters a phase of asynchronous cell division, where each new cell formed is different from its parent in respect to cell size, gene expression, and hemoglobin content. These precursors are called erythroblasts and by each new cell division the CFU-E forms proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts and orthochromatic erythroblasts (Figure 1). At this stage, the erythroblast exits the cell cycle, extrudes its nucleus and undergoes cellular rearrangements to allow the transition from the reticulocyte to a fully functional mature red blood cell (reviewed in (Ji *et al.* 2011)). Reticulocyte maturation is a complicated process involving reduced cell size, membrane rearrangement and loss of all residual organelles. These rearrangements are

necessary for the mature red blood cell to cope with the strenuous task of circulating in the blood stream. Erythroid differentiation occurs in so called erythroblastic islands, where erythroid precursors are in close proximity to a central macrophage that facilitates their maturation. These macrophages not only support erythroid differentiation via cell surface molecules, but are also believed to engulf and degrade extruded nuclei from maturing erythroblasts, transfer iron to developing erythroblasts and facilitate proliferation of erythroblast precursors (Heideveld et al. 2017; Hom et al. 2015).

Erythroid differentiation is an extensively studied process largely due to the ability to isolate distinct erythroid populations during differentiation taking advantage of differently expressed surface markers. Flow cytometry based protocols have been developed allowing isolation of distinct maturation stages of differentiating erythroid precursors in both mice and humans. The initial protocol combining the markers CD71 and Ter119 to isolate erythroblasts in the mouse fetal liver (Zhang, Socolovsky, et al. 2003) has later been revised and updated using the non-erythroid specific marker CD44 (Chen et al. 2009; Liu et al. 2013). The same was also done in human cells monitoring the markers α-integrin and Band3 (Hu et al. 2013). Later, isolation of CFU-Es and BFU-Es was reported using a combination of multiple markers both in humans and mice (Li et al. 2014; Flygare et al. 2011; Pronk et al. 2007; Tusi et al. 2018).

REGULATION OF ERYTHROPOIESIS

Every second throughout life the human body produces around 2 million red blood cells. Every red blood cell has a lifespan of 120 days in humans and 40 days in mice. New red blood cells need to be constantly generated from erythroid precursors in the bone marrow to maintain sufficient oxygen tension. Additionally, the erythroid system is highly adaptable to situations of stress, such as extensive blood loss. The remarkable and adaptable process of red blood cell replenishment is orchestrated by a complex, yet well described, network of transcription factors, as well as extrinsic regulators such as hormones and other humoral factors. The following sections will highlight some of these regulators, primarily relevant for the work presented in this thesis (Figure 1).

Extrinsic regulation

In 1878, Paul Bert proposed that low oxygen tension at high altitude increases the number of red blood cells in circulation (Koulnis et al. 2014). Indeed, oxygen tension regulates blood cell production via inhibiting degradation of the hypoxia-inducible factor 1-alpha (Hif1α) which promotes secretion of erythropoietin (Epo) by the kidney. For erythropoiesis to occur, Epo is required to bind the Epo receptor (EpoR) of developing erythroid precursors and stimulate their survival and differentiation (Goldwasser et al. 1971; Dandrea et al. 1989; Erslev 1953). Although erythroid precursors respond to many different cytokines, erythroid cells absolutely require stimulation of the receptors c-Kit and EpoR by the ligands stem cell factor (SCF) and Epo for efficient erythropoiesis to occur.

EpoR and c-Kit signaling

Secretion of Epo by the kidney induces EpoR signaling in maturing erythroid precursors and activates an array of signaling pathways that stimulate survival, differentiation and proliferation, including PI3-kinase/Akt, Ras/Raf/MAPK and Stat5 signaling pathways. While the early BFU-Es and CFU-Es survive and proliferate independent of EpoR signaling, differentiation and survival of erythroblasts transitioning from CFU-E to proerythroblasts are completely Epo dependent (Wu et al. 1995; Lin et al. 1996; Landschulz et al. 1992). Mice lacking *Epo* or the *EpoR* die *in utero* due to severely impaired fetal liver erythropoiesis, underscoring the absolute necessity of Epo signaling for terminal erythroid differentiation (Lin et al. 1996; Kieran et al. 1996; Wu et al. 1995). EpoR signaling is mediated through the tyrosine kinase Janus Kinase 2 (JAK2), and this kinase is indispensable for EpoR signaling, as the *JAK2* knock out mouse phenotype is identical to that of *EpoR* knock out mice (Neubauer et al. 1998; Parganas et al. 1998).

The erythroid system is constantly ready to rapidly adjust red blood cell output in response to blood loss, and therefore erythroid precursors are formed abundantly and then regulated by balancing pro- and anti- apoptotic signals, primarily after the CFU-E stage (Koulnis *et al.* 2014). Unless stimulated by Epo, erythroid precursors will display increased apoptotic signals, primarily via pro-apoptotic members of the Bcl-2 family, *e.g.* Bax, Bak and Bid. EpoR signaling prevents apoptosis by multiple signaling pathways, inducing activation of the pro-survival molecule Bcl-$_{XL}$ induced by Stat5, which is considered one of the main survival pathways for erythroid progenitors. Indeed, *Bcl-$_{XL}$* knock out mice show, among other symptoms, extensive apoptosis of hematopoietic cells and

die *in utero* around E13.5 (Motoyama et al. 1995). *Stat5-/-* mice show a less severe phenotype, but still display impaired erythropoiesis due to increased apoptosis and insufficient levels of Bcl-$_{XL}$ during fetal development (Socolovsky et al. 1999). Whereas *Stat5-/-* erythroblasts show increased differentiation instead of renewal, cells transduced with *Bcl-$_{XL}$* are able to terminally differentiate even in the absence of Epo, indicating a pivotal role of Bcl-$_{XL}$ during terminal erythroid differentiation (Dolznig et al. 2006).

Early hematopoietic progenitors are among many cell types expressing the transmembrane tyrosine kinase receptor c-Kit, which dimerizes and undergoes autophosphorylation when bound to its ligand stem cell factor (SCF) (Miettinen *et al.* 2005). Activation of the c-Kit receptor mediates signaling through many different signaling pathways including the Src pathway (Tan et al. 2003). c-Kit expression is maintained by the BFU-E, but it continuously declines from the CFU-E stage and onwards during erythroid differentiation. Signaling via c-Kit is crucial for early erythroid precursors, as it stimulates proliferation and survival, and delays terminal differentiation (Muta et al. 1995; Wessely, Mellitzer, et al. 1997). Synergistic signaling through both EpoR and c-Kit is necessary for efficient erythroid formation (Arcasoy et al. 2005). Also, there seems to be cooperativity between Epo and SCF in erythroid development, since changes in gene expression caused by either Epo or SCF never have opposite effects (Kolbus et al. 2003).

Glucocorticoids and stress erythropoiesis

In order to rapidly adjust to sudden insults to the erythroid system, as well as to maintain steady state erythropoiesis, the production of mature red blood cells is dependent on the self-renewal of committed BFU-Es. The state of self-renewal is characterized by intense proliferation of BFU-Es and CFU-Es, followed by differentiation where proliferation is decreased and differentiation, including increased hemoglobin content and cellular rearrangements take place to extrude the nucleus and form mature red blood cells. Upon sudden decline in red blood cells in the peripheral blood, a phenomenon known as stress erythropoiesis occurs. This is a physiological response to severe anemia, by which the body tries to compensate for inadequate levels of red blood cells. In mice, stress erythropoiesis takes place primarily in the spleen (Hara et al. 1976). This process increases the erythroid output dramatically, and requires signaling by the glucocorticoid receptor (GR), activated by its ligands, glucocorticoids (Bauer et al. 1999). The GR resides in the cytoplasm associated with a multimeric chaperone complex comprises of heat shock proteins Hsp70 and 90 to prevent it from degradation and facilitate ligand interaction (Bresnick et al. 1989). Glucocorticoids are essential endocrine regulators of cell growth,

metabolism, apoptosis are synthesized in the adrenal cortex in humans. Synthesis and secretion of the most common endogenous glucocorticoid cortisol is regulated by the pituitary gland. Once bound to glucocorticoids, the GR translocates to the nucleus where it acts as a homodimeric transcription factor binding via its DNA binding domain to glucocorticoid response elements (GRE) of its target genes (Strahle et al. 1987), or functions as a monomeric protein that co-operates with other factors to induce transcription (reviewed in (Oakley et al. 2013)). Also, GR can function as a transcriptional repressor by occupying DNA sites where other transcription factors would normally bind. The effects of GR activation are highly cell type specific, best illustrated by the fact that activated GR stimulates erythropoiesis, but induces apoptosis in lymphoid cells (Bauer et al. 1999; Wyllie 1980). Signalling through the GR has widespread implications in a living organism, but this section focuses solely on the erythroid lineage.

As discussed previously, erythropoiesis is a process where self-renewal and proliferation is balanced against differentiation and terminal maturation of red blood cells to generate optimal oxygen tension in an organism. In the presence of glucocorticoids, early erythroid progenitors like BFU-Es are maintained in a self-renewing state and differentiation is delayed (Dolznig et al. 2006). Indeed, glucocorticoids enhance the formation of erythroid BFU-E and CFU-E colonies *in vitro*, and addition of glucocorticoids increases the erythroid output by at least 20-fold (Flygare et al. 2011; von Lindern et al. 1999; Wessely, Deiner, et al. 1997). In an erythroid context, GR regulates two key transcription factors involved in erythropoiesis, Myb and Gata1. While glucocorticoid administration inhibits Gata1 upregulation and thereby prevents differentiation (see section Genes and intrinsic factors), it induces Myb activity in early erythroid precursors. A constitutively active form of Myb has similar effects as glucocorticoid administration in erythroid culture, indicating that Myb expression is key for self-renewal and proliferation in GR treated erythroid precursors (Wessely, Deiner, et al. 1997; Chang et al. 1993). The GR also increases the expression of genes associated with the progenitor stage, such as increases the expression of c-Kit and Tal1 (Ebert et al. 2005; Wessely, Deiner, et al. 1997).The GR cooperates with both Epo and the c-Kit receptor ligand SCF to enhance the erythroid output *in vitro* (von Lindern et al. 1999; Kolbus et al. 2003; Dolznig et al. 2006; Wessely, Deiner, et al. 1997). For instance, self-renewal and inhibition of differentiation of erythroid precursors require cooperation between glucocorticoids and SCF. Although promoting colony formation on its own, glucocorticoid administration together with Epo enhances erythroid output (Golde et al. 1976).

Glucocorticoid administration has widespread effects in the body, affecting multiple signalling pathways. Interestingly, the GR has been found to physically

bind to and sequester the tumor suppressor p53 in the cytoplasm, preventing p53 from entering the nucleus to regulate gene expression (Sengupta et al. 2000). This negative cross talk between GR and p53 also exists in erythroid cells, since *p53-/-* erythroblasts proliferate more and are less differentiated in the presence of glucocorticoids, compared to wild type counterparts (Ganguli et al. 2002).

Genes and intrinsic factors

Gata1

Gata1 is an X-linked erythroid zinc-finger transcription factor highly expressed in erythroid cells and necessary for both erythropoiesis and formation of platelets form megakaryocytic precursors. Additionally, it is expressed in megakaryocytic, eosinophilic and mast cell precursors. It was first discovered by its ability to bind regulatory sequences in the globin gene via GATA-binding motifs ((T/A)GATA(A/G)) (Tsai, Martin, et al. 1989; Evans et al. 1989). Of all factors promoting erythropoiesis, Gata1 is considered as the central mediator of erythroid gene expression. Indeed there are few, if any, erythroid specific genes independent of GATA-motifs (Orkin 1992; Weiss et al. 1995). Mice lacking *Gata1* expression die of anemia *in utero* at day E10.5-11.5 and *Gata1-/-* embryonic stem cells are unable to form erythroblasts beyond the proerythroblast stage (Fujiwara et al. 1996; Pevny et al. 1995). Indeed, Gata1 is required for proerythroblasts not to undergo apoptosis, but overexpression hinders terminal differentiation at later stages of erythroblast maturation, best illustrated by anemia in Gata1 overexpressing mice (Whyatt et al. 2000). These studies highlight the intricate regulation of Gata1 gene expression needed for efficient erythropoiesis. Gata1 is known to interact physically with other proteins, such as Fog1 (Tsang et al. 1997), Lmo2 (Osada et al. 1995) and Klf1 (Merika et al. 1995) among others. Mutations in Gata1 disrupting DNA binding or co-factor associations cause anemia and/or thrombocytopenia (Del Vecchio et al. 2005; Phillips et al. 2007). Also, Gata1 mutations causing transcription of a truncated form called Gata1s are associated with the congenital erythroid disorder Diamond-Blackfan anemia (DBA) (Sankaran et al. 2012).

