UNDERSTANDING THE ROLE OF RIBOSOMAL PROTEINS
AND FLVCR1 ABERRANT SPlicing IN DIAMOND
BLACKFAN ANEMIA

By

Abigail Brenda Fernandes

A thesis submitted in conformity with the requirements
for the degree of Masters of Science

Department of Molecular Genetics
University of Toronto

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Understanding The Role Of Ribosomal Proteins And FLVCR1 Aberrant
Splicing In Diamond Blackfan Anemia

Abigail Fernandes
Master of Science
Department of Molecular Genetics
University of Toronto
2012

ABSTRACT

Diamond Blackfan Anemia is a rare congenital disease that is primarily characterized
by reduced erythroid progenitors. DBA pathogenesis has been associated with genes
encoding ribosomal proteins (RPs) which are important in translation. However, this fails to
explain why erythropoiesis is specifically disrupted. Our lab previously found that aberrant
splicing of the human transcript encoding heme exporter, FLVCR1, is involved in DBA
pathogenesis; and that RPS19 implicated in 25% of DBA patients, regulates FLVCR1
transcript splicing.

This thesis investigated the role of another DBA associated gene encoding RPS17, in
the regulation of FLVCR1 splicing and disrupted erythropoiesis in DBA. My findings further
support the role of FLVCR1 aberrant splicing in DBA and provide evidence suggesting that
RPS17 may not be a candidate DBA gene. Furthermore, my study implicates a potential role
for RPS19 transcript levels in defective erythroid differentiation observed in DBA.
Acknowledgments

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To my family and friends, thank you for being persistently confident in me and cheering me on from the sidelines.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>BACE-1</td>
<td>Beta- secretase-1</td>
</tr>
<tr>
<td>BFU-E</td>
<td>Erythroid burst forming unit</td>
</tr>
<tr>
<td>CFU-E</td>
<td>Erythroid colony forming unit</td>
</tr>
<tr>
<td>CHH</td>
<td>Cartilage- hair hypoplasia</td>
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<tr>
<td>DBA</td>
<td>Diamond Blackfan Anemia</td>
</tr>
<tr>
<td>DKC</td>
<td>Dyskeratosis congenital</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbeccos’ minimal essential medium</td>
</tr>
<tr>
<td>eADA</td>
<td>Erythrocyte adenosine deaminase</td>
</tr>
<tr>
<td>eIF-2</td>
<td>Eukaryotic initiation factor- 2</td>
</tr>
<tr>
<td>EPOR</td>
<td>Erythropoietin receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FeLV</td>
<td>Feline leukemia virus</td>
</tr>
<tr>
<td>FLVCR1</td>
<td>Feline Leukemia Virus subgroup C receptor 1</td>
</tr>
<tr>
<td>GA</td>
<td>Guanine and adenosine residues</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney cell- line 293T</td>
</tr>
<tr>
<td>HEL</td>
<td>Human erythroleukemia cells</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogenous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>HO</td>
<td>Heme oxygenase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IBMFS</td>
<td>Inherited bone marrow failure syndromes</td>
</tr>
<tr>
<td>IL-3</td>
<td>Interleukin- 3</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
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<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
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<tr>
<td>Luc</td>
<td>Luciferase</td>
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<td>MEL</td>
<td>Murine erythroleukemia</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>MLV</td>
<td>Moloney murine leukemia</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pen</td>
<td>Penicillin</td>
</tr>
<tr>
<td>PGK</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PTC</td>
<td>Premature termination codon</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RP</td>
<td>Ribosomal protein</td>
</tr>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
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<td>rRNA</td>
<td>Ribosomal RNA</td>
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<td>Shwachman Bodian Diamond syndrome gene</td>
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<td>Shwachman Diamond Syndrome</td>
</tr>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SR</td>
<td>Serine-arginine</td>
</tr>
<tr>
<td>Strep</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>TCS</td>
<td>Treacher Collins syndrome</td>
</tr>
<tr>
<td>TERC</td>
<td>Telomerase RNA component</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>THTR1</td>
<td>Thiamine transporter protein</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus glycoprotein</td>
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</table>
CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview

Diamond Blackfan Anemia (DBA) is a rare congenital disease that is primarily characterized by reduced erythroid progenitor cells. The only identified genes that have been implicated in DBA are those encoding ribosomal proteins (RP) which are important in translation (Doherty et al., 2010). It remains unknown how mutated RPs specifically disrupt erythropoiesis, while other lineages are rarely affected. Interestingly, disruption of the protein encoding a heme exporter, FLVCR1, reduces early erythroid progenitors, both in vivo and in vitro, which mimics the hematological features in DBA (Keel et al., 2008, Quigley et al., 2004, Rey et al., 2008). Rey and colleagues (Rey et al., 2008) found enhanced aberrant splicing of human FLVCR1 transcript in human DBA erythroid cells (negative for RPS19 mutations) compared to normal erythroid cells. These authors also found that reduction of RPS19 in human K562 cells leads to aberrant FLVCR1 splicing and disrupted erythroid differentiation (Rey et al., 2008).

I hypothesize that ribosomal proteins implicated in DBA lead to aberrant FLVCR1 splicing and disrupted erythropoiesis in DBA. This thesis will investigate the role of RPS17, associated with DBA, on FLVCR1 splicing and erythropoiesis, in comparison to RPS19 (positive control) and RPS3A, which has not been found mutated in DBA patients. This introductory chapter provides a review of DBA, the ribosomal proteins implicated, the erythropoietic models of DBA and the role of FLVCR1 in this disease.
1.2 Diamond Blackfan Anemia (DBA)

1.2.1 Characteristics

Diamond Blackfan Anemia (DBA), first identified in 1938 (Diamond et al., 1938) is part of a rare group of inherited bone marrow failure syndromes (IBMFS) (Young et al., 1994), characterized by congenital anomalies, birth defects (Gripp et al., 2001) and a predisposition to cancer (Lipton et al., 2001; Lipton & Ellis, 2010). DBA is a heterogeneous congenital disease occurring in about five to seven infants per million. It is sporadic in many cases while familial with autosomal dominant inheritance in others (Orfali et al., 2004).

1.2.2 Clinical features of DBA

Hematological features of DBA which usually manifest within the first year of life include macrocytic and normochromic anemia in addition to depleted bone marrow red blood cell precursors due to defective proliferation and differentiation (Diamond et al., 1976). Furthermore, there are high levels of folic acid serum levels, erythropoietin, fetal haemoglobin levels, vitamin B12 and erythrocyte adenosine deaminase (eADA) levels (Chiabrando & Tolosano, 2010). A defining characteristic of DBA however, is impaired erythroid differentiation, with moderate to severe reduction of erythroid burst forming (BFU-E) and colony forming (CFU-E) units, respectively (Nathan et al., 1978). While these cells are essential precursors of erythroblasts and erythrocytes, other hematopoietic cells are rarely affected (Miyake et al., 2005) (Figure 1-1).
A defining characteristic of Diamond-Blackfan Anemia (DBA) is a moderate to severe reduction in erythroid burst-forming and colony-forming units (BFU-E and CFU-E respectively). These cells are essential erythroid progenitors of erythroblasts and erythrocytes. Other hematopoietic cells are rarely affected.

Approximately 40% of DBA patients display additional abnormalities including congenital heart defects, limb malformations and growth retardation (Miyake et al., 2005). Many patients have urogenital, skeletal, and kidney abnormalities in addition to short stature, webbed neck and craniofacial abnormalities (Chiabrando & Tolosano, 2010; Choesmel et al.,

**Figure 1-1: Cell lineages of the hematopoietic stem cell system**

A defining characteristic of Diamond-Blackfan Anemia (DBA) is a moderate to severe reduction in erythroid burst-forming and colony-forming units (BFU-E and CFU-E respectively). These cells are essential erythroid progenitors of erythroblasts and erythrocytes. Other hematopoietic cells are rarely affected.
Additionally, DBA patients are predisposed to developing hematopoietic and non-hematopoietic malignancies. These include “acute myeloid leukemia, myelodysplastic syndrome, Hodgkin and non-Hodgkin lymphomas, acute lymphoblastic leukemia, osteogenic sarcoma, breast cancer, hepatocellular carcinoma, melanoma, fibrohistiocytoma, gastric cancer and colon cancer” (Lipton, 2006; Vlachos et al., 2008; Doherty et al., 2010). It is however unclear how these abnormalities are associated with defective erythropoiesis.

1.2.3 Treatment of DBA

Common treatments for this otherwise fatal disease include corticosteroids and red blood cell transfusions. However, corticosteroids are usually ineffective since many patients develop resistance or may require high doses of steroids which can have deleterious side effects such as growth retardation, cataracts and Cushingoid features (Flygare & Karlsson, 2007). Furthermore, secondary hemochromatosis (iron overload) which tends to arise in transfusion-dependent patients is responsible for 23% of deaths reported in the DBA American Registry (Lipton et al., 2006). The only current curative form of treatment is bone marrow transplantation; but human leukocyte antigen (HLA) matching and preference given to DBA patients with severe complications such as leukemia, often poses a problem (Da Costa et al., 2010; Hamaguchi et al., 2002). Some treatments have been effective in a subset of DBA patients. A short course of interleukin-3 (IL-3) treatment in steroid-dependent DBA patients led to a 50% chance of sustained remission (Ball et al., 1995). Cyclosporin, an immunosuppressant, was also found to be effective in 50% of steroid-resistant DBA patients (El-Beshlawy et al., 2002). Moreover, administration of leucine, which stimulates protein synthesis (Anthony et al., 2000; Kimball & Jefferson, 2006), achieved improved reticulocyte
count, hemoglobin levels and BFU-Es in one steroid resistant, transfusion dependent DBA
patient leading to transfusion independence and remission (Pospisilova et al., 2007).
Evidently, treatments to date are only effective in a small subset of patients. Therefore, this
thesis will try to elucidate the mechanism underlying DBA with the long-term goal of
developing an effective treatment for this disease.