Tal1-Lmo2-Ldb1-E2A complex

The expression of *Tal1* largely mirrors that of *Gata1* since it is expressed in erythroid cells and megakaryocytes. *Tal1-/-* embryos die *in utero* due to inability to form any of the hematopoietic lineages. These embryos also lack expression of Gata1 (Begley et al. 1995). The LIM domain-containing protein Lmo2 is involved during certain types of T-cell leukemia and its loss of function is identical to *Tal1-/-*, consistent with the physical interaction between the two (Osada et al. 1995). Tal1 and Lmo2 forms a pentameric complex with Gata1 and another LIM domain protein Ldb2. Together with the ubiquitous E2A this complex regulates the expression of multiple erythroid genes carrying E-box/GATA motifs (Wadman et al. 1997).

Klf1

Klf1 is a zinc-finger transcription factor that binds the promoter of globins to regulate their expression. Disruption of *Klf1* or its binding sequence of the β-globin promoter, results in thalassemia (Orkin et al. 1982; Nuez et al. 1995; Perkins et al. 1995). Moreover, *Klf1-/-* mice show decreased levels of β-globin and increased levels of the fetal γ-globin, indicating that Klf1 is involved in globin switching from fetal to adult erythropoiesis (Perkins et al. 1996). Klf1 is also known to physically associate with Gata1 and regulate fetal to adult globin switching via Bcl11a (Bauer et al. 2013; Merika et al. 1995). In contrast to the Gata1-containing pentameric complex that regulates many erythroid genes, the erythroid transcription factor Klf1 is involved in the regulation only of a small set of erythroid specific genes, especially α- and β-globins.

Tgfβ

Transformation growth factor β (Tgfβ) is a negative regulator of many hematopoietic lineages and also inhibits early erythropoiesis. Epo-containing semi-solid media cannot promote BFU-E colony formation unless Tgfβ is inhibited (Dybedal et al. 1995; Sing et al. 1988). Further, when BFU-Es are subdivided in early and late fractions, the earliest BFU-Es express the Tgfβ receptor type III in low amounts, later BFU-Es in higher amounts, but CFU-Es lack expression of this receptor, indicating a transient upregulation of Tgfβ receptor III on late BFU-Es during a short time (Gao et al. 2016). Tgfβ administration has an inhibitory effect on colony formation and expansion of the early BFU-Es, however when added at later stages Tgfβ accelerates full terminal erythroid differentiation (Zermati et al. 2000). Inhibition of Tgfβ

responsive activin receptor IIA results in improvement of ineffective erythropoiesis in β-thalassemic mice, elevating Epo levels and reducing iron overload (Dussiot et al. 2014) and an activin receptor IIB inhibitor promoted erythroid differentiation (Suragani et al. 2014).

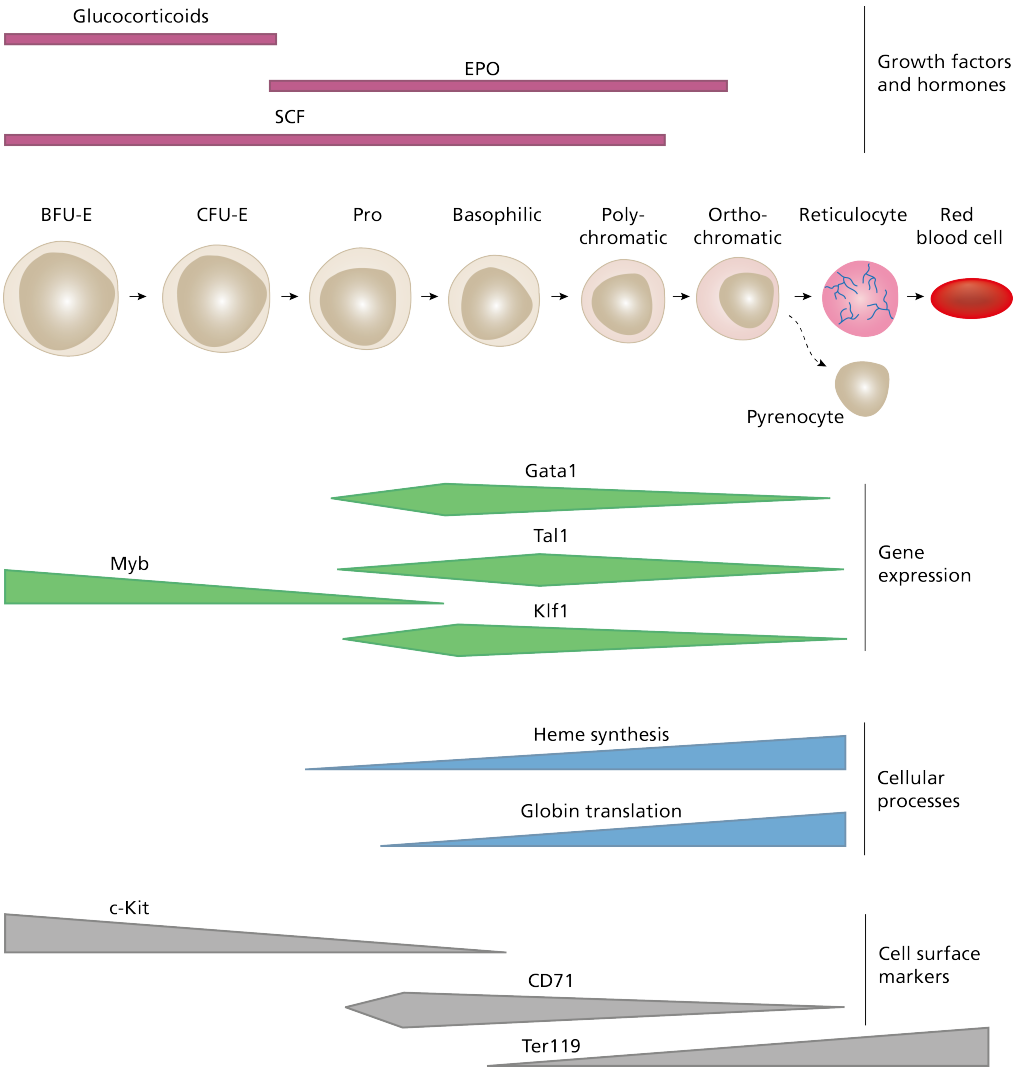


Figure 1. Erythroid differentiation showing genes expressed, growth factors regulating it and the occurrence of important cellular processes. Also, surface markers expressed during maturation utilized for flow cytometric analysis are shown.

HEME AND HEMOGLOBIN IN ERYTHROPOIESIS

Hemoglobin

Hemoglobin is the major component in red blood cells making up about 95% of their dry weight and is responsible for carrying O_2 to tissues and bringing back CO_2 for expiration. The hemoglobin tetramer is made up of two α- and two β-globins carrying one heme molecule each, requiring a 2:2:4 ratio of the three residues to form stable hemoglobin complexes (Schechter 2008; Perutz et al. 1960). The significance of maintaining equimolar concentrations of α- and β-globin is best illustrated by thalassemias, where underproduction of β- and sometimes α-globin disturbs the globin stoichiometry and results in ineffective erythropoiesis and iron overload (Rund et al. 2005). A majority of the body iron is incorporated in heme, since hemoglobin is estimated to contain ~75% of total body iron in a healthy adult (Abbaspour et al. 2014).

Hemoglobin binds O_2 reversibly and cooperatively, where O_2-binding to one heme group increases the affinity of O_2 for remaining heme groups. The affinity between heme and O_2 decreases with declining oxygen tension, ensuring O_2 deposition to tissues with low oxygen pressure and preventing release to already saturated tissues. A conformational change in the secondary structure of hemoglobin between the oxygenated state and deoxygenated state makes this tissue O_2 exchange possible. When O_2 has been released, the heme molecules bind CO_2 and nitric oxide which are then transported back to the lungs for expiration (Schechter 2008).

Heme and iron

Heme is a porphyrin molecule carrying a central iron molecule and it has diverse biological functions of which the most relevant for erythropoiesis is the oxygen carrying property when incorporated into hemoglobin. Erythroid precursors differentiating from the CFU-E to the proerythroblast stage upregulate the transferrin receptor (CD71) and start importing iron (Li et al. 2014; Liu et al. 2013). In humans, iron is obtained from the diet and is taken up by enterocytes in the duodenum. From the enterocytes, iron is transported into circulation by the transporter ferroportin. If iron levels in circulation are elevated, the liver-secreted hormone hepcidin mediates the degradation of ferroportin to prevent iron overload (reviewed in (Coffey et al. 2017)). Another recently discovered regulator of iron homeostasis is erythroferrone, which is synthesized by erythroblasts when stimulated by Epo. Erythroferrone counteracts hepcidin by

releasing iron into circulation and making it available for heme- and haemoglobin synthesis in differentiating erythroblasts (Kautz et al. 2014). Iron regulates translation and stability of mRNAs containing iron response elements on *e.g.* ferritin and CD71. Iron also promotes translation of the erythroid specific rate limiting enzyme in heme metabolism delta-aminolevulinate synthase 2 (Alas2), initiating heme synthesis (Cox et al. 1991). The synthesis of heme is a well-studied process occurring in both the cytoplasm and mitochondria, where porphyrin precursors are modified by eight enzymes to form the heme backbone (Figure 2). The eighth and final step of heme biosynthesis involves iron incorporation catalysed by ferrochelatase. Heme is able to induce gene expression of multiple targets that carry heme response elements and in erythroid cells heme induces protein translation by inhibiting negative regulators of globin translation, Bach1 and heme-regulating eIF2α kinase (HRI) (Han et al. 2001; Tahara et al. 2004). Heme synthesis precedes globin translation and newly formed heme induces globin translation. Differentiating erythroid cells primarily translate α- and β-globins for hemoglobin formation and therefore any changes in protein translation would affect globin levels. Alas1, which is the equivalent enzyme of Alas2 in non-erythroid cells, is subject to negative feedback by heme and heme thereby regulates its own formation. In contrast, erythroid-specific Alas2 is not subject of feedback inhibition by heme, but rather by Gata1 (Cox et al. 1991; Elferink et al. 1988).

Heme is an essential component for life in mammals, but also has a dark side. Due to its lipophilic nature, free heme causes damage to cell membranes and induces production of reactive oxygen species. To avoid heme overload the cell either incorporates heme into hemoproteins or transport heme out of the cell to be scavenged in circulation by hemopexin (reviewed in (Ryter et al. 2000; Chiabrando et al. 2014)). Therefore, it is essential for erythroid cells in particular to keep the levels of α-, β-globin and heme in a ratio that enables hemoglobin formation, avoiding surplus of any of the molecules to avoid cellular toxicity due to heme overload or globin inclusions (Chiabrando et al. 2014). In the short time during erythroid differentiation where heme exceeds globin, erythroid precursors utilize the erythroid specific heme exporter feline leukemia virus subgroup C receptor-related protein 1 (Flvcr1) for heme export out of the cell. This receptor is essential for the progression from CFU-E to proerythroblast and final erythroid differentiation (Quigley et al. 2004; Doty et al. 2015). Mice lacking *Flvcr1* die *in utero* due to impairment in erythropoiesis. Interestingly, *Flvcr1*-/- mice not only recapitulate the erythroid failure seen in DBA, but also display malformations similar to those observed in DBA patients (Keel et al. 2008).