1.3 Ribosomal proteins implicated in DBA

While the molecular cause underlying DBA has yet to be elucidated, approximately
55% of DBA patients have heterozygous mutations in genes encoding ribosomal proteins
belonging to both the 40S subunit and the 60S subunit of the ribosome. Mutations have been
found in genes encoding RPS19 (25%), RPS17 (1.5%), RPS24 (2.4%), RPS7 (<1%), RPS10
(2.6%), RPS26 (6.4%), RPL35A (3%), RPL5 (6.6%) and RPL11 (4.8%) (Figure 1-2)
(Cmejla et al., 2007; Doherty et al., 2010; Draptchinskaia et al., 1999; Farrar et al., 2008;
Gazda et al., 2006; Gazda et al., 2008). The genetic cause in the remaining 45% of DBA
patients is currently unknown. Due to the involvement of these ribosomal proteins, DBA has
been characterized as a ribosomal and translational disorder. However, it is unclear how
these ribosomal proteins specifically disrupt erythropoiesis.
Figure 1-2: Percentage of mutated genes encoding ribosomal proteins (RP) in DBA patients to date.
Ribosomal proteins mutated in DBA patients are structural components of the 40S subunit and the 60 subunit of the ribosome.

1.3.1 Ribosome biogenesis

Approximately 80 ribosomal proteins in conjunction with 4 ribosomal RNAs (rRNAs) and more than 150 accessory proteins are coordinately synthesized to form the 40S and 60S subunits of the ribosome (Doudna & Rath, 2002; Robledo et al., 2008). Ribosome biogenesis begins in the nucleolus where a polycistronic 45S pre-rRNA transcript transcribed by RNA polymerase I, is processed into 18S rRNA (of the 40S subunit) and 5.8S and 28S rRNAs (of the 60S subunit) through various chemical modifications and cleavage reactions. RNA polymerase III transcribes the 5S rRNA (of the 60S subunit) (Kressler et al., 1999; Venema & Tollervey, 1999). Ribosomal proteins then associate with their respective rRNAs.
to form pre-40S and pre-60S pre-ribosomes, which assemble in the nucleolus and are exported to the cytoplasm through nuclear pore complex where maturation is completed (Campagnoli et al., 2008; Henras et al., 2008; Zemp & Kutay, 2007). The small 40S ribosomal subunit and the large 60S ribosomal subunit make up the mature 80S eukaryotic ribosome which is essential for protein synthesis in every cell (Figure 1-3).

**Figure 1-3: Ribosome biogenesis**
The eukaryotic ribosome is composed of 4 ribosomal RNAs (rRNAs), approximately 80 ribosomal proteins and over 150 accessory proteins. In the nucleolus, the 45S rRNA precursor is transcribed and processed along with the 5S rRNA to produce mature 40S and 60S ribosomal subunits.
1.3.2 Ribosomal Protein S19 (RPS19)

Twenty-five percent of DBA patients have a heterozygous mutation in the gene encoding the ribosomal protein S19 (RPS19) which consists of six exons spanning 11kb and is located on chromosome 19q13.2 (Martinez Barrio et al., 2009; Miyake et al., 2005). The first exon is included in the 5’ untranslated region (UTR), which is non-coding, whereas the remaining five exons encode a 145 amino acid, 16kDa protein (Boria et al., 2010). RPS19 is highly conserved among species (Martinez Barrio et al., 2009) and is one of 33 ribosomal proteins that form the 40S ribosomal subunit with the 18S rRNA (Idol et al., 2007; Lutsch et al., 1990). Within the cell, RPS19 is located in the nucleus where ribosome synthesis occurs, as well as the cytoplasm as a component of the ribosome (Angelini et al., 2007; Da Costa, Tchernia et al., 2003). Moreover, RPS19 is highly expressed in tissues with high rates of proliferation such as bone marrow, glandular organs, fetal brain and placenta while expression decreases in mature tissues (Gazda et al., 2006).

RPS19 is important for the production of 18S rRNA and 40S ribosomal subunits. Reduction of RPS19 levels lead to the accumulation of the 18S rRNA precursors (such as 21S pre-rRNA), decreased 18S rRNA and small 40S ribosomal subunits and increased large 60S ribosomal subunits (Choesmel et al., 2008; Flygare et al., 2007; Idol et al., 2007; Robledo et al., 2008). Defective 18S rRNA maturation and 40S ribosomal subunit biogenesis was also increased in DBA patient fibroblasts and primary CD34+ bone marrow cells (Flygare et al., 2007; Robledo et al., 2008).

To date, there are approximately 129 reported heterozygous RPS19 mutations including translocations, deletions, insertions, splice defects, truncating and rearrangement mutations. 40% of reported mutations are missense mutations leading to single amino acid
substitutions (Campagnoli et al., 2008). Of these, 74% of them are arginine mutations such as Arg62Trp (R62W), Arg62Gln (R62Q) and Arg56Gln (R56Q) (Campagnoli et al., 2008). RPS19 mutations can lead to reduced RPS19 mRNA and/or protein levels, impaired ribosomal association, disrupted nucleolar localization, reduced stability and reduced affinity to interactors (Campagnoli et al., 2008; Devlin et al., 2010; Gregory et al., 2007; Schuster et al., 2010).

### 1.3.3 Ribosomal Protein 17 (RPS17)

RPS17 consists of five exons spanning 3.7 kb and is located on human chromosome 15 (Chen & Roufa, 1988). The five exons encode a 135 amino acid protein of 15.5 kDa (Boria et al., 2010). Similarly to RPS19, depletion of RPS17 in HeLa cells lead to reduced 40S ribosomal subunits, reduced mature 80S ribosomes and increased free 60S subunits (Robledo et al., 2008).

Unlike RPS19, RPS17 mutations are rare and have been reported in three DBA cases to date. Gazda et al. (2008) discovered a deletion of GA residues at position 200 and 201 of RPS17 in one DBA patient without any malformations. Then, Cmejla and colleagues (Cmejla et al., 2007) found that one DBA patient had a heterozygous mutation in the translation initiation codon (c.2T>G) resulting in a less stable mutated mRNA. Similarly, another mutation (c.1A>G) in the translation initiation codon of RPS17 was discovered (Song et al., 2010). Both of these missense mutations arose de novo and are expected to produce a short four amino acid peptide due to a downstream ATG initiation codon located at position +158 (Cmejla et al., 2007; Song et al., 2010). Compared to 242 control chromosomes, the RPS17 translation initiation codon did not harbour any sequence
variations, therefore dismissing the possibility of it being a rare polymorphism (Collins & Schwartz, 2002).

1.3.4 Extraribosomal functions of ribosomal proteins

Ribosomal proteins are important in the correct folding, cleavage and processing of rRNA and ribosomal assembly (Steitz & Moore, 2003). In addition to their structural role, some ribosomal proteins also have extra-ribosomal functions. It has therefore been postulated that ribosomal proteins implicated in DBA specifically disrupt erythropoiesis through an extra-ribosomal function (Choesmel et al., 2008; Morimoto et al., 2007; Shimamura, 2008).

RPS19 is one of the many ribosomal proteins that have extraribosomal functions. Based on the RPS19 interactome determined by Orru and colleagues (Orru et al., 2007), RPS19 interacts with transcription factors, DNA/RNA-binding proteins, NT-Pases, kinases, hydrolases, splicing factors, transporters etc. The authors found that RPS19 also interacts with proteins that play a role in rRNA pseudouridylation, exporting small subunits to the cytoplasm and other components of the 40S ribosome (Campagnoli et al., 2008; Orru et al., 2007). RPS19 also interacts in vitro with fibroblast growth factor-2 (Soulet et al., 2001), which is important in regulating rRNA transcription (Sorensen et al., 2006), PIM-1, a serine-threonine kinase (Chiocchetti et al., 2005) and a novel nuclear S19 binding protein (S19BP) whose function is unknown (Maeda et al., 2006).

RPS19 is located on the external surface of the 40S subunit, near RPS24, RPS3A, RPS13 and RPS16 in a region known to interact with the eukaryotic initiation factor eIF-2 (Lutsch et al., 1990) which is important for translation. Additionally, RPS19 has been found
to play a role in “macrophage- dependent apoptotic cell clearance” by acting as a chemotactic factor for monocytes when released by apoptotic cells (Shrestha et al., 1999).

Several ribosomal proteins also have RNA binding properties that enable them to bind to their own pre- mRNA and mRNA to regulate their expression levels. Human ribosomal proteins S13, L30 and S26 are able to regulate their own pre-mRNA splicing by binding to their pre-mRNA and compromising the access of proteins involved in alternative splicing (Ivanov et al., 2005; Macias et al., 2008; Malygin et al., 2007). Human RPL3 regulates its own pre-mRNA splicing by binding to and recruiting heterogenous nuclear ribonucleoprotein (hnRNP) (Russo et al., 2010), a nuclear RNA- binding protein involved in alternative splicing (Krecic & Swanson, 1999; Martinez-Contreras et al., 2007). Human RPS26 and yeast RPL30 can also bind to their own mRNA; RPL30 inhibits the translation of its mRNA through this interaction (Dabeva & Warner, 1993; Ivanov et al., 2003; Ivanov et al., 2005; Li, Vilardell, & Warner, 1996; Macias et al., 2008; Malygin et al., 2007). Furthermore, RPS19 has the ability to bind to its own mRNA at the 5’ UTR, which is decreased by introduction of the RPS19 DBA missense mutations W52R and R62W (Schuster et al., 2010). In addition to binding to their own mRNA, some ribosomal proteins such as RPL26 and RPS13a have the ability to bind to other mRNAs (Takagi et al., 2005).