There are additional mechanisms to protect cells from heme toxicity. In circulation, hemopexin acts as a heme scavenger and intracellularly α1-microglobulin (A1M) protects against heme induced cell damage and production of reactive oxygen species by scavenging heme (Olsson et al. 2013). A1M is a humoral house-keeping protein mainly synthesized by the liver (Tejler *et al.* 1978) and present in blood plasma, liver and kidneys (Akerstrom 1983). This endogenous protein is known to have reductase properties for e.g. the hemoproteins cytochrome c in the mitochondria and methemoglobin (Allhorn *et al.* 2005). A1M binds heme with a K_d of 10^{-6} M and is able to both facilitate transport of heme out of the cell and cleave heme (Allhorn et al. 2002; Larsson et al. 2004). Moreover, A1M protects the human erythroid K526 cell line against heme-induced toxicity, implying a heme scavenging function relevant to erythroid cells (Olsson et al. 2008).

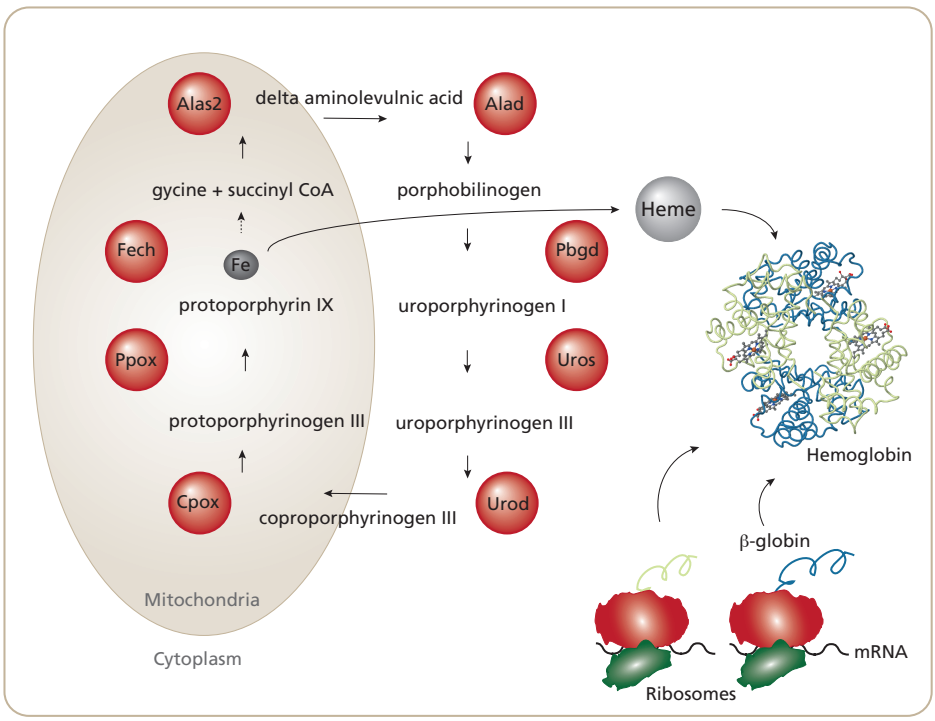


Figure 2. Heme synthesis is a stepwise process involving eight enzymes. Two α- and two β-globins carrying one heme molecule each will form the tetrameric hemoglobin molecule.

RIBOSOME BIOGENESIS

When the genetic code is being translated into protein, messenger RNA (mRNA) is transcribed from DNA and then further used as template to form proteins. This fundamental and energy consuming process is carried out by ribosomes and it is estimated that each cell in mammalians carry around 10 million ribosomes. Ribosomal biogenesis takes places in a specialized part of the nucleus known as the nucleolus where transcription of the 47S pre-RNA by RNA polymerase I and 5S transcribed by RNA polymerase III take place. Newly transcribed pre-RNA is subject to post translational modifications *e.g.* methylation and pseudouridinylation at functionally important regions by small nucleolar RNAs (snoRNA) (reviewed in (Roundtree et al. 2017)). The 47S pre-RNA fragment is further processed into 18S that will later form the small 40S subunit, and into 5.8S, and 28S, that will comprise the large 60S subunit of the ribosome together with 5S (Figure 3). During biogenesis, the ribosomal fragments are transported to the cytoplasm and ribosomal proteins are incorporated into the ribosomal structure in the cytoplasm (Kressler et al. 2017).

Apart from its core rRNA structure, the ribosome is comprised of several ribosome-associated proteins, and many of these are ribosomal proteins located on both the small and large subunit of the ribosome, contributing to optimal ribosomal function. Ribosomal proteins are essential for the function of the ribosome and they are generally rapidly translated and transported into the nucleolus. Interestingly, ribosomal proteins are synthesized in excessive amounts than necessary for ribosome incorporation and surplus ribosomal proteins are constantly degraded by the proteasome, underscoring the necessity for the cell to have a pool of synthesized ribosomal proteins readily accessible for urgent changes in ribosome biogenesis (Lam et al. 2007). The ribosomal protein levels are mainly regulated at the translational level, where mTOR induces their translation via recognition of a terminal oligopyrimidine tract (TOP) sequence at the mRNA (Jefferies et al. 1997).

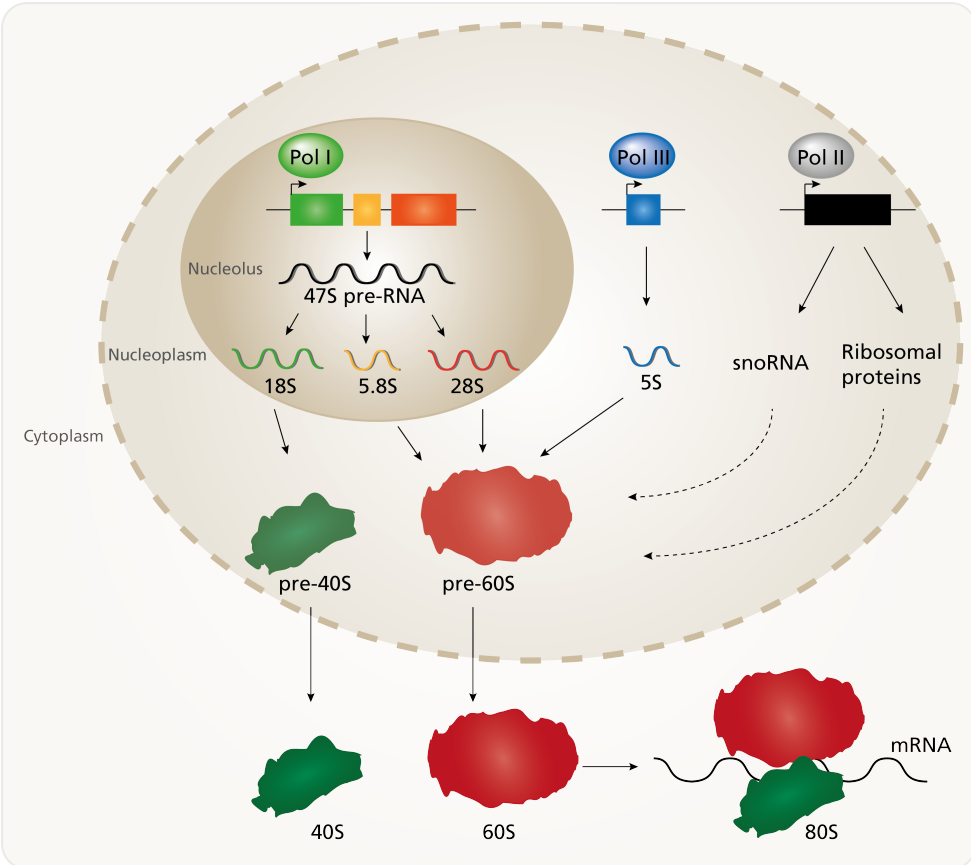


Figure 3. Ribosomal biogenesis in eukaryotes. RNA polymerase I and III transcribe rRNA that is post-transcriptionally modified by snoRNA and further processed into mature 40S and 60S subunits together with ribosomal proteins. During transcription, 40S and 60S form the mature 80S ribosome to translate mRNA into proteins.

Ribosome biogenesis must be tightly regulated and adjusted, primarily to avoid malignant transformation which greatly relies on high ribosomal fidelity to support extensive cell growth. Hence, ribosome biogenesis is tightly coupled to cell proliferation rate. Rapidly proliferative cells, such as early committed erythroid progenitors depend on active ribosome biogenesis, whereas stressed or damaged cells halt their production of ribosomes. The first study linking disrupted ribosomal biogenesis to induction of the tumor suppressor p53 showed that a dominant negative form of the ribosomal assembly factor Bop1 led to p21-mediated cell cycle arrest via p53 due to a disrupted nucleolus (Pestov et al. 2001). p53 activity, as a result of aberrant ribosomal biogenesis, was further linked to nucleolar integrity in a study where the nucleolus was visualized after a variety of cellular stresses, such as UV radiation, 5-fluorouracil

or actinomycin D treatment, concluded that p53 upregulation occurs only at disruption of the nucleolus (Rubbi et al. 2003). Additionally, a p53 response can be triggered by blocking rRNA transcription, interfering with ribosome assembly or by knockdown of many ribosomal proteins (reviewed in (Bursac et al. 2014)). Many of the ribosomal proteins associated with the mature ribosome have extra-ribosomal functions, primarily in surveilling impairments in ribosome biogenesis to prevent malignant transformation. One of the first discoveries of extra-ribosomal functions of ribosomal proteins was the ability of Rpl5 and Rpl11 to stabilize p53 by inhibiting its negative regulator Mdm2 (Dai et al. 2004; Lohrum et al. 2003; Zhang, Wolf, et al. 2003). Since then, multiple ribosomal proteins have been shown to modulate p53 activity via Mdm2 (reviewed in (Zhou et al. 2015)). Activation of p53 at aberrant ribosomal biogenesis is a surveillance mechanism to prevent uncontrolled growth which is the hallmark of malignant transformation. However, as will be discussed in the context of DBA, it can also be deleterious to non-malignant cells with ribosomal protein mutations.

EIF2α AND HRI IN CAP-DEPENDENT PROTEIN TRANSLATION

Translation of DNA into proteins takes place at the ribosome and simplified; this process can be mediated through cap-dependent and cap-independent systems. In cap-dependent translation, eukaryotic translation initiation factors associate with 40S ribosomal subunit at the cap of the 5´UTR of mRNAs, scans for a translational start codon and recruits the 60S ribosomal subunit for protein translation. The best studied form of cap-independent translation is internal ribosomal entry sites (IRES)-mediated translation where ribosomes are bypassing the initial start codon scanning when recruited to the mRNA. This and other alternative forms of cap-independent translation are believed to be important in stress situations where cap-dependent translation is inhibited (reviewed in (James et al. 2018)). Once ribosomes are recruited to the mRNA, tRNA carrying amino acids are paired to the RNA strand creating amino acid structure that is later folded to become a functional protein.

Protein translation is a vast field, and this section focuses on cap-dependent translation by eIF2α and how that is regulated by heme availability through HRI. Simplified, GTP-bound eIF2α, β and γ forms a complex with eIF1, eIF3, eIF4 and 40S and initiates translation at the 5'UTR of mRNAs. For each round of initiation, GTP is dephosphorylated to GDP and in order for translation to proceed, GTP must be regenerated by eIF2B. However, phosphorylation at serine51 of eIF2α locks it in a GDP-bound inactive state and prevents another round of translational initiation, thereby putting protein translation to halt (Figure 4).

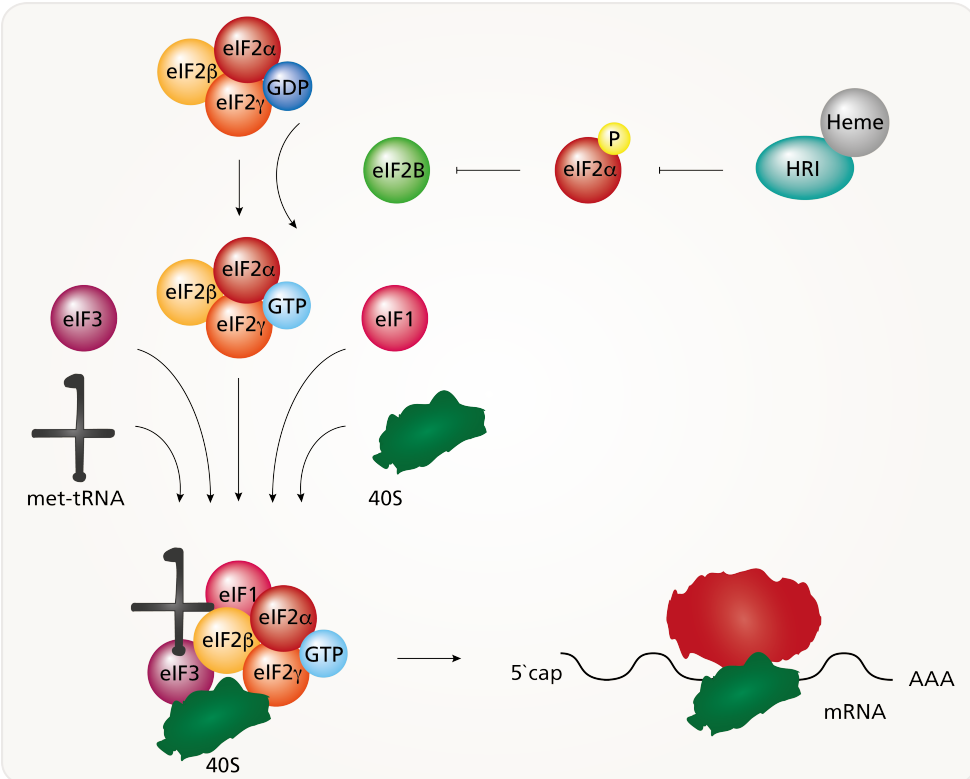


Figure 4. Cap-dependent translation. The initiation complex requires GTP initiate translation with tRNA and the 40S ribosomal. HRI regulates eIF2α by phosphorylation and inhibits protein translation by preventing GDP recycling into GTP by eIF2B at low heme levels.

eIF2α activity can be regulated by four different stress sensing kinases; PKR, PERK, mGCN2, and HRI that phosphorylate eIF2α at detection of double stranded RNA, ER stress, amino acid deprivation and heme, respectively. HRI is considered an erythroid specific protein expressed in differentiating erythroblasts and reticulocytes (Pal et al. 1991). HRI is a kinase, able to bind heme primarily at the N-terminus but also to some extent at the kinase insertion region. At low heme levels, HRI is subject to autophosphorylation which leads to its activation and subsequent eIF2α phosphorylation (Chefalo et al. 1998; Fagard et al. 1981; Gross et al. 1978). Additionally, HRI binds ATP which facilitates both autophosphorylation and phosphorylation of eIF2α (Rafie-Kolpin et al. 2000). Once heme is bound to HRI it inhibits ATP binding and thereby prevents phosphorylation of eIF2α (Chen et al. 1991).. Through this mechanism HRI protects erythroid cells from accumulation of excess globins causing precipitation and proteotoxicity, since HRI knock down models show increased protein (globin) translation (Han et al. 2001; Han et al. 2005; Crosby et al. 2000).

The *HRI-/-* mouse has normal steady state erythropoiesis but exhibits marked anemia upon iron deficiency (Han et al. 2001). Maintained HRI expression is necessary for adaptive gene expression during erythroid differentiation in iron deficiency, indicating a protective role for HRI at insufficient heme levels, rather than a steady state function (Liu et al. 2008). Reduced *HRI* levels have also been shown to exacerbate the symptoms of the erythroid disorders β-thalassemia and erythroid protoporphyria, further strengthening its role in aberrant erythropoiesis (Han et al. 2005). Additionally, HRI is able to induce transcriptional arrest and phosphorylate eIF2α by oxidative stress, a process independent of heme (Lu et al. 2001). Apart from inhibiting protein translation, HRI activation is able to not only reduce protein translation by phosphorylation of eIF2α, but also activate translation of certain proteins *e.g.* ATF4, illustrating that phosphorylation of eIF2α elicits a wider range of functions at cellular stress (Suragani et al. 2012; Paolini et al. 2018).

DIAMOND-BLACKFAN ANEMIA

In 1938, the American pediatricians Louis Diamond and Kenneth Blackfan first described a childhood hypoplastic anemia that later would carry both of their names (Diamond et al. 1938). The first gene found mutated in this disease was a ribosomal protein of the small ribosomal subunit *RPS19*, discovered 60 years later by a Swedish research group (Draptchinskaia et al. 1999). Since this discovery, 19 additional ribosomal proteins have been found mutated in this disorder. Researchers have long been puzzled and intrigued by how aberrant ribosomal formation mainly leads to erythropoietic failure, rather than having widespread physiological consequences. Research on this rare disease has unraveled great complexity of both underlying genetics, disease mechanisms and clinical symptoms. Despite intensive research, a lot is still to learn about the mechanisms underlying Diamond-Blackfan anemia.

PATHOLOGY

Clinical manifestations

DBA is a congenital disease diagnosed early in life with 90% of all patients diagnosed before their first birthday (Vlachos et al. 2010). The diagnostic criteria proposed by Diamond *et al* have been largely unchanged and include anemia (most often presenting prior to the first birthday) with reticulocytopenia and near normal neutrophil and platelet counts, macrocytosis (increased red cell volume) and a normocellular bone marrow where erythroid precursors are scarce or absent (Diamond et al. 1976). Patients meeting these criteria are considered to have "classical" DBA, but since DBA is a disease of great heterogeneity, many patients also display "non-classical" DBA with varying severity of the symptoms (Vlachos et al. 2008). Around 80-85% of all DBA patients show elevated levels of erythrocyte adenosine deaminase (eADA) activity, as well as increased levels of fetal hemoglobin and Epo (Glader et al. 1983; Vlachos et al. 2008; Da Costa et al. 2018). While the underlying cause for elevated eADA and HbF remain elusive, the increased levels of Epo is likely

a physiological response to compensate for inefficient erythropoiesis. Intriguingly, patients with DBA have a 20% chance of entering spontaneous remission from the hematological symptoms in their lifespan, and the reason behind this is still unclear (Vlachos et al. 2008).

DBA is primarily a hematological disease, however a more complex array of symptoms associated with DBA have been described. Apart from hematological manifestations, approximately just under half of all patients suffer from malformations in other organ systems, most commonly craniofacial and thumb anomalies, but also urogenital and cardiac malformations (Arbiv et al. 2018; Ball et al. 1996; Willig, Draptchinskaia, et al. 1999; Lipton et al. 2006; Willig, Niemeyer, et al. 1999). Low birth weight is reported in ~25% of all cases. DBA is a heterogenous disease with varying penetrance, resulting in a great variance in the severity of the symptoms. Some patients even display malformations but have normal or very mild hematological parameters and these patients are considered to have "non-classical" DBA.

Patients suffering from DBA have a ~5-fold increased risk for malignant transformation throughout life, with a median age of 15 years at onset, compared to 68 years for the general population. The most common malignant transformations include acute myeloid leukemia, colon carcinoma, osteosarcoma and genitourinary cancers (Vlachos et al. 2012; Vlachos et al. 2008; Lipton et al. 2001; Alter et al. 2018). It can seem rather contradictory that a hypoproliferative disease like DBA bears an increased risk of a hyperproliferative malignant transformation, however this feature is shared with many other syndromes affecting ribosome biogenesis. The reason behind this predisposition is not fully known, however it is known that many types of cancer cells display haploinsufficiency for ribosomal proteins (Ajore et al. 2017). A hypothesis has been put forward suggesting that hematopoietic clones with increased proliferation capacity will have a selective advantage over surrounding cells that are pro-apoptotic or poorly cycling. Hence, any clones escaping the proliferative defect would easily take over the system and give rise to malignancy (De Keersmaecker et al. 2015).

Inheritance and genetics

The incidence of DBA is estimated to be 5-7 cases per million births, with a higher incidence in the Northern Europe, with no ethnic or gender bias (Ball *et al.* 1996; Willig, Niemeyer*, et al.* 1999; Willig *et al.* 1998). A ribosomal protein of the small subunit RPS19, was the first mutation identified in DBA patients (Draptchinskaia et al. 1999) and since then 20 ribosomal protein genes (*RPS7, RPS10, RPS15, RPS17, RPS19, RPS24, RPS26, RPS27, RPS27a, RPS28, RPS29,*

RPL5, RPL9, RPL11, RPL15, RPL18, RPL26, RPL27, RPL31, and RPL35a) have been found mutated in this disease, and collectively explain the genetic background of approximately ~70-75% of all patients (Draptchinskaia et al. 1999; Mirabello et al. 2017; Wlodarski et al. 2018; Wang et al. 2015; Landowski et al. 2013; Gazda et al. 2012; Doherty et al. 2010; Farrar et al. 2008; Cmejla et al. 2009; Cmejla et al. 2007; Mirabello et al. 2014; Gazda et al. 2006; Gripp et al. 2014; Gazda et al. 2008; Farrar et al. 2014). *RPS19* still remains the most commonly mutated gene in DBA and accounts for ~25% of all cases. Mutations in ribosomal proteins lead to perturbation of ribosomal biogenesis of either the 40S or 60S subunit and disrupts the normal stoichiometry of ribosomal subunits, leading to ribosomal stress (Choesmel et al. 2007). Experimentally, deficient ribosome biogenesis was confirmed as an underlying cause of ribosomal protein mutations in DBA and insertion of corrected *Rps19* confirmed that the symptoms were indeed due to insufficiency of ribosomal proteins (Hamaguchi et al. 2003; Flygare et al. 2005; Flygare et al. 2007; Flygare et al. 2008; Jaako et al. 2014; Debnath et al. 2017). Further, all patients have one affected allele, indicating that haploinsufficiency of ribosomal proteins is sufficient to develop DBA and that complete loss is most likely not compatible with life.

DBA is a heterogenous disease both with regard to the differences in clinical manifestations as well as the array of ribosomal protein genes mutated. Studies have investigated genotype-phenotype correlations and found that certain physical malformations are more common than others in for different gene mutations (Arbiv et al. 2018; Boria et al. 2010). For instance, patients carrying RPL5 mutations very often display craniofacial malformations, whereas this is absent in patients with mutations in *RPS19*, indicating that *RPL5* plays a role in the development of cell systems distinct from the hematopoietic lineages. Around half of all DBA cases are autosomal dominantly inherited, however there are examples of families with affected children to unaffected parents and different severities between anemia and response to treatment, even within families have been observed (Orfali et al. 2004).