Interestingly, two ribosomal proteins have been shown to be involved in erythropoiesis. CHOP, a transcription factor of the CCAAT- enhancer binding (C/EBP) family of proteins (Coutts et al., 1999), is up regulated during erythroid differentiation (Ron & Habener, 1992). Cui and colleagues (Cui et al., 2000) have demonstrated that an in vitro and in vivo interaction of RPS3A and CHOP inhibits CHOP’S ability to enhance erythroid differentiation of induced Rauscher murine erythroleukemic cells. Secondly, down regulated
RPS5 mRNA transcript and protein expression in murine erythroleukemia (MEL) cells delayed the onset of erythropoiesis as well as cell cycle entry (Matragkou et al., 2008).

1.4 Erythropoietic models of DBA

1.4.1 RNAi mediated approach to study DBA

Since mutated ribosomal proteins in DBA patients are associated with anemia, several groups (Da Costa et al., 2003; Ebert et al., 2005; Flygare et al., 2005; Flygare et al., 2007; Miyake et al., 2008) have studied the role of RPS19 (DBA mutant) on erythropoiesis using cell culture models. siRNA and shRNA mediated knockdown of RPS19 in human CD34+ cultured resulted in defective cell proliferation, reduced erythroid differentiation, reduced colony formation and increased apoptosis (Flygare et al., 2005; Miyake et al., 2008). These DBA characteristics were also observed in RPS19 reduced human erythroid leukemic cell lines such as human K562, TF-1 and UT-7 cells. A lentiviral expression vector expressing RPS19 rescued the DBA phenotypes seen in human erythroid cells (Miyake et al., 2005).

The importance of RPS19 in erythropoiesis was also demonstrated through the rescue of BFU-E and CFU-E formation in CD34+ cells of RPS19 depleted DBA patients following RPS19 over expression (Hamaguchi et al., 2002). In addition, RPS19 transgene over expression improved the proliferation of defective erythroid progenitor cells in RPS19 deficient DBA patients (Hamaguchi et al., 2002). It has therefore been proposed that RPS19 haplo- insufficiency partly explains the mechanism of DBA pathogenesis in patients with RPS19 deletions and nonsense mutations (Ebert et al., 2005; Gazda et al., 2004). In a murine cell culture model of DBA, RPS19 disruption led to the down- regulation of erythroid
signalling proteins and decreased expression of the transcription factor MYB, and its target KIT which are crucial for erythropoiesis (Sieff et al., 2010). Similar to RPS19, defective disrupted pre-rRNA maturation was observed in both RPS24 mutated DBA patient lymphoblastoid cells as well as RPS24 knockdown HeLa cells (Choesmel et al., 2008). In vitro studies show that RPS19 is directly linked to DBA and has a causal role in disrupting erythroid differentiation.

1.4.2 In vivo models of DBA

Homozygous RPS19 null mice die in utero by the blastocyst stage. In contrast, heterozygous RPS19 mice are normal (Matsson et al., 2004; Matsson et al., 2006). A transgenic mouse conditionally expressing the RPS19R62W missense mutation successfully mimicked DBA through a dominant negative mechanism by exhibiting growth retardation, reduced erythroid progenitors (BFU-Es and CFU-Es) and disrupted terminal erythroid maturation (Devlin et al., 2010). This supports the hypothesis that DBA patients with RPS19 missense mutations which don’t affect RPS19 protein folding, cause DBA pathogenesis through a dominant negative mechanism (Angelini et al., 2007; Gregory et al., 2007).

Antisense morpholinos targeting RPS19 in zebrafish also result in growth impairment and similar haematological features to DBA patients (Danilova et al., 2008; Lai et al., 2009; Uechi et al., 2008). Erythropoietic defects are evident through decreased erythroid progenitors and expression of erythroid differentiation markers such as gata-1 and c-myb. Furthermore, morphological and developmental defects such as a reduction in the size of head, eyes, forebrain and eye field were also evident (Danilova et al., 2008; Uechi et al.,
In vivo models of DBA demonstrate that RPS19 has a crucial role in erythropoiesis, and in some cases, morphology and development.

1.5 Other ribosomal disorders

Like DBA, there are other diseases that are also associated with defective proteins responsible for ribosome biosynthesis. These include X-linked “dyskeratosis congenita (DKC), cartilage-hair hypoplasia (CHH), Treacher Collins syndrome (TCS), 5q- syndrome and Shwachman- Diamond syndrome (SDS)” (Campagnoli et al., 2008) (Table 1-1). Interestingly, these ribosomal disorders all lead to distinct haematological features implying that disrupted translation is not the mechanism underlying DBA, which specifically targets the erythroid lineage.

1.5.1 Shwachman-Diamond Syndrome

Shwachman-Diamond syndrome (SDS), an autosomal recessive disease, is primarily characterized by neutropenia and pancreatic insufficiency (Bodian et al., 1964; Shwachman et al., 1964). Other features include thrombocytopenia, skeletal abnormalities and increased risk of leukemia (Orfali et al., 2004). Approximately 90% of SDS patients have biallelic mutations in the Shwachman-Bodian-Diamond syndrome (SBDS) gene (Boocock et al., 2003; Ellis & Massey, 2006). No homozygous SBDS mutations have been found in SDS patients and furthermore, SBDS null mice are embryonic lethal in murine models (Zhang et al., 2006). This suggests that some level of functional protein encoding SBDS is required. While the function of SBDS is unknown, it has been suggested to play a role in RNA processing, ribosome biogenesis and protein synthesis (Flygare & Karlsson, 2007). SBDS
has been found to co-localize with rRNA and proteins associated with the 60S subunit, including RPL1, RPL2, RPL14 and RPL3P (Hesling et al., 2007; Luz et al., 2009; Ng et al., 2009; Savchenko et al., 2005). Furthermore, SBDS disruption leads to increased 60S ribosomal subunit precursors (Moore et al., 2010) and reduced mature 60S subunit levels (Menne et al., 2007), thereby supporting its association with the large 60S subunit of the ribosome (Burwick et al., 2011; Ganapathi et al., 2007). Sdo1p, the S. cerevisiae ortholog of SBDS, was strongly implicated in the translational activation of ribosomes and ribosome biogenesis since it participates in the release and recycling of the 60S ribosomal biogenesis factor and nucleolar shuttling factor, Tif6, from pre-60S subunits (Menne et al., 2007). Furthermore, in vitro disruption of SBDS in HEK293T cells reduced global protein synthesis (Ball et al., 2009) similar to preliminary results from mouse embryonic fibroblasts from SDS- model mice (Zhang et al., unpublished data) (Ball et al., 2009).
<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene defect</th>
<th>Role</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diamond Blackfan anemia</td>
<td>RPS19, RPS17, RPS24, RPS7, RPS10, RPS26, RPL5, RPL11, RPL35a</td>
<td>Structural ribosomal proteins of the small 40S and large 60S of the ribosome</td>
<td>Bone marrow failure, anemia, craniofacial defects, limb abnormalities, short stature, predisposition to cancer</td>
</tr>
<tr>
<td>Shwachman-Diamond syndrome</td>
<td>SBDS</td>
<td>Associated with the 60S ribosomal subunit; unknown molecular function</td>
<td>Bone marrow failure, neutropenia, pancreatic insufficiency, short stature, predisposition to cancer</td>
</tr>
<tr>
<td>X-linked dyskeratosis congenital</td>
<td>DKC1</td>
<td>DKC1: dyskerin, nucleolar protein involved in rRNA maturation; associated with telomerase complex components including TERT, TERC</td>
<td>Bone marrow failure, cytopenias, abnormal skin pigmentation, nail dystrophy, cancer predisposition</td>
</tr>
<tr>
<td>Cartilage-hair hypoplasia</td>
<td>RMRP</td>
<td>Small nucleolar RNA encoding the RNA component of the RNase mitochondrial RNA processing complex; cleaves rRNA precursors; mitochondrial DNA replication; cell cycle-regulated mRNA turnover</td>
<td>Short stature, skeletal defects, hypoplastic anemia, defective cellular immunity, gastrointestinal dysfunction, predisposition to cancer</td>
</tr>
<tr>
<td>Treacher Collins syndrome</td>
<td>TCOF1</td>
<td>Encodes a nucleolar phosphoprotein, treacle, which affects ribosomal DNA transcription, pre-rRNA methylation</td>
<td>Craniofacial abnormalities</td>
</tr>
<tr>
<td>5q- syndrome</td>
<td>RPS14</td>
<td>Structural ribosomal protein of the small 40S subunit</td>
<td>Myelodysplastic syndrome, severe macrocytic anemia, normal/elevated platelets, hypoglobulated micromegakaryocytes, predisposition to cancer</td>
</tr>
</tbody>
</table>

Adapted from Liu & Ellis, 2006; Narla & Ebert, 2010
1.6 Feline leukemia virus subgroup C induces DBA-like disease in cats

Feline leukemia viruses (FeLVs) are classified into three major subgroups: A, B and C. Each subgroup uses a specific cell surface transporter proteins as a receptor for infection. The FeLV-C subgroup has a broad host range (Hoover & Mullins, 1991; Jarrett et al., 1973; Neil et al., 1991), is formed from mutations of FeLV-A (Neil et al., 1991; Rigby et al., 1992) and induces feline pure red cell aplasia in cats (Abkowitz et al., 1987). This feline disease specifically affects the erythroid lineage (Abkowitz et al., 1987; Dornsife et al., 1989; Jarrett et al., 1984; Neil et al., 1991; Onions et al., 1982) by reducing erythroid burst-forming (BFU-E) and erythroid colony forming units (CFU-E) in vivo and in bone marrow cultures (Abkowitz et al., 1987; Onions et al., 1982; Rojko et al., 1986; Testa et al., 1983). The hematologic features of feline pure red cell aplasia in cats infected with FeLV-C are strikingly similar to DBA. The feline disease is suggested to be caused by the disruption of the cellular function of the FeLV-C receptor (FLVCR1) that is used for virus entry (Rey et al., 2008).

1.6.1 The FeLV subgroup C receptor FLVCR1

Feline leukemia virus subgroup C receptor (FLVCR1) (Quigley et al., 2000; Tailor et al., 1999) belongs to a group of major facilitator superfamily (MFS) of transporters (Pao et al., 1998). The human FLVCR1 gene consists of 10 exons, is located in human chromosome 1q31.3 (Quigley et al., 2000) and is a multi-membrane spanning cell surface protein that is found to function as a heme exporter (Figure 1-4) (Quigley et al., 2004).
A. Exons (E) of human FLVCR1 gene are color coded and exon number is shown below. The length of each exon in base pairs (bp) is shown above.

B. Predicted cell surface topology of the encoded FLVCR1 protein. The heme exporter, FLVCR1, contains 12 membrane spanning segments with six extracellular loops. N and C termini are intracellular.

Figure 1-4. Organization of FLVCR1 gene and encoded protein.

Heme, a complex consisting of iron and protophorpyrin IX ring, is the prosthetic group of several proteins such as cytochromes, myoglobin and hemoglobin (Ponka et al., 1982; Tolosano et al., 2010). High rates of heme biosynthesis are required for producing hemoglobin in erythroblasts for red blood cell production (Metcalf, 2008; Zhu & Emerson, 2002). However, high concentrations of heme are toxic to cells due to oxidative cell damage (Kumar & Bandyopadhyay, 2005; Ryter & Tyrrell, 2000); therefore balanced levels are critical. Heme is highly produced in erythroid progenitor cells, particularly erythroid colony-
forming units (CFU-E) (Ogawa et al., 2001). FLVCR1, which is also highly expressed in CFU-Es (Quigley et al., 2004), has been suggested to act as a safety valve by exporting excess heme out of cells (Ogawa et al., 2001). This was observed in rat renal epithelial NRK cells where FLVCR1 overexpression led to a decrease of intracellular heme. In contrast, reduced FLVCR1 expression increased intracellular heme concentration in feline embryonic fibroblasts (Quigley et al., 2004). Furthermore, disrupted heme export of FLVCR1 led to apoptosis and failed erythroid differentiation of BFU-Es and CFU-Es in lineage-depleted human umbilical cord blood cells and human K562 cells (Quigley et al., 2004; Quigley et al., 2004). FLVCR1 therefore has a crucial role in early erythroid progenitors, since its disruption mimics the erythropoietic defect observed in DBA.

1.6.2 Human homolog of FLVCR1 in DBA

FLVCR1 null mice die in utero due to impaired erythropoiesis, however like DBA patients, they display craniofacial and limb deformities as well as disrupted development of erythroid colony-forming units (CFU-E) (Ball et al., 1996; Keel et al., 2008). Furthermore, postnatal depletion of FLVCR1 results in severely anemic mice (Keel et al., 2008). FLVCR1 disruption in human hematopoietic stem cells recapitulated the hematologic features of DBA by disrupting early erythropoiesis and not myelopoiesis (Rey et al., 2008). While four DBA families have been linked to human chromosome 1q31 (O'Hara et al., 1990; Quigley et al., 2005), the FLVCR1 gene locus (Quigley et al., 2000), no FLVCR1 gene mutations were found (Quigley et al., 2005). Instead, Rey and colleagues found enhanced alternatively spliced variants of the FLVCR1 transcript in DBA patient erythroid cells negative for RPS19 gene mutations, compared to normal patient erythroid cells (Figure 1-5).
Figure 1-5: Alternatively spliced FLVCR1 variants isolated from immature erythroid cells of DBA patients and normal patients.

Alternatively spliced FLVCR1 isoforms were isolated from five DBA patients (D1-D5) and 10 normal patients (N1-N10). FLVCR1 exons are shown in boxes labelled E1-E10. FLVCR1 spliced variants are denoted as FLVCR1 (full length FLVCR1), E2- (exon 2 deletion), E2-E6- (exons 2 and 6 deleted), E3- (exon 3 deletion), E3-E6- (exons 3 and 6 deleted). An asterisk denotes a premature termination codon (PTC). Diagrams of the predicted surface topology of potential FLVCR1 proteins encoded by the alternatively spliced FLVCR1 transcripts are also shown. Normally spliced FLVCR1 transcript encodes a protein predicted to contain 12 transmembrane spanning segments with six extra-cellular loops (numbered 1-6). The DBA and normal patient samples from which the FLVCR1 transcripts were isolated are also denoted. (This figure is from Rey et al., 2008)

The FLVCR1 exon 3 spliced out variant (E3-) caused an in-frame deletion encoding FLVCR1 protein that was disrupted in cellular and surface expression as well as receptor function (Rey et al., 2008). Keel and colleagues showed that murine FLVCR1 E3- transcript completely disrupts heme export function (Keel et al., 2008) therefore raising the possibility of defective heme export of human FLVCR1 E3- transcript. The FLVCR1 E2- transcript was not studied but creates a premature stop codon (PTC) and was therefore was most likely
degraded through the nonsense-mediated decay pathway (Chang et al., 2007). Furthermore, the FLVCR1 E2’ transcript could encode a truncated protein lacking E3 required for heme export (Keel et al., 2008).

Interestingly, RPS19 reduced K562 cells exhibited enhanced aberrant splicing of FLVCR1 exons 2 and 3, which reduced FLVCR1 protein levels (Figure 1-6) (Rey et al., 2008), thereby raising the possibility that FLVCR1 aberrant splicing and FLVCR1 protein insufficiency potentially contribute to the anemia in DBA. These findings strongly suggest that the disruption of the human homolog of FLVCR1 could be one of the underlying mechanisms for DBA pathogenesis.
Figure 1-6: Enhanced aberrant splicing of FLVCR1 transcript and reduced FLVCR1 protein expression in RPS19 reduced human K562 cells (This figure is from Rey et al., 2008).

A. RPS19 protein expression relative to β-actin in RPS19 reduced human K562 cells, R1 and R2. shLuc cells were used as a control.

B. Percent (%) of FLVCR1 transcripts containing exon 2 (E2) and exon 3 (E3) in RPS19 reduced K562 cells

C. Disrupted FLVCR1 protein expression relative to β-actin in RPS19 reduced human K562 cells, R1 and R2. shLuc cells were used as a control.
1.7 Objective of study

1.7.1 Rationale

Diamond Blackfan Anemia (DBA) is a congenital anemia that disrupts the production of erythroid progenitor cells. To date, approximately 25% of DBA patients have a heterozygous mutation in the ribosomal protein (RP) S19 gene, and 30% of patients with normal RPS19 have mutations in other RP genes including RPS24, RPS17, RPS7, RPS10, RPS26, RPL5, RPL11 and RPL35a (Doherty et al., 2010). This suggests that DBA is caused by a disruption of the ribosome and a defect in protein translation. However, this fails to explain why erythropoiesis is specifically disrupted.

Our lab identified the heme exporter and retroviral receptor, FLVCR1, as a major contributor for the erythropoietic defect in DBA. FLVCR1 disruption in vitro and in vivo mimicked the disrupted erythropoiesis observed in DBA (Ebert et al., 2005; Quigley et al., 2005; Rey et al., 2008). Rey and colleagues observed enhanced aberrant splicing of the FLVCR1 transcript in DBA patient erythroid cells through the exon skipping of FLVCR1 exons 2 and 3. The enhanced FLVCR1 aberrant splicing disrupted FLVCR1 protein expression, cell surface localization, and function that is critical for erythropoiesis (Rey et al., 2008). Furthermore, down-regulation of RPS19, in human K562 cells, led to enhanced FLVCR1 aberrant splicing of exons 2 and 3, thus showing for the first time a relationship between a ribosomal protein mutated in DBA (RPS19) and FLVCR1 splicing. What remains unclear is whether the other ribosomal proteins associated with DBA also lead to aberrant FLVCR1 splicing and disrupted erythropoiesis. In this thesis, I studied the role of RPS17 (implicated in DBA) and RPS3A (not implicated in DBA) on erythropoiesis and on FLVCR1 splicing. I used human K562 cells that were disrupted in expression of the specific ribosomal
proteins above, and analyzed RP transcript expression, protein expression, FLVCR1 aberrant splicing and erythroid differentiation. Human K562 cells are megakaryocyte-erythroid progenitors that can be induced to differentiate into the erythroid lineage when incubated with hemin (Lozzio & Lozzio, 1975).

1.7.2 Hypothesis and aims

The central hypothesis of my project is that ribosomal proteins RPS17, RPS24, RPL5 and RPL11 implicated in DBA lead to aberrant FLVCR1 splicing of exons 2 and 3, and disrupted erythropoiesis; similar to the effects of RPS19. However, this thesis will outline the results obtained from studying RPS17 (associated with DBA), in comparison to another DBA mutant, RPS19 (positive control), and RPS3A (not associated with DBA).