Surprisingly, exome sequencing has identified that a subset of DBA patients carry non-ribosomal protein mutations in *GATA1*, *TSR2*, and *Epo* (Ludwig et al. 2014; Sankaran et al. 2012; Klar et al. 2014; Parrella et al. 2014). In a few DBA cases, mutations in *GATA1* was found to alter splicing, which led to favouring of the short splice form GATA1s, containing exons 1 and 3, but lacking exon 2 (Sankaran et al. 2012). Interestingly, mutations in a *GATA1* zinc-finger region results in thrombocytopenia whereas mutations favouring splicing of the truncated form GATA1s causes anemia (Nichols et al. 2000; Hollanda et al. 2006). Therefore, it has been speculated that affecting different regions and thereby functions of GATA1 gives rise to different clinical symptoms (Sankaran

et al. 2012). TSR2 is not a well-studied protein, however haploinsufficiency leads to aberrant ribosome formation and reduced levels of both multiple ribosomal proteins and GATA1 expression, possibly linking these two mutations in DBA (Khajuria et al. 2018). TSR2 is predicted to be a binding partner of RPS26, which is mutated in some DBA cases, however this remains to be experimentally confirmed (Gripp et al. 2014).

p53 activation

Increased p53 activity causes cell cycle arrest and apoptosis in both model systems and patients with DBA, while inactivation of p53 often ameliorates and reverses the symptoms (Dutt et al. 2011; Taylor et al. 2012; Jaako et al. 2015; Sjogren et al. 2015; Danilova et al. 2008). Studies in zebrafish have found p53-independent hematological defects in DBA, further underscoring the complexity of the disease (Jia et al. 2013; Torihara et al. 2011; Singh et al. 2014). However, studies in other diseases with affected ribosome function, so called ribosomopathies, also identify increased p53 activity to contribute to the pathophysiology (Barlow et al. 2010; Jones et al. 2008). p53 is also known to be upregulated in response to impaired ribosome biogenesis in general, reviewed in (Bursac et al. 2014).

p53 is an intensively studied protein that protects cells against stresses, primarily DNA damage, hypoxia, ribonucleotide depletion and oncogenic stress. In order to allow tissue repair from such insults, p53 promote cell cycle arrest and should the insults persist beyond repair, apoptosis is induced (Opferman et al. 2006). The ability of p53 to induce cell cycle arrest and apoptosis is highly dependent on its ability to activate gene expression of *e.g.* the cell cycle arrest modulators p21 and Ccng and the pro-apoptotic Bax (Fischer 2017). Since p53 is subject to extensive post translational modifications, its activity is often measured by its downstream targets. p53 is negatively regulated by Mdm2 (HDM2 in humans), and the link between p53 activity and DBA pathology is further strengthened by the fact that ribosomal proteins mutated in DBA RPL5, RPL11, RPS7 and RPS26 regulate p53 via inhibition of HDM2 (Ofir-Rosenfeld et al. 2008; Bursac et al. 2012; Chen et al. 2007). RPS26 has also shown to bind and initiate translation of p53 mRNA and stimulate its translation (Takagi et al. 2005). Apart from this, multiple ribosomal proteins that have yet not been associated with DBA also regulate p53 activation via Mdm2 inhibition, strongly suggesting that defective ribosome assembly is a serious cellular insult capable of activating p53 and promoting cell cycle arrest and apoptosis.

Molecular mechanisms behind DBA

Over the past decades, studies elucidating disease mechanisms behind DBA has increased the understanding of the pathophysiology tremendously. It is now well established that erythroid cells from DBA patients and DBA models show reduced proliferation, most profoundly in the earlier committed erythroid precursors, as well as altered cell cycle kinetics and increased apoptosis (O'Brien et al. 2017; Perdahl et al. 1994; Lipton et al. 1986; Tsai, Arkin, et al. 1989; Ohene-Abuakwa et al. 2005; Miyake et al. 2008). One study identified reduced expression of the chaperone Hsp70 patients with mutations in *RPL5* and *RPL11*, but not *RPS19*. This study also observed a decrease in Gata1 expression and activation of p53, that could be reversed by restored *Hsp70* expression, indicating that Hsp70 could be an important disease modifying gene in some cases of DBA (Gastou et al. 2017). Another study identified elevated heme levels in a DBA patient along with reduced α-globin mRNA, prompting further investigation to validate a disrupted balance between heme and globin in erythroid cells (Yang et al. 2016).

Ribosome biogenesis and protein translation in DBA

Efficient and correct ribosome biogenesis is crucial to any protein producing cell and deviations in this machinery increase susceptibility for malignant transformation. Indeed, both loss-of-function mutations in mediators associated with proper ribosome assembly, as well as overexpression of many of these components increase the risk of malignant transformation, highlighting the importance of keeping ribosome biogenesis tightly regulated. p53 is highly involved in surveilling ribosome biogenesis and is able to repress transcription by RNA polymerase I and III and thereby reducing levels of rRNA (Zhai et al. 2000; Cairns et al. 1998; Chesnokov et al. 1996). Since mutations in ribosomal proteins cause aberrant ribosome biogenesis in DBA, protein translation would likely be affected when ribosome function is compromised. Many studies confirm aberrant polysome profiles in models of DBA, indicating distorted mRNA translation, and also observe a decrease in general protein synthesis, especially IRES-mediated translation (Jaako et al. 2011; Garcon et al. 2013; Choesmel et al. 2007; Cmejlova et al. 2006; Zhang et al. 2014; Horos et al. 2012). More interestingly, certain mRNA transcripts have been found less translated in DBA patients, *e.g.* the Hsp70 co-chaperone BAG1 and the RNA-binding protein CSDE1, as well as the key erythroid transcription factor GATA1 (Horos et al. 2012; Ludwig et al. 2014; Khajuria et al. 2018). One study also found the enzyme BCAT1 to be decreased in a lymphoid cell line derived from DBA patients (Pereboom et al. 2014). Interestingly, this enzyme is involved in

the biogenesis and degradation of branched amino acids, such as leucine. Leucine and other branched amino acids are known activators of the mTOR pathway, promoting protein translation. Studies in DBA models of both mice and fish have shown that L-leucine treatment increases erythropoiesis, however it is unclear if the treatment affects the mTOR and/or p53 pathways (Jaako et al. 2012; Narla et al. 2014; Payne et al. 2012). L-leucine is currently in clinical trial to be investigated as a potential treatment for DBA and preliminary data shows that oral administration of L-Leucine it is safe and that it improves growth velocity in children with DBA (Vlachos et al. 2018).

Mutations in ribosomal proteins is known to cause aberrant ribosome biogenesis in DBA, resulting in accumulation of pre-RNA particles and decreased amounts of mature 40S or 60S ribosomal subunits, depending on which subunit the haploinsufficient ribosomal protein is associated with (Choesmel et al. 2007). This in turn is believed to cause fewer ribosomes correctly assembled, and as a consequence of this some mRNAs are insufficiently translated. One recent study proposes that *GATA1* mRNA is highly affected by the reduced numbers of functional ribosomes from human cellular models of DBA. The 5´UTR structure and total mRNA length of GATA1 mRNA deviates from many other hematopoietic transcription factors, making it more sensitive to low levels of functional ribosomes (Khajuria et al. 2018). This finding contributes to the understanding of the erythroid specificity behind DBA. In line with theories reviewed previously (Mills et al. 2017), the authors argue that the major factor affecting erythropoiesis are reduced numbers of functional ribosomes causing reduced Gata1 expression, rather than ribosome heterogeneity.

Disease models

Many animal models of DBA pathology have been generated over the years (reviewed in (McGowan et al. 2011)). Although the phenotype in mice do not fully recapitulate the disease phenotype in humans, these models have served as great tools of gaining mechanistic insights into DBA pathology, considering the low availability of patient samples. However, generating mouse models for DBA has not been without challenges. In one mouse model of *Rps19* deficient DBA, the mice were able to compensate for heterozygosity, but were embryonic lethal when *Rps19* was removed from both alleles (Matsson et al. 2004; Matsson et al. 2006). Other models of *Rps19* and *Rps20* deficiency have poorly recapitulated hematological pathology, but resulted in hyperpigmented skin due to increased p53 which stabilizes SCF expression in melanocytes of the epidermis (McGowan et al. 2008).

Rps19 deficiency in mice has also been generated by a dominant negative form of Rps19 and these mice display growth retardation, decreased numbers of early erythroid precursors, anemia and reticulocytopenia (Devlin et al. 2010). Jaako *et al* used the approach of an inducible shRNA to reduce *Rps19* levels. These mice show scarcity of erythroid precursors, anemia, distorted polysome profiling, increased apoptosis, and decreased bone marrow cellularity (Jaako et al. 2011). This mouse model has been utilized throughout the work of this thesis, and is depicted in Figure 5. In addition, many zebrafish models have been generated by silencing ribosomal protein expression using morpholinos (Zhang et al. 2014; Danilova et al. 2008; Ear et al. 2015; Chakraborty et al. 2009).

Cellular models of DBA have also been established, and shRNA mediated knock down has shown to be a particularly useful approach, pioneered by the lab of Stefan Karlsson. These studies ultimately confirm that the erythroid defect seen in DBA is indeed due to ribosomal haploinsufficiency (Flygare et al. 2005; Miyake et al. 2005; Miyake et al. 2008; Ebert et al. 2005; Flygare et al. 2007). The lab of Mitchell Weiss generated induced pluripotent stem cells from DBA patients (Garcon et al. 2013; Ge et al. 2015) and fibroblasts from DBA patients have been reprogrammed into hematopoietic progenitors. This technique further increased expansion of the otherwise rare erythroid progenitors from DBA patients (Doulatov et al. 2017).

The inducible *Rps19* deficient mouse model used throughout the studies presented in this thesis have reduced *Rps19* expression due to RNA interference with short hairpin RNA (shRNA) causing degradation of *Rps19* mRNA (Figure 6). This system is taking advantage of a conserved gene silencing response in eukaryotes where double stranded RNA mediates mRNA degradation. This was first discovered in 1998 and later awarded the Nobel Prize in 2006 (Fire et al. 1998). In the context of the inducible *Rps19* deficient mouse, a microRNA is processed into a 50-70 nucleotide structure short hairpin RNA (shRNA), in which two strands showing near full complementarity are held together by a loop structure (Meister et al. 2004). Then, one of the Dicer enzymes cleave the double stranded shRNA into two smaller RNA strands of about ~20 nucleotides (Grishok et al. 2001; Hutvagner et al. 2001). One of these RNA strands is degraded and the other incorporated into the nuclease RISC complex. The RISC complex then uses this RNA strand as a template to find single stranded RNA, such as mRNA, to hybridize with (Martinez et al. 2002; Martinez et al. 2004). If complementarity is high the mRNA is degraded and no protein is translated, whereas lower complementarity leads to translational repression. Hence, the endogenous machinery enabling RNAi is altering gene expression without interfering with the genetic structure of the target gene (Figure 5).