I therefore aim to investigate whether other ribosomal proteins such as RPS17 also lead to aberrant FLVCR1 splicing and disrupted erythroid differentiation. This will be done by testing the significance of reduced RPS17 (associated with DBA) on FLVCR1 aberrant splicing and erythropoiesis in comparison to reduced RPS19 (positive control) and reduced RPS3A (not associated with DBA), in human K562 cells. This will help delineate the relationship between ribosomal proteins implicated in DBA and FLVCR1 aberrant splicing and help identify which ribosomal protein is responsible for DBA. Furthermore, I will determine whether FLVCR1 aberrant splicing is specific to DBA by comparing it to another ribosomal disorder, Shwachman Diamond syndrome (SDS). This will be done by examining the role of reduced Shwachman-Bodian-Diamond syndrome (SBDS) gene, mutated in SDS patients, on FLVCR1 splicing and erythropoiesis in human K562 cells.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines

Human embryonic kidney 293T cells (HEK293T) packaging cells were maintained in Dulbecco’s minimal essential medium (DMEM) with high glucose (4500 mg/L) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin (Pen/Strep). Human TE671 and HeLa cells were maintained in DMEM with low glucose (1000 mg/L) supplemented with 10% FBS and Pen/Strep. K562 human erythroleukemia cells were provided by Dr. Yigal Dror (The Hospital for Sick Children) and maintained in RPMI media with L-glutamine and supplemented with 10% heat-inactivated FBS and Pen/Strep. All cells were maintained at 37°C and 5% CO₂ conditions.

2.2 Construction of expression vectors

Human FLVCR1 and feline THTR1 (thiamine transporter) each fused to an hemagglutinin (HA) epitope tag in pFBneo retroviral expression vectors were generated as previously described (Brown et al., 2006). FLVCR1 HA and THTR1 HA (containing the epitope tag) were digested out of the pFBneo G418-resistant retroviral vector (Agilent) (Figure 2-1a) and cloned into a pMSCV blasticidin-resistant retroviral expression vector (kindly provided by Dr. Scott Grey-Owen, University of Toronto) (Figure 2-1b) to generate pMSCV-FLVCR1 HA and pMSCV-THTR1 HA, respectively. These expression constructs were subsequently used in the rescue experiments.
A.

B.

Figure 2-1: pFBneo and pMSCV retroviral vectors used to over express FLVCR1 and THTR1.
A) pFBneo G418- resistant retroviral vector (Agilent) used to over express human FLVCR1 in RPS19- reduced K562 cells.
B) pMSCV blasticidin- resistant retroviral vector (kindly provided by Dr. Scott Grey-Owen) used to over express human FLVCR1 and feline THTR1 in RPS19- reduced K562 cells.
2.3 Generation of RP knockdown cells

2.3.1 Ribosomal protein shRNA constructs

Frozen bacterial glycerol stocks of two small hairpin RNA (shRNA) clones in lentiviral pLKO.1 puromycin-resistant plasmids (Figure 2-2) targeting each ribosomal protein S17 and S3A along with a luciferase-targeted shRNA lentiviral pLKO.1 puromycin-resistant plasmid (control) were obtained (Sigma-Aldrich, St. Louis, MO). The shRNA antisense oligonucleotides were as follows:

shLuc: 5’- GACATTTCTGAAGTACTCAGCG - 3’
shRPS17-1: 5’ - AATTGTCTCTCCTTTTCTCTCT - 3’
shRPS17-3: 5’ - AATAATCTCTCCTGATCAGG - 3’
shRPS3A-2: 5’ - TTTCCAATTCAAACCTTGGG - 3’
shRPS3A-3: 5’ - ATTTCCATCATTTTCCCGG - 3’

The bacterial stocks of these shRNA clones were streaked on Luria broth (LB) agar plates with ampicillin (100µg/ml). 100mls of LB agar liquid cultures with ampicillin (100µg/ml) were inoculated with well-isolated bacterial colonies for 12-16 hours at 37°C under agitation. Inoculated cultures were centrifuged and shRNA lentiviral plasmid DNA was purified using the Qiagen Plasmid Midi kit, and concentration was determined using ultraviolet (UV) spectroscopy.

In order to knockdown ribosomal protein S19, two shRNAs, shRPS19-1 (5’-AAGAGCTTGCTCCCTACGA-3’) and shRPS19-2 (5’-ACTGACACCTCAGGGACA-3’), along with a control shRNA targeting luciferase (5’-CTTACGCTGAGTACTTCGA-3’) in
pRETRO-SUPER retroviral expression vector (Figure 2-3), were kindly provided by Dr. Colin A. Sieff (Ebert et al., 2005).
Figure 2-2: Map of pLKO.1 puromycin- resistant lentiviral vector with schematic of expressed shRNA sequence and structure.

Transient shRNA- mediated knockdown of ribosomal proteins S17 and S3A were achieved using the replication incompetent pLKO.1 puromycin- resistant lentiviral vector with shRNA targeting the gene of interest.
Figure 2-3: pRETROSUPER puromycin-resistant retroviral vector used to knockdown RPS19 in human K562 cells.
Transient shRNA-mediated knockdown RPS19 and luciferase (control) was achieved using the pRETROSUPER puromycin-resistant retroviral vector, kindly provided by Dr. Colin A. Sieff.
2.3.2 Lentivirus and retroviral production

Using PolyFect transfection reagent (Qiagen), shLuc, shRPS17-1, shRPS17-3, shRPS3A-2 and shRPS3A-3 were individually transfected into previously seeded HEK293T cells with a packaging plasmid (psPAX2) and an envelope plasmid (pMD2.G) to produce lentiviral particles. psPAX2 (Figure 2-4a) is a 2nd generation packaging plasmid that has a CAG promoter to efficiently express packaging proteins gag, pol, tat and rev (Salmon & Trono, 2006). pMD2.G (Figure 2-4b) encodes the G- protein of vesicular stomatitis virus glycoprotein (VSV-G) envelope protein which enables viral entry (Salmon & Trono, 2006). HEK293T transfected cells were incubated at 37°C in 5% CO₂ for 48 hours. Supernatant containing the lentiviral particles was harvested by filter sterilization through a 0.45µm filter and stored at -80°C until used for infections.

shLuc, shRPS19-1, shRPS19-2, pFBneo-FLVCR1 HA, pMSCV-FLVCR1 HA and pMSCV-feTHTR1 HA were individually transfected into HEK293T cells with the Moloney murine leukemia virus gag and pol genes (MLV gag-pol) and VSV-G to produce retroviral particles.
Figure 2-4: Packaging and envelope plasmids used with the pLKO.1 shRNA vector to knockdown RPS17 and RPS3A in human K562 cells.

A) psPAX2 packaging plasmid used a CAG promoter to efficiently express proteins gag, pol, tat and rev for viral packaging.

B) pMD2.G envelope plasmid was used to express G protein of vesicular stomatitis virus glycoprotein (VSV-G) envelope protein to enable viral entry.
2.3.3 Lentiviral and retroviral infection

To achieve RPS19, RPS17 and RPS3A knockdown as well as over expression of FLVCR1 and THTR1 in human K562 cells, 1ml of filtered retrovirus and lentivirus was incubated with 5 x 10^5 human K562 cells for four hours at 37°C with gentle agitation at every hour, in the presence of polybrene (8ug/ml). After centrifugation for 5 minutes at 1000 rpm (with a Jouan CR3i T40 rotor), virus supernatant was replaced with 1ml of RPMI and centrifuged as previously. Medium was aspirated and infected cells were then cultured in RPMI and incubated at 37°C and 5% CO₂. Puromycin (1.5µg/ml) was added to RPS17 and RPS3A shRNA- transduced K562 cells 24 hours later. G418 (1.5mg/ml) was added to pFBneo-FLVCR1 HA infected cells, and blasticidin (8µg/ml) was added to pMSCV-FLVCR1 HA and pMSCV-THTR1 HA infected cells 24 hours post infection.

To over- express FLVCR1 and THTR1 in human TE671 and HeLa cells, 1ml of 0.45µm- filtered retrovirus was incubated with previously seeded TE671 and HeLa cells in a 24- well plate for four hours at 37°C and 5% CO₂ in the presence of polybrene (8ug/ml). Virus was then replaced with DMEM with low glucose at 37°C and 5% CO₂. Forty- eight hours post infection, cells were washed, treated with trypsin and transferred to a 100mm plate. Four hours later, blasticidin (8µg/ml) was added.
2.4 RNA extraction and quantitative real-time PCR

Using Qiagen’s RNeasy minikit, total RNA was extracted 7 days post RPS19 shRNA infection and 4 days post RPS17 and RPS3A shRNA infections in human K562 cells. These time-points were found optimal for efficient knockdown of each gene. cDNA was then synthesized using Superscript III reverse transcriptase (Invitrogen) and the oligo(dT) primer.

mRNA transcript expression was measured by quantitative real-time PCR (qRT-PCR) using SYBR green technology and the standard curve method. Standard curves for each gene were determined by 1:10-1:10000 dilutions of untreated human K562 cells to quantify initial template quantities. Samples were run in triplicates on a 96 well plate. In order to determine total mRNA transcript expression, primers targeting exon 1 of each gene were used (Figure 2-5a). In order to detect splicing of FLVCR1 exon 2 (E2) and/or exon 3 (E3), I used primers targeting the junction between exons 2 and 3 (Figure 2-5b). The percentage of FLVCR1 mRNA transcripts containing exons 2 and 3 were determined by the ratio of FLVCR1 exon 2-3 expression to FLVCR1 exon 1 expression (total FLVCR1 mRNA transcript expression). The mRNA transcript expression of each gene was normalized to β-actin mRNA expression. The primers used for qRT-PCR are listed in Table 2-1.
A.

**Figure 2-5: Schematic of primers used in quantitative real time PCR to quantify total mRNA transcript levels of the gene of interest, and aberrant splicing of FLVCR1 transcripts.**

A) Exon 1 forward and reverse primers (E1/αE1) were used in qRT-PCR to quantify total mRNA transcript expression of β-actin, RPS17, RPS19 and RPS3A. Dashed line represents subsequent exons not displayed.

B) Exon 1 forward and reverse primers (E1/αE1) were used in qRT-PCR to quantify total FLVCR1 mRNA transcript expression. Primers targeting the junction of exons 2 and 3 (E2/αE3) were used to quantify FLVCR1 transcripts containing E2 and E3. The proportion of mRNA amplified from E2/αE3 primers to that of E1/αE1 primers was used to determine the percentage of FLVCR1 transcripts containing exons 2 and/or 3. Dashed line represents subsequent exons not displayed.
Table 2-1: Primer sets used in qRT-PCR to detect mRNA expression

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Amplicon (bps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>F 5’- TCAGTGAATTTGAGAAGGAG- 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5’- AGGAAGAAGGGCTGGAAGAG- 3’</td>
<td>175</td>
</tr>
<tr>
<td>RPS17</td>
<td>F 5’- TTTACCAAGGACCCGCAACAT- 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5’- ATATATGCGATCTCCTCCTGCAC- 3’</td>
<td>142</td>
</tr>
<tr>
<td>RPS19</td>
<td>F 5’- ATCTCCACCACCTGTTCTCTCCAG- 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5’- CGTTTCCCTCTCCCCGGAGTGAAT- 3’</td>
<td>113</td>
</tr>
<tr>
<td>RPS3A</td>
<td>F 5’- TTGCTCTCTGACCAGCACCAT- 3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5’- GGATCAACCACCTTCTCTTTGCTCC- 3’</td>
<td>92</td>
</tr>
<tr>
<td>FLVCR1 exon 1</td>
<td>F 5’- TCATGGTGTACATGCTGGCCTA- 3’</td>
<td></td>
</tr>
<tr>
<td>FLVCR1 exons 2 &amp; 3</td>
<td>R 5’- AAGAGATGTTGTGCCACACTGCAGGCG- 3’</td>
<td>152</td>
</tr>
<tr>
<td>FLVCR1 exons 2 &amp; 3</td>
<td>F 5’- GAACATCAGCTGTTGCCACA - 3’</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>R 5’- TGTCTTTGAAGAGCTGCTTGA - 3’</td>
<td></td>
</tr>
</tbody>
</table>
2.5 Confirming primer specificity

Polymerase chain reaction (PCR) was used in order to confirm that the primers (β-actin, RPS17, RPS19, RPS3A, FLVCR1 E1-1 and FLVCR1 E2-3) used in qRT-PCR were indeed specific in amplifying the product of interest. The total reaction contained 5µls of 10x PCR buffer minus Mg, 1µl of 10mM dNTP mixture, 1.5µl of 50mM MgCl₂, 2.5µl of each 10µM primer, 0.5µl of 5U/ul Taq DNA Polymerase, 5ul of K562 WT template cDNA and RNase/DNase free water to a total volume of 50µl. The conditions of the PCR were follows:

1. Initial denaturation: 94°C for 10mins
2. 35 cycles of
   - Denaturation: 94°C for 45 seconds
   - Annealing: 55°C for 30 seconds
   - Extension: 72°C for 1 minute, 30 seconds
   - Final extension: 72°C for 10 minutes

The total volume of PCR products were run on a 1% agarose gel. 5µl of the PCR products of RPS19, FLVCR1 E1-1 and FLVCR1 E2-3 primers were used as template DNA for a second round of PCR to amplify more product, which was run on an agarose gel to provide a stronger signal (Figure 2-6). The single ~100bp bands were excised out and purified using Qiagen’s Gel Extraction kit. The eluted DNA was then subjected to sequencing (The Centre for Applied Genomics) using the forward primers for the gene of interest.
Figure 2-6: Polymerase chain reaction (PCR) and DNA sequencing was used to confirm specificity of primers used to detect mRNA transcript expression.

Lanes (1: β-actin, 2: RPS17, 3: FLVCR1 E2-3 PCR #1, 4: FLVCR1 E2-3 PCR #2 to amplify more product) are PCR products ran on a 1% agarose gels. Bands were then excised, purified and sent for sequencing.
2.6 Western blot analysis

Western blots were used to examine protein expression levels of ribosomal proteins, and HA tagged- FLVCR1 and THTR1 proteins.

RPS19- reduced K562 cells were harvested 7 days post shRNA infection and RPS17- and RPS3A- reduced K562 cells were harvested 4 days post shRNA infections. These time-points were found optimal for efficient knockdown of each gene. Cells were centrifuged at 1000rpm (with a Jouan CR3i T40 rotor) for 5 minutes at room temperature. Medium was aspirated and cells were incubated on ice for 10 minutes with 40µl of cell lysis buffer containing 20 mM Tris-HCl pH7.5, 1% Triton X-100, 0.05% sodium dodecyl sulphate (SDS), sodium deoxycholate (5mg/ml), 150 mM NaCl and 1mM phenylmethylsulfonyl fluoride (PMSF). Lysates were centrifuged at 14000rpm (with a Beckman Microfuge 22R F301.5 rotor) for 15 mins at 4°C in order to remove cellular genomic DNA and the supernatant was stored at -80°C.

Approximately 30µg of protein, as determined by the Bradford assay, was run on a 12% sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) gel for 100 minutes at 110V. Proteins were transferred on to a nitrocellulose membrane (Pall Corp.) and blocked for one hour at room temperature in 5% skim milk in Phosphate Buffered Saline with 0.1% Tween 20 (PBST). Blots were then incubated with the following human primary antibodies at 4°C overnight: 1:2500 of anti- β-actin monoclonal antibody (Sigma- Aldrich, St. Louis, MO), 1:1000 of anti- RPS17 monoclonal antibody (Abcam), 1:1000 anti- RPS19 monoclonal antibody (Abcam), 1:1000 anti- RPS3A polyclonal antibody (ProteinTech), and 1:1000 anti- HA antibody (Covance). Blots were washed with 1x PBST and incubated with 1:7000 dilution of either rabbit anti- mouse IgG horseradish peroxidise conjugate or goat
anti-rabbit IgG horseradish peroxidise conjugate, for 1.5 hours at room temperature. Signals were detected using chemiluminescent horseradish peroxidise (HRP) antibody detection reagent (Denville Scientific Inc.), followed by exposure to Hyperfilm (GE Healthcare). Protein expression was quantified by densitometry analysis using Image J software provided by the National Institutes of health (NIH).

2.7 Analysis of erythroid differentiation

shRNA- transduced human K562 progenitor cells were induced to differentiate with 30uM hemin (Sigma- Aldrich, St. Louis, MO) for 5 days. 4mM hemin stocks consisted of 13 mg of hemin and 0.2 ml of 0.5M NaOH. 0.25 ml of 1M Tris HCl (pH 7.8) was then added, the solution was diluted to 5 ml with distilled water, filter sterilized and stored at -20°C until use (Addya et al., 2004). Differentiated human K562 cells were detected by benzidine staining. Cells were centrifuged at 500rpm (with a F-45-12-11 rotor) for 5 minutes, media was aspirated and 40 µl cold phosphate buffered saline (PBS) was added to wash cells. 40 µl of benzidine solution (0.2% (w/v) benzidine dihydrochloride (Sigma- Aldrich, St. Louis, MO), 2.92% (v/v) acetic acid) was activated with 0.6% (v/v) hydrogen peroxide and added to cells (Addya et al., 2004). At least 300 cells were counted for each cell line following treatment using light microscopy to quantify benzidine positive stained cells which were indicative of hemoglobin’s pseudo-peroxidase activity in K562 cells, and therefore erythroid differentiation.
2.8 Statistics

Means and standard errors (SE) based on independent experiments were used to describe results regarding mRNA transcript expression, aberrant FLVCR1 splicing of exons 2 and 3 and erythroid differentiation. Student’s t-test was used to determine the statistical significance between two means. Welch’s t-test was used to determine the statistical significant between two means with unequal sample size. P values equal to or less that 0.05 were considered statistically significant.
CHAPTER 3: RESULTS

3.1 Established ribosomal protein reduced human K562 cells

RPS19 or FLVCR1 disruption individually disrupted erythropoiesis at the BFU-E to CFU-E stage which mimics the disrupted erythropoiesis observed in DBA (Ebert et al., 2005; Quigley et al., 2005; Rey et al., 2008). Our lab previously reported that DBA erythroid cells over-express aberrantly spliced FLVCR1 transcripts encoding proteins that are disrupted in expression and cell surface localization (Rey et al., 2008). Moreover, down regulation of RPS19 in human K562 cells resulted in enhanced aberrant splicing of FLVCR1 exon 2 (E2) and exon 3 (E3) (Rey et al., 2008). This identified a relationship between RPS19 (a RP mutated in DBA) and FLVCR1 aberrant splicing, in the context of E2 and E3. The goal of this aim was to investigate whether other ribosomal proteins mutated in DBA, such as RPS17, also lead to FLVCR1 aberrant splicing and disrupted erythropoiesis.