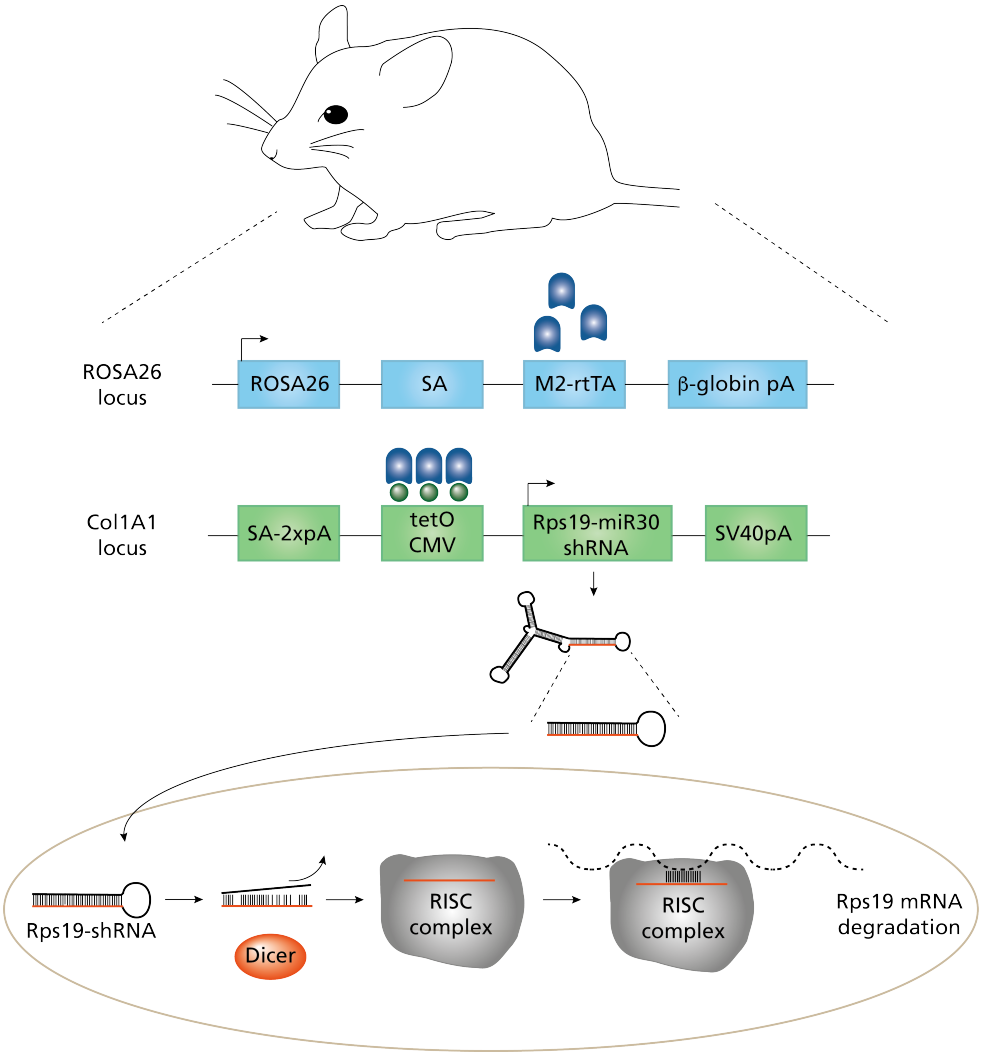


Figure 5. A Doxycycline inducible *Rps19* deficient mouse model where a constitutively active trans activator together with Doxycycline enables transcription of a microRNA. This RNA is further processed into a shRNA, cleaved by Dicer and degrades *Rps19* mRNA together with the RISC complex to induce reduced levels of Rps19.

Other ribosomopathies and bone marrow failure disorders

DBA is at the same time both considered a ribosomopathy and a bone marrow failure disorder. Diseases affecting ribosomal biogenesis in general are considered ribosomopathies, whereas bone marrow failure syndromes are disorders where the bone marrow fails to sustain the body's need for cells from the bone marrow. Strikingly, most ribosomopathies at the same time display hematological abnormalities, such as the del(5q) myelodysplastic syndrome, Schwachman-Diamond syndrome, and dyskeratosis congenita which are also characterized as bone marrow failure disorders (Ruggero et al. 2014). Other ribosomopathies, such as cartilage hair hypoplasia, is primarily diagnosed by short stature, but patients also display immunodeficiency to a varying degree (Liu et al. 2006). The only ribosomopathy not displaying hematological symptoms is Treacher-Collins syndrome, where patients are born with facial malformations improved only by recurrent reconstructive surgery. Most patients carry mutations in the gene TCOF1 coding for the protein Treacle, which is involved in initiating RNA polymerase I transcription (Valdez et al. 2004). Treacle is essential for migration and survival of neural crest cells, giving rise to facial structure during development. These cells show increased p53 activity in Treacher-Collins syndrome, and the symptoms can be completely rescued when p53 is knocked out during embryogenesis (Jones et al. 2008). Treacher Collins syndrome share common ground with DBA when it comes to increased p53 activity and the presence of craniofacial malformations, but it is not known why patients with Treacher-Collins syndrome do not show hematological abnormalities.

Bone marrow failure occurs when the bone marrow fails to produce sufficient numbers of one or many different blood lineages to meet the need of the body. This is seen in inherited diseases such as Fanconi anemia (Garaycoechea et al. 2014), DBA, Schwachman-Diamond syndrome, and dyskeratosis congenita, but also in acquired diseases such as acquired aplastic anemia. Common for all these conditions is a predisposition to malignant transformation and occurrence of somatic malformations (Dokal et al. 2010).

TREATMENT OF DBA

Current clinical treatments

There are currently three mainstay therapies for DBA; glucocorticoid treatment, blood transfusion accompanied with iron chelation, and allogenic bone marrow transplantation. Since the middle of the last century, DBA has been treated with glucocorticoids, first described in 1951 by Gasser (Gasser 1951). Initially, around 80% of all patients respond to the treatment, but many become resistant or do not tolerate the side effects and over time only around 40% of all patients remain on glucocorticoid treatment. To date there is no genotype-phenotype correlation that can predict the response to glucocorticoid treatments. Although effective in promoting erythroid expansion, glucocorticoids come with many and sometimes severe side effects such as pathologic fractures, cataracts, diabetes mellitus, hypertension and avascular necrosis, all of which have been observed at high rates in DBA patients (Vlachos et al. 2008; Lipton et al. 2006). Patients also need prophylactic pneumonia treatment, since mortality is increased due to the immunosuppressant effects of glucocorticoid treatment (Vlachos et al. 2010). Moreover, glucocorticoid treatment impact growth and physical as well as neurological development of infants, and therefore other treatment options are preferred for younger children (Vlachos et al. 2010). Recently, an update from a DBA registry of 90 patients in China showed that a combination therapy of glucocorticoids and the immune suppressant cyclosporine A was used as a second line option successful for some patients not responding to glucocorticoids alone (Wan et al. 2016). Experimental studies have also found cyclosporine A treatment beneficial for some patients (Leonard et al. 1989; El-Beshlawy et al. 2002).

The other mainstay treatment for DBA is blood transfusions, usually given every 3-5 weeks. The major issue receiving repeated blood transfusions is iron accumulation in internal organs, resulting in cardiac hemosiderosis, hepatic cirrhosis, diabetes mellitus, hypothyroidism and hyperparathyroidism (Vlachos et al. 2010). Apart from allogenic bone marrow transplantation, iron overload is the most common cause of death in DBA patients and patients receiving this treatment have a reduced life expectancy. To minimize iron overload of internal organs, blood transfusion therapy is always accompanied by chelation therapy (Vlachos et al. 2008).

The only curative treatment for DBA is allogenic bone marrow transplantation where hematopoietic stem cells from a matched donor are replacing the

patient's bone marrow after irradiation. Although sibling matched donor bone marrow transplantation before 9 years of age has a survival rate of 90%, it is still a procedure with substantial risk for the patient and require life-long immunosuppressive therapy (Vlachos et al. 2010).

Experimental and future treatments

Over the years many attempts have been made to find alternative treatment regimens for DBA, in the hope of finding a therapy with less side effects than the current ones (reviewed in (Sjogren *et al.* 2012). Most of the treatments have only been beneficial for occasional patients, such as treatment with the iron chelator Deferasirox, recombinant Epo, interleukin-3, intravenous IgG, anti-nausea drug Metoclopramide and the anticonvulsant valproic acid (Abkowitz et al. 2002; Ball et al. 1995; Bastion et al. 1994; Fiorillo et al. 1991; Ganser et al. 1990; Gillio et al. 1993; Jabr et al. 2004; Leblanc et al. 2007; Niemeyer et al. 1991; Sopo et al. 1990; Sumimoto et al. 1992; Taher et al. 2009). Lenalidomide, an FDA approved drug used to treat the anemia of del(5q) myelodysplastic syndrome where one allele of *RPS14* is missing, has shown to increase the CFU-E population in *Rps19* deficient cells (Narla *et al.* 2011; Stahl *et al.* 2017).

Currently the dietary supplement L-leucine and the activin trap Sotatercept are in clinical trials to treat DBA. Sotatercept is a molecule containing the extracellular part of the activin receptor a bound to an IgG fc fragment, preventing its lysosomal degradation. In short, Sotatercept binds activin and prevents it from activating endogenous activin receptor signaling. Sotatercept treatment increases hemoglobin levels as well as reverses anemia and clinical trials are ongoing for myelodysplastic syndrome and β-thalassemia, where it seems to be well tolerated (Komrokji et al. 2018; Cappellini et al. 2013; Cappellini et al. 2015; Porter et al. 2014). Anemia was improved in a zebrafish model of DBA treated with Sotatercept (Ear et al. 2015). L-leucine is an essential amino acid promoting protein translation via mTOR (Stipanuk 2007). Improvement of anemia by L-leucin administration was observed in animal studies and has been seen in DBA patients (Payne et al. 2012; Jaako et al. 2012; Narla et al. 2014; Pospisilova et al. 2007).

Although most studies elucidating new treatment strategies for DBA have been pharmacological, studies by Stefan Karlsson's lab have paved the way for a gene therapy based approach. Cellular models of *Rps19* deficient DBA have shown reversal of DBA symptoms upon correction of *Rps19* delivered by both lentiviral and retroviral vectors (Hamaguchi et al. 2003; Flygare et al. 2008). Further, an inducible *Rps19* deficient mouse model has been successfully treated with lentiviral vectors containing *Rps19* and follow up studies improving the safety of this delivery system has been made (Debnath et al. 2017; Jaako et al. 2014).

AIMS AND RESULTS OF THE STUDIES

PAPER I

Glucocorticoids improve erythroid progenitor maintenance and dampen Trp53 response in a mouse model of Diamond–Blackfan anaemia

Glucocorticoids have been used to treat DBA for decades and although GR agonists are well known to stimulate erythroid precursor self-renewal, proliferation and delay differentiation, less is known about the disease-specific mechanisms behind glucocorticoid treatment in DBA. This study identified that glucocorticoids significantly decrease the elevated p53 activity observed in *Rps19* deficient erythroid cells, allowing them to survive and be maintained in a more immature erythroid state, which facilitates proliferation and thereby increases erythroid output.

PAPER II

Heme-regulated eIF2α kinase can be targeted to normalize intracellular heme toxicity in Diamond-Blackfan anemia

This study observed elevated levels of unbound intracellular heme in purified *Rps19* deficient erythroblasts, possibly contributing to the disease phenotype. Knock down of the heme-regulated eIF2α kinase HRI, which regulates the balance between heme and globin translation for optimal hemoglobin formation, increases proliferation of erythroid precursors and lowers both the elevated heme levels and p53 activity.

PAPER III

Human α1-microglobulin reduces excess intracellular heme and improves proliferation in Diamond-Blackfan anemia

The endogenous molecule A1M is identified to be the only molecule increasing proliferation in *Rps19* deficient erythroid precursors in a small screen of compounds affecting iron- or heme availability. The proliferative effect of A1M treatment is significant in both *Rps19* deficient mice and in cells from DBA patients. A1M treatment reduces elevated levels of unbound intracellular heme in *Rps19* deficient erythroblasts, without affecting either heme synthesis or the p53 pathway.