I used retroviral-mediated RPS19 knockdown K562 cells as a positive control (cell line re-generated by Francisca Aidoo), which disrupted erythropoiesis and caused enhanced aberrant splicing of FLVCR1 transcript (Aidoo, F., M.Sc. thesis, 2012). I investigated the effects of RPS17 knockdown using lentiviral-mediated delivery of RPS17-specific shRNAs in human K562 cells and tested erythroid cell differentiation of these cells, aberrant splicing of FLVCR1 transcripts, and expression of ribosomal proteins such as RPS3A and RPS19. I also investigated the effects of RPS3A, which is not associated with DBA, using lentiviral-mediated delivery of RPS3A-targeted shRNAs in human K562 cells. A shRNA targeting luciferase, termed shLuc, was used as a control.

The human K562 erythroleukemic cell line represents megakaryocyte-erythroid bone marrow progenitor cells (Lozzio & Lozzio, 1975). These cells have widely been used to
model erythropoiesis since they undergo erythroid differentiation by increasing globin mRNA expression upon induction with hemin (Sassa, 1976; Rutherford et al., 1979; Leder & Leder, 1975).

RPS19- targeted shRNA sequences, shRPS19-1 and shRPS19-2 (Ebert et al., 2005), were used to knockdown RPS19 in human K562 cells (generated by Francisca Aidoo). The shRPS19-1 and shRPS19-2 shRNAs have been previously used to successfully knockdown RPS19 expression in human K562 cells, HEL cells (human erythroleukemia cell line) and human bone marrow CD34+ cells (Ebert et al., 2005; Rey et al., 2008). RPS19 knockdown K562 cells were reduced in RPS19 protein expression as determined by Western blotting, and reduced in RPS19 transcript expression as determined by qRT-PCR. RPS19 mRNA expression was significantly reduced 2.7- fold in shRPS19-1 K562 cells and 1.7- fold in shRPS19-2 K562 cells (Figure 3-1b). This correlated with a 4.2- fold decrease and a 3- fold decrease of RPS19 protein levels in shRPS19-1 and shRPS19-2 K562 cells, respectively (Figure 3-1a). It has been found that DBA patients with RPS19 mutations (such as PTCs) have a two- to four fold decrease in RPS19 mRNA and two to three fold decrease in RPS19 protein levels (Gazda et al., 2004). The RPS19 knockdown K562 cells used in this study is therefore representative of RPS19 mutated DBA patients.

RPS17 shRNA- expressing human K562 cells, shRPS17-1 and shRPS17-3, were successfully reduced in RPS17 mRNA transcript and protein expression compared to luciferase shRNA- expressing K562 cells, shLuc (control). qRT-PCR was used to confirm significant mRNA knockdown revealing a 7.9- fold decrease and a 12- fold decrease in transcript levels of shRPS17-1 and shRPS17-3 K562 cells, respectively (Figure 3-2b). Reduced mRNA transcript levels correlated with reduced protein expression, as determined
by Western blotting. Densitometry analysis revealed that RPS17 protein levels were reduced by 3.4-fold in shRPS17-1 K562 cells and by 7.3-fold in shRPS17-3 K562 cells compared to the control (Figure 3-2a).

To confirm whether the effects of RPS19 and RPS17 knockdown are specific to DBA associated RPs, RPS3A was used as a control since it has not been found mutated in DBA patients to date (Cmejla et al., 2001). Two RPS3A reduced K562 cell lines were successfully knocked down in RPS3A mRNA and protein expression and were termed shRPS3A-2 and shRPS3A-3. Compared to the control (shLuc), total RPS3A mRNA transcript expression was significantly reduced 2.2-fold and 3.6-fold in shRPS3A-2 and shRPS3A-3 K562 cells, respectively (Figure 3-3b). RPS3A protein expression was reduced 4.5-fold in both RPS3A knockdown K562 cells (Figure 3-3a).
Figure 3-1: RPS19 knockdown in human K562 cells

A) Western blot confirmed RPS19 knockdown using 30ug of protein from RPS19 shRNA transduced human K562 cells, termed shRPS19-1 and shRPS19-2. RPS19 reduction was compared to control (shLuc: shRNA targeting luciferase gene). Western blot is representative of 6 independent experiments (Figure from Aidoo, F., M.Sc. thesis, 2012).

B) Quantitative real-time PCR (qRT-PCR) using RPS19 primers targeting exon 1 confirmed knockdown of RPS19 mRNA transcript levels. The data (normalized to β-actin) represents the mean ± SE of 6 independent experiments. Statistical significance was determined using the Student’s t-test as marked with an asterisk, (p≤0.04).
Figure 3-2: RPS17 knockdown in human K562 cells.
A) Western blot confirmed RPS17 knockdown using 30ug of protein from RPS17 shRNA transduced human K562 cells, termed shRPS17-1 and shRPS17-3. RPS17 reduction was compared to control (shLuc: shRNA targeting luciferase gene). Western blot is representative of 3 independent experiments.
B) Quantitative real-time PCR (qRT-PCR) using RPS17 primers targeting exon 1 confirmed knockdown of RPS17 mRNA transcript levels. The data (normalized to β-actin) represents the mean ± SE of 3 independent experiments. Statistical significance was determined using the Student’s t-test as marked with an asterisk, (p≤0.007).
**Figure 3-3: RPS3A knockdown in human K562 cells.**

A) Western blot confirmed RPS3A knockdown using 30ug of protein from RPS3A shRNA transduced human K562 cells, termed shRPS3A-2 and shRPS3A-3. RPS3A reduction was compared to control (shLuc: shRNA targeting luciferase gene). Western blot is representative of 6 independent experiments.

B) Quantitative real-time PCR (qRT-PCR) using RPS3A primers targeting exon 1 confirmed knockdown of RPS3A mRNA transcript levels. The shRPS3A-2 K562 cells’ data (normalized to β- actin) represents the mean ± SE of 4 independent experiments. The shRPS3A-3 K562 cells’ data (normalized to β- actin) represents the mean ± SE of 6 independent experiments. Statistical significance was determined using Welch’s t-test as marked with an asterisk, (p≤0.01).
3.2 Disrupted erythropoiesis and aberrant FLVCR1 splicing is specific to RPS19- reduced K562 cells

3.2.1 RPS19 disruption leads to impaired erythropoiesis while RPS17 and RPS3A disruption does not

A defining feature seen in DBA patients is disrupted differentiation of erythroid progenitor cells (Diamond et al., 1976). The gene encoding ribosomal protein S19 is mutated in 25% of DBA patients and is the only DBA- implicated ribosomal protein whose role on erythropoiesis has been studied both in vitro and in vivo (Matsson et al., 2004; Matsson et al., 2006). RPS19 disruption was shown to interfere with the normal progression of erythroid differentiation in many cell culture models of DBA (Flygare et al., 2005; Miyake et al., 2008; Hamaguchi et al., 2002; Sieff et al., 2010).

My DBA cell culture model of RPS19 reduced human K562 cells recapitulates the disrupted erythropoiesis seen in DBA patients and in vitro studies of DBA (Gazda et al., 2004; Flygare et al., 2005; Miyake et al., 2008; Hamaguchi et al., 2002; Sieff et al., 2010). The disruption of RPS19 at the protein and mRNA transcript level led to a significant disruption in erythroid differentiation by 63.74% and 64.67% in shRPS19-1 and shRPS19-2 K562 cells, respectively (Figure 3-4) (Aidoo, F., M.Sc. thesis, 2012).

Interestingly, knockdown of RPS17 protein expression in human K562 cells (shRPS17-1 and shRPS17-3) did not disrupt erythroid differentiation as determined by benzidine staining (see Materials & Methods section 2.7) (Figure 3-5). Normal erythropoiesis was also observed in RPS3A reduced K562 cells, shRPS3A-2 and shRPS3A-3, compared to the control, shLuc (Figure 3-6).
These results suggest and corroborate the positive role of RPS19 in erythroid differentiation. Furthermore, RPS3A, which is not associated with DBA, did not disrupt erythropoiesis and therefore served as an appropriate negative control for DBA. More importantly, this is the first study of RPS17’s role on erythropoiesis revealing that a disruption of RPS17 in human K562 cells leads to normal erythropoiesis.

**Figure 3-4: RPS19 knockdown in human K562 cells leads to disrupted erythroid differentiation** (Figure from Aidoo, F., *M.Sc. thesis*, 2012).

RPS19 knockdown K562 cells were induced to differentiate using 30µm hemin. Benzidine staining was used to detect hemoglobinized cells. The shRPS19-1 K562 cells’ data (normalized to β- actin) represents the mean ± SE of 7 independent experiments. The shRPS19-2 K562 cells’ data (normalized to β- actin) represents the mean ± SE of 8 independent experiments. Statistical significance was determined using the Welch’s t-test as marked with an asterisk, (p≤0.0002).
**Figure 3-5: RPS17 knockdown in human K562 cells does not disrupt erythroid differentiation.**
RPS17 knockdown K562 cells were induced to using 30μm hemin. Benzidine staining was used to detect hemoglobinized cells. Data (normalized to β-actin) represents the mean ± SE of 3 independent experiments.

**Figure 3-6: RPS3A knockdown in human K562 cells does not disrupt erythroid differentiation.**
RPS3A knockdown K562 cells were induced to differentiate using 30μm hemin. Benzidine staining was used to detect hemoglobinized cells. Data (normalized to β-actin) represents the mean ± SE of 3 independent experiments.
3.2.2 Aberrant FLVCR1 splicing correlates with disrupted erythropoiesis

Our lab previously observed enhanced aberrant splicing of exon 2 (E2) and exon 3 (E3) of FLVCR1 transcripts in DBA patients and in RPS19 down regulated K562 cells (Rey et al., 2008). qRT-PCR was used to determine the FLVCR1 transcripts containing E2 and E3. Primers targeting E1 of FLVCR1 were used to determine total FLVCR1 mRNA transcript expression and primers for E2 and E3 were used to determine the percent of FLVCR1 mRNA transcripts containing E2 and E3 (Figure 2-5b).