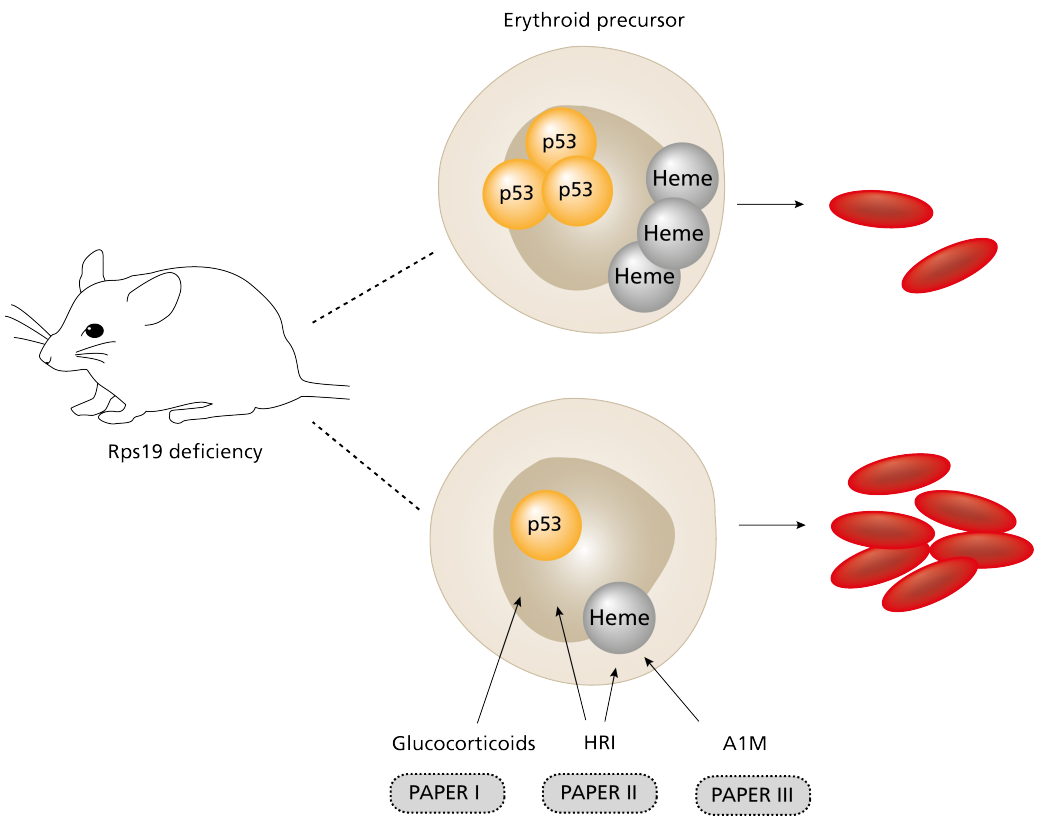


Figure 6. Summary of strategies presented in this thesis to improve the anemia of a *Rps19* deficient DBA mouse model.

TOWARDS DISEASE SPECIFIC TREATMENTS FOR DIAMOND-BLACKFAN ANEMIA

TISSUE SPECIFICITY OF DBA – A KEY TO NEW TREATMENT?

The cardinal question of why a ribosomal protein haploinsufficiency causes an erythroid specific disease has for long been puzzling and engaging researchers and clinicians studying DBA. Despite the heterogeneity in disease phenotype, the common feature of DBA patients is erythroid failure. The main reason for dissecting the mechanisms behind the erythroid specificity - apart from intense curiosity - is that increased and detailed understanding of the molecular mechanisms behind DBA is necessary for the development of new treatments. Why does haploinsufficiency of certain ribosomal proteins specifically cause anemia? Is the anemia a response to defective ribosome biogenesis or a consequence of defective translation of certain transcripts essential for erythropoiesis? When discussing the erythroid specificity behind ribosomal protein haploinsufficiency in DBA, two main hypotheses can be used to explain the erythroid specificity of the disease. The first hypothesis is that the anemia is primarily caused by increased activity of negative regulators of cell growth in general and/or erythropoiesis in particular, such as p53. The second hypothesis explains the erythroid failure as a consequence of decreased activity of key factors positively stimulating proliferation and/or survival during erythropoiesis, such as Gata1. Any of these mindsets could bear the answer, or likely a combination of the two. Altered protein translation has been observed in DBA and would be included both these hypotheses, as it both can be affected by increased stress signals as well as by lack of positive signals stimulating proliferation and protein translation.

Defective ribosome biogenesis impairs erythropoiesis

Defective ribosome assembly is known to induce surveillance mechanisms and elevated p53 activity through nucleolar stress. Whereas it has been shown that disrupted nucleolus is a prerequisite for a p53-mediated nucleolar stress response in irradiated cells, p53 activity is increased by ribosomal stress even if the nucleolus is intact (Rubbi et al. 2003; Fumagalli et al. 2009). In the ribosomopathy Treacher-Collins syndrome, neural crest cells giving rise to craniofacial structures during development show increased p53 activity and apoptosis, which leads to craniofacial malformations (Dixon et al. 2006). The disease phenotype of a mouse model of this syndrome is completely reversed in a *p53*-/-background, showing that elevated p53 activity is indeed responsible for the disease phenotype (Jones et al. 2008). Although less dramatic, reduced p53 activity reversed erythroid symptoms in a mouse model of DBA, strongly suggesting that p53 activity is closely linked also to diseases of defective ribosome biogenesis (Jaako et al. 2015; Jones et al. 2008). When this work started, p53 was suspected, however not yet fully recognized, to be an important player in DBA pathology which later was confirmed by multiple studies. For this reason, multiple p53-responsive genes were included in the custom made screen we carried out to identify disease-modifying genes in DBA (Paper II).

If aberrant ribosome biogenesis leads to p53 activation, why are not all cell types equally affected? The cell type specific effects of p53 has mostly been studied in the context of irradiation-induced p53 activation. There seem to be differences in both p53 sensitivity and base line p53 activity between different tissues (Fei et al. 2002; Komarova et al. 1997). As an example, liver cells in these studies showed very little increase in p53 activity, whereas intestine and thymus were more prone to upregulate p53 after irradiation. Why are certain cell types in greater need for protection by p53? Also, p53 is known to induce both apoptosis and cell cycle arrest, depending on cell type and severity of insult (reviewed in (Gudkov et al. 2003). p53 sensitivity has been suggested to be linked to proliferative potential, and to the cell's need of replacement. Neural cells that have exited the cell cycle and are not easily replaced seem less prone to p53-induced apoptosis, but rapidly cycling hematopoietic cells that are replaced frequently show an increased sensitivity to elevated p53 activity (reviewed in (Gudkov et al. 2003)).

If the hypothesis of increased sensitivity to p53 in rapidly cycling erythroid precursors holds true, it still remains to be answered what mechanisms cause the increased sensitivity. As mentioned previously, early Epo-dependent erythroid precursors require EpoR signaling to circumvent pro-apoptotic signaling, an arrangement allowing erythropoiesis to rapidly adapt to excessive

blood loss (Koulnis et al. 2014). Hence, without stimulation of the EpoR, early erythroid precursors are already in a pro-apoptotic state, which could explain why additional apoptotic stimuli by p53 causes an increased and cell type specific sensitivity to apoptosis. One could also speculate if there are cell type specific signaling pathways that activate p53 in erythroid cells, or that converge with other pathways to amplify p53 activity under cellular stress. Still, it remains to be answered why other highly proliferating cell types, such as the gut epithelium that is sensitive to p53 activation, remain unaffected in patients with ribosome protein haploinsufficiency. Interestingly, the inducible *Rps19* deficient mouse model used for this work was generated in two strains by two distinct shRNA against *Rps19* and mice induced by one of the shRNA showed swollen intestine upon reduced levels of Rps19 (unpublished personal communication from the lab of Stefan Karlsson and (Jaako et al. 2011)).

Work presented in this thesis propose that glucocorticoids that have been used to treat DBA patients for decades indeed work through dampening the p53 activation in *Rps19* deficient erythroid cells, thereby increasing survival. Other studies have shown that glucocorticoids also enhance erythroid output by upregulation of genes required for BFU-E self-renewal, something confirmed by data in Paper I in this thesis (von Lindern et al. 1999; Wessely, Deiner, et al. 1997; Flygare et al. 2011). Paper I strengthens the hypothesis that glucocorticoids affect erythroid precursors on two fronts; both by reducing anti-proliferative and pro-apoptotic p53 activity, as well as by stimulating proliferation and/or self-renewal of early erythroid progenitors, leading to increased erythroid output. However, glucocorticoids primarily promote self-renewal at the expense of erythroid differentiation and therefore glucocorticoid treatment might be even more successful if combined with treatment promoting efficient erythroid differentiation, *e.g.* by increasing Gata1 levels (Jeannesson et al. 1997). An additional approach to develop future DBA treatment could be to inhibit Tgfβ and/or Activin signaling with Sotatercept, which has proven effective in diseases of inefficient erythropoiesis such as β-thalassemia and myelodysplastic syndrome (Ear et al. 2015; Ear et al. 2013). There are other examples where removal of a negative regulator increase erythroid proliferation, *e.g.* when p53 is removed from erythroid precursors (Ganguli et al. 2002). Although targeting inhibitors of erythropoiesis seems to be a promising strategy, it remains to be seen if such an approach is sufficient to compensate for increased negative regulation of erythropoiesis via p53 and lack of sufficient erythroid signaling through reduced levels of Gata1.

Impact of altered translation of erythroid specific transcripts

Many models of ribosomal haploinsufficiency observe aberrant translation of certain mRNA transcripts, *e.g.* the Hsp70 co-chaperone Bag1, the RNA-binding protein Csde1, Bcat1 involved in amino acid biogenesis and the erythroid transcription factor Gata1 which is essential for erythroid differentiation (Horos et al. 2012; Ludwig et al. 2014; Pereboom et al. 2014; Khajuria et al. 2018). Interestingly, Bag1 is a positive regulator of DNA synthesis and is inhibited by increased levels of Hsp70 *e.g*. after heat shock (Song *et al.* 2001). Csde1 regulates the translation of certain mRNA involved in ribosomal biogenesis as well as erythroid terminal differentiation (Moore *et al.* 2018). In the case of Gata1, reduced expression of either *RPS19*, *RPL5* or *RPL11* all result in reduced expression of full length *Gata1*, indicating that this might be a general effect of ribosomal protein haploinsufficiency in DBA (Ludwig et al. 2014). However, decreased Gata1 protein levels have not been observed in other studies of reduced ribosome protein expression (Horos et al. 2012). Hence, studies elucidating Gata1 protein levels in DBA patients carrying different ribosomal protein mutations would confirm a general reduction of Gata1 as a cause behind DBA pathology. Adding to the knowledge of why some mRNA transcripts are particularly affected by aberrant ribosome biogenesis caused by reduction of TSR2, Khajuria *et al* show that the length and secondary structure of the 5`UTR of Gata1 mRNA makes it more sensitive to reduced levels of functional ribosomes, making this transcript less translated than other hematopoietic transcription factors. In this study, overexpression of *Gata1* increases the erythroid output. Although this is an indication that sufficient Gata1 levels can improve the DBA phenotype, Gata1 overexpression does not fully rescue the phenotype. It can therefore be speculated if Gata1-independent mechanisms are also involved (Khajuria et al. 2018). This finding is strongly favoured by the fact that mutations in *GATA1* leads to transcription of the truncated version GATA1s in a few DBA patients (Sankaran et al. 2012). Another transcription factor important for erythropoiesis, which is reduced in DBA, is the transcription factor Myb (Sieff et al. 2010). Myb is involved in the development of other hematopoietic lineages, but is also essential to erythropoiesis best illustrated by embryonic death of *Myb-/-* mice due to severely impaired erythropoietic differentiation (Mucenski et al. 1991).

There is still a lot to unravel about aberrant mRNA translation in DBA patients. To begin with, a comprehensive study on the protein levels of important erythroid transcription factors in general and Gata1 in particular would shed light on if reduced Gata1 protein levels indeed is the link between ribosome protein mutations and erythroid failure. It would be especially interesting to quantify protein levels of erythroid transcription factors, such as Gata1, Klf1,

Tal1 and Myb, but also other proteins important during erythroid differentiation such as EpoR, the transferrin receptor and globin proteins. Such a study could answer if many different ribosomal protein mutations affect the same transcript(s) or if alteration in different transcripts still lead to the same end result, *i.e.* defective erythropoiesis. Comprehensive studies like this would shed light on any genotype-phenotype correlations on a molecular level, although no proof concludes a phenotype-genotype correlation when it comes to hematological symptoms. Such a study could also better understand the molecular basis behind non-hematological malformations observed in nearly half of all DBA patients, since there is a genotype-phenotype correlation when it comes to these symptoms (Arbiv et al. 2018).

IMPORTANCE OF MECHANISTIC INSIGHT BEHIND DBA

Where does the erythroid defect occur in DBA?

As discussed previously, erythropoiesis consists of both a self-renewing phase characterized by extensive proliferation and by a differentiation phase where proliferation is much more restricted. Are the reduced numbers of red blood cells due to defective erythroid differentiation, or reduced function (or possibly absolute numbers) of BFU-Es and CFU-Es? Since future treatment strategies would be quite different if targeting self-renewal and expansion or terminal differentiation, this question is of great importance. The BFU-E colony forming potential of DBA patient bone marrow is markedly reduced, whereas colony formation of precursors of myeloid lineages remains intact, indicating a severe effect on the erythroid lineage but not on other lineages (Bagnara et al. 1991; Hamaguchi et al. 2003). The hematopoietic stem cell function was reduced in a mouse model of DBA together with reduced numbers of megakaryocytic progenitors as well as granulocytic-macrophage progenitors (Jaako et al. 2011). Although DBA patients display normal numbers of $CD34^+$ hematopoietic progenitors, their proliferative capacity is significantly reduced (Hamaguchi et al. 2003). Whereas one study found the BFU-E and CFU-E colony forming ability markedly reduced in DBA patients that had been diagnosed and was in treatment (Nathan et al. 1978), another study from the same author claimed that the reduced BFU-E activity was not enough to explain the drastically reduced red blood cell output in newly diagnosed and untreated DBA patients (Lipton et al. 1986). The authors conclude that DBA is not merely

a disorder of deficient early erythroid progenitors, but also a disease of defective differentiation rather than impairment of erythroid precursor quantity. This was confirmed by studying erythroid differentiation of peripheral blood from DBA patients in a 2-phase culture system. Here, the reduced colony numbers from DBA patient cells was only moderate and did not correspond to the dramatic decrease in hemoglobinized cells (Ohene-Abuakwa et al. 2005). However, the size of erythroid colonies from DBA patients were markedly reduced, indicating that erythroid precursors might be generated in near sufficient numbers, but fail to proliferate and generate enough differentiated and hemoglobinized red blood cells. Other studies observed a greater reduction in CFU-E colony formation than BFU-E colony formation and conclude that the ability to sustain erythropoiesis declines from the BFU-E stage and onwards (Tsai, Arkin, et al. 1989). Further, a dramatic reduction in erythroid colony formation in bone marrow from DBA patients has been observed, with almost no colonies formed (Freedman et al. 1976) and defective proliferation was observed in early CD34 expressing hematopoietic progenitors. Cellular model systems of DBA, mostly shRNA-based, confirm impairment of terminal erythroid differentiation (Ebert et al. 2005). Combined, these studies point towards a great heterogeneity in erythroid precursor characteristics, even when studying patient cell material. However, it seems clear that BFU-Es and CFU-Es are affected in DBA and display both reduced numbers and proliferative capacity, but to what degree this explains the erythroid failure in DBA could be debated. When considering deficiency of erythroid precursors or disrupted differentiation as the main cause behind DBA pathology, it might be beneficial to consider the disease causing both quantitative and qualitative defects in erythroid progenitors (BFU-Es and CFU-Es), as well as by defective differentiation of these precursors, rather than carrying an either-or-perspective.

Models to test new drug candidates

Generating a model system that completely recapitulates all DBA symptoms has been challenging, illustrated by embryonic lethality of *Rps19-/-* and lack of symptoms in *Rps19+/-* conventional knock out mice (Matsson et al. 2004; Matsson et al. 2006). The inducible *Rps19* deficient mouse model used throughout the studies presented in this thesis does not in all aspects recapitulate the progression of the human disease. When *Rps19* deficiency is induced, these mice both recapitulate the erythroid phenotype of DBA, but additionally also show reduced numbers of megakaryocytic progenitors and develop bone marrow failure much more rapidly than DBA patients (Jaako et al. 2011). Despite these deviations, this mouse has proved very useful as a

model system for DBA, as patient samples are rare. From a pharmacological point of view it also holds great advantage over cell based systems, as the *in vivo* setting always recapitulates the effects of treatment better than *in vitro* systems. In Paper I we successfully treat this mouse model with the glucocorticoid Prednisolone, similar to the treatment given to patients. We thereby show that this mouse model can be used to evaluate new therapies against DBA. Complementary to animal models, induced pluripotent stem cell models of both *Rps19* and *Rpl11* deficiencies have been generated and proven useful for initial drug screening and has also been transplanted into immunocompromised mice, creating a humanized model for DBA (Doulatov et al. 2017).

Ribosome heterogeneity or absolute ribosome number behind DBA pathology?

Advances in measuring global gene expression has been a great leap forward for medical science, however the translation between gene expression and the protein levels are not always fully corresponding to each other, especially during dynamic transitions in cell homeostasis such as by external stimuli or by cellular stress (reviewed in (Liu et al. 2016)). In a disease like DBA, where translation from mRNA to protein is a part of the underlying pathology, gene expression levels might deviate even more from protein quantifications. Therefore, it would be beneficial with a shift towards quantifying protein levels rather than only looking at gene expression in models of DBA.

The field of ribosome biogenesis research has transitioned from the notion that ribosomes translate whatever is in front of them, to the notion that ribosomes are heterogenous in function and are involved in regulating translation (reviewed in (Genuth et al. 2018)). A change in the perception of ribosome biogenesis, assembly and regulation as a highly dynamic process has taken place, concluding that ribosome composition indeed varies both intracellularly and between tissues. Heterogeneity in ribosome composition has been illustrated by examining multiple ribosomal proteins on ribosomes from mouse embryonic stem cells and found that some of them show a decreased representation in polysomal fractions compared to free ribosomes within the cell. This indicates that ribosomes can indeed lack one (or possibly more) core ribosomal proteins and still be functional and actively translate proteins (Shi et al. 2017). Indeed, the expression of ribosomal proteins are known to vary greatly between tissues and cell types. For instance, one study identified that Rps29 and Rps27L show an antagonistic expression pattern in lymphoid and myeloid cell types, whereas the opposite pattern is found in

lymphoma/lymphoid leukemia cell lines. The authors hypothesize that there is a connection between regulation of ribosomal protein expression and the hematopoietic developmental program (Guimaraes et al. 2016). Also, this study observed an increase in the expression of certain ribosomal proteins in certain tissues compared to total mRNA in all tissues. For example, Rpl3L is expressed at higher levels in skeletal muscle than in all tissues combined. Further, deletion of Rpl38 resulted in unchanged global protein synthesis, but defective translation of several Hox genes, resulting in aberrant skeletal patterning. This study exemplifies that certain ribosomal proteins could be involved in promoting transcription of selected genes (Kondrashov et al. 2011).

One study from Vijay Sankaran's group set out to dissect how aberrant ribosome biogenesis might affect translation of transcripts essential to erythropoiesis. They found that the mRNA of Gata1 is sensitive to decreased levels of functional ribosomes, due to its 5´UTR length and complexity. This results in reduced levels of Gata1 protein and thereby deficient erythropoiesis, linking erythroid specificity of aberrant ribosome biogenesis to translation of the key erythroid transcription factor Gata1. They find no heterogeneity in ribosomal protein expression and conclude that the absolute ribosome numbers and not ribosome heterogeneity is critical for DBA pathology (Khajuria et al. 2018). However, considering the studies that observe ribosomal heterogeneity both within and between cell types, one should not rule out that ribosome heterogeneity, especially when it comes to ribosome protein stoichiometry, could further explain the erythroid specificity of DBA. Also, it might explain why some DBA patients display physical abnormalities and why this is much more prevalent in patients haploinsufficient for certain ribosomal proteins, such as *Rpl5* and *Rpl11*. In the future, the detailed understanding of DBA pathology at a molecular level could benefit tremendously from incorporating emerging concepts from the ribosome biogenesis field highlighting intra- and intercellular heterogeneity in ribosome function to answer questions about erythroid specificity, physical abnormalities and even differences in global mRNA translation possibly contributing to disease heterogeneity.

TARGETING HEME IN FUTURE DBA TREATMENT

Development of new, more efficient and more disease-specific treatments often go hand in hand with increased knowledge of the molecular mechanisms behind a disease. Iron uptake is necessary for erythroid cells to synthesize heme and is a highly regulated process. Iron homeostasis is mainly regulated by hepcidin, a humoral factor involved in degrading the heme exporter ferroportin, resulting in retention of intracellular iron in enterocytes. Once stimulated by Epo, erythroblasts produce the hormone erythroferrone that counteracts the function of hepcidin and thereby contributes to iron homeostasis (Kautz et al. 2014). Indeed, DBA patients receiving blood transfusions show increased levels of hepcidin and the intracellular iron storage protein ferritin, indicating increased intracellular iron. This is not observed as much in glucocorticoid responding patients, indicating that the treatment strategy of stimulating erythropoiesis rather than pure alleviation of the symptoms (as with blood transfusions) has major impact on iron homeostasis in DBA patients (Pospisilova et al. 2014). Since iron is known to induce expression of Alas2 and thereby initiate heme synthesis, targeting hepcidin or its counteracting factor erythroferrone could be beneficial for DBA patients. However, in Paper III we treated erythroid precursors from bone marrow with the iron chelator Deferoxamine and this had no effect on proliferation of Rps19 deficient cells or healthy controls. Interestingly, Gata1 protein levels are reduced by iron deficiency and since Gata1 regulate the expression of Alas2 and thereby heme synthesis, one can hypothesize that there is mutual regulation between heme synthesis and Gata1 (Liu et al. 2008; Cox et al. 1991; Elferink et al. 1988). Mutual regulation between heme synthesis and Gata1 would connect studies where reduced levels of Gata1 contributes to the DBA phenotype (Sankaran et al. 2012; Ludwig et al. 2014; Khajuria et al. 2018) to our study in Paper II, where increased heme synthesis is seen in the *Rps19* deficient model of DBA. Although studies in Paper II and III never investigated levels of Gata1, it would be interesting to elucidate if there is a dysregulation between Gata1 and heme synthesis in DBA and to what extent such an imbalance would contribute to the disease.

Work presented in this thesis suggest that *Rps19* deficient erythroblasts have elevated levels of intracellular unbound heme, which is likely toxic to cells and contributes to the disease phenotype (Paper II and III). One study showed increased heme levels erythroid cells from one DBA patient, however the study does not address the cause behind the elevated heme levels. Paper II observe elevated heme synthesis in *Rps19* deficient erythroblasts upregulating multiple heme enzymes. More studies are needed to confirm that the elevated intracellular heme levels are due to increased heme synthesis, or possibly due

to decreased globin translation or a combination of the two. Regardless, targeting unbound intracellular heme is here presented as a new and disease specific therapeutic strategy. Additionally, this work highlight the possible therapeutic potential of heme scavenging, in this case by treatment with A1M (Paper III). Additionally, the elevated heme levels observed in *Rps19* deficient erythroid cells implies a cell type specific phenomena, since erythroid precursors synthesized great amounts of heme. Hence, the data presented in Paper II and III contribute to understanding the erythroid specificity behind DBA.

DBA is an intriguing disease, where some mechanisms underlying the disease are unraveled, but much is yet to discover. Alongside the strive to fully elucidate why the disease so severely affects erythropoiesis is the need for new treatments for the patients. This thesis has identified a p53-dempening effect of current glucocorticoid treatment and also discovered elevated heme levels in erythroblasts with *Rps19* deficiency. The studies presented here show that knock down of the heme-sensing kinase *HRI* lowers both elevated heme levels and dampens p53 activity in Rps19 deficient erythroid precursors. We also discover that treatment with the humoral protein A1M significantly improves erythroid proliferation in both mice and DBA patients, and decreases elevated intracellular heme levels. In all, work presented in this thesis contribute to the understanding of mechanisms underlying DBA pathology and proposes new treatment strategies for DBA.

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It is very easy to overestimate the importance of our own achievements in comparison with what we owe others
Dietrich Bonhoeffer (1906-1945)

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Diamond-Blackfan anemia is a rare congenital red cell disorder where patients suffer from chronic anemia. All current treatments have severe side effects and therefore there is a great need for more disease specific therapies. To develop new treatments, it is vital to gain a deeper understanding of the mechanisms underlying the disease. The work presented in this thesis has identified mechanisms behind current treatment, as well as found new disease mechanisms and explored novel treatment strategies for Diamond-Blackfan anemia.

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