Total FLVCR1 transcript (E1-1) expression was not disrupted in the RPS19 reduced K562 cells, (shRPS19-1 and shRPS19-2) compared to the control, shLuc (Figure 3-7a) (Aidoo, F., M.Sc. thesis, 2012). However, down- regulation of RPS19 in K562 cells significantly enhanced aberrant splicing of FLVCR1 transcript by 40-60% (Figure 3-7b) (Aidoo, F., M.Sc. thesis, 2012) which recapitulated data observed by Rey and colleagues (Rey et al, 2008).

Interestingly, human K562 cells reduced in RPS17 (shRPS17-1 and shRPS17-3) showed significantly increased expression of total FLVCR1 transcript (Figure 3-8a) with no aberrant splicing of E2 and E3 (Figure 3-8b). Thus RPS17 reduced K562 cells have constitutively spliced FLVCR1 transcripts which correlates with normal erythropoiesis (Figure 3-5).

Knockdown of RPS3A (shRPS3A-2 and shRPS3A-3) also did not induce aberrant FLVCR1 splicing (Figure 3-9b). Total FLVCR1 mRNA transcript expression increased in RPS3A knocked down K562 cells, significantly so in shRPS3A-3 K562 cells. The normal levels of constitutive FLVCR1 splicing in these shRPS3A-2 and shRPS3A-3 K562 cells correlated with normal erythropoiesis (Figure 3-6).
Figure 3-7: RPS19 knockdown in human K562 cells leads to aberrant FLVCR1 splicing of exons 2 and 3 (Figure from Aidoo, F., M.Sc. thesis, 2012).

**A)** Total FLVCR1 transcript expression (Exon 1-1) was quantified by qRT-PCR using primers targeting exon 1. FLVCR1 exons 2 and 3 expression was quantified by qRT-PCR using primers targeting the junction of exons 2 and 3 of FLVCR1. Data (normalized to β-actin) represents the mean ± SE of 4 independent experiments. Statistical significance was determined using the Student’s t-test as marked with an asterisk, (p≤0.005).

**B)** Bars represent percent (%) of FLVCR1 mRNA transcripts containing exons 2 and 3 relative to shLuc (control), measured by the ratio of FLVCR1 exons 2 and 3 to FLVCR1 exon 1 expression. Data (normalized to β-actin) represents the mean ± SE of 4 independent experiments. Statistical significance was determined using the Student’s t-test as marked with an asterisk, (p≤0.01).
Figure 3-8: RPS17 knockdown in human K562 cells does not lead to aberrant FLVCR1 splicing of exons 2 and 3.

A) Total FLVCR1 transcript expression (Exon 1-1) was quantified by qRT-PCR using primers targeting exon 1. FLVCR1 exons 2 and 3 expression (Exon 2-3) was quantified by qRT-PCR using primers targeting the junction of exons 2 and 3 of FLVCR1. Data (normalized to β-actin) represents the mean ± SE of 4 independent experiments in shRPS17-1 K562 cells and 3 independent experiments in shRPS17-3 K562 cells. Statistical significance was determined using Welch’s t-test, *= p≤0.04, **= p≤0.0009.

B) Bars represent percent (%) of FLVCR1 mRNA transcripts containing exons 2 and 3 measured by the ratio of FLVCR1 exons 2 and 3 to FLVCR1 exon 1 expression. Data (normalized to β-actin) represents the mean ± SE of 4 independent experiments in shRPS17-1 K562 cells and 3 independent experiments in shRPS17-3 K562 cells.
Figure 3-9: RPS3A knockdown in human K562 cells does not lead to aberrant FLVCR1 splicing of exons 2 and 3.

A) Total FLVCR1 transcript expression (Exon 1-1) was quantified by qRT-PCR using primers targeting exon 1. FLVCR1 exons 2 and 3 expression (Exon 2-3) was quantified by qRT-PCR using primers targeting the junction of exons 2 and 3 of FLVCR1. Data (normalized to β-actin) represents the mean ± SE of 4 independent experiments in shRPS3A-2 K562 cells and 5 independent experiments in shRPS3A-3 K562 cells. Statistical significance was determined using Welch’s t-test, as marked with an asterisk, p≤0.04.

B) Bars represent percent (%) of FLVCR1 mRNA transcripts containing exons 2 and 3 relative to shLuc. Data (normalized to β-actin) represents the mean ± SE of 4 independent experiments in shRPS3A-2 K562 cells and 5 independent experiments in shRPS3A-3 K562 cells.
3.2.3 Reduced RPS19 mRNA transcript expression correlates with aberrant FLVCR1 splicing and disrupted erythropoiesis

Interestingly, only RPS19 reduced K562 cells, (shRPS19-1 and shRPS19-2), led to aberrant FLVCR1 splicing and disrupted erythropoiesis. Previous studies have shown that RPS19 mutated DBA erythroid cells are down-regulated in other ribosomal proteins (Gazda, Kho et al., 2006; Koga et al., 2006). To assess the effect of RPS19, RPS17 and RPS3A shRNA expression in K562 cells on expression of ribosomal proteins, I analysed protein and mRNA transcript levels of RPS19, RPS17 and RPS3A in the RP reduced K562 cells.

RPS19 reduced K562 cells dramatically reduced levels of RPS3A and RPS17 protein expression by 89.6-100% and 93-99%, respectively (Figure 3-10a). Similarly, RPS17 reduced K562 cells showed reduced levels of RPS19 and RPS3A protein expression (Figure 3-11a), and RPS3A reduced cells showed reduced RPS19 and RPS17 protein expression levels (Figure 3-12a). Interestingly, RPS19 protein expression was down-regulated in both RPS17 and RPS3A reduced K562 cells to similar levels observed in RPS19 reduced cells. However, as shown in Figures 3-4 and 3-7, aberrant FLVCR1 splicing and disrupted erythropoiesis was only observed in RPS19 shRNA-expressing K562 cells. My findings suggest that reduced RPS19 protein expression was not responsible for aberrant FLVCR1 splicing and disrupted erythropoiesis.

I then determined RP transcript levels in RPS19, RPS17 and RPS3A shRNA-expressing K562 cells using qRT-PCR. RPS19 reduced K562 cells did not affect RPS17 (Figure 3-10b) and RPS3A (Figure 3-10c) transcript expression levels. Interestingly, RPS17 reduced K562 cells did not affect RPS3A transcript expression (Figure 3-11c); however, RPS19 mRNA levels were significantly enhanced compared to RPS19 expression in shLuc
control (Figure 3-11b). Similarly, RPS3a reduced K562 cells did not affect RPS17 mRNA levels (Figure 3-12b), but significantly increased RPS19 transcript expression (Figure 3-12c). Taken together, my results show a strong correlation between reduced RPS19 transcript expression, enhanced FLVCR1 aberrant splicing and disrupted erythropoiesis.
Figure 3-10: RPS19 knockdown in human K562 cells reduces RPS17 and RPS3A protein expression levels, but not mRNA transcript expression levels.

A) RPS19 knockdown in human K562 cells leads to reduced protein expression of RPS17 and RPS3A. Western blotting was used to detect RPS19, RPS17 and RPS3A protein expression in RPS19 knockdown K562 cells 7 days post shRNA transduction. β-actin was used as a loading control.

B) RPS17 mRNA transcript expression is not significantly reduced in shRPS19-1 K562 cells and is comparable to shLuc control in shRPS19-2 K562 cells. qRT-PCR using RPS17 primers targeting exon 1 of RPS17 was used to determine total RPS17 mRNA transcript expression. Data (normalized to β-actin) represents mean ± SE of 4 independent experiments.

C) RPS3A mRNA transcript expression is maintained in shRPS19-1 and shRPS19-2 K562 cells. RPS3A primers targeting exon 1 of RPS3A was used to determine total RPS3A mRNA transcript expression. Data (normalized to β-actin) represents mean ± SE of 3 independent experiments.
**Figure 3-11: RPS17 knockdown in human K562 cells reduces RPS19 and RPS3A protein expression levels, but increases RPS19 mRNA transcript expression.**

A) RPS17 knockdown in human K562 cells leads to reduced protein expression levels of RPS19 and RPS3A. Western blotting was used to detect RPS17, RPS19 and RPS3A protein expression in RPS17 knockdown K562 cells 4 days post shRNA transduction. β-actin was used as a loading control.

B) RPS19 mRNA transcript expression is increased in shRPS17-1 K562 cells and significantly increased in shRPS17-3 K562 cells. qRT-PCR using RPS19 primers targeting exon 1 of RPS19 was used to determine total RPS19 mRNA transcript expression. Data (normalized to β-actin) represents the mean ± SE of 5 independent experiments in shRPS17-1 K562 cells and 6 independent experiments in shRPS17-3 K562 cells. Statistical significance was determined using Student’s t-test, as marked with an asterisk, p≤0.001.

C) RPS3A mRNA transcript expression is maintained in RPS17 knocked down K562 cells compared to the shLuc control. RPS3A primers targeting exon 1 of RPS3A was used to determine total RPS3A mRNA transcript expression. Data (normalized to β-actin) represents mean ± SE of 4 independent experiments.
Figure 3-12: RPS3A knockdown in human K562 cells reduces RPS19 and RPS17 protein expression, but increases RPS19 mRNA transcript expression.

A) RPS3A knockdown in human K562 cells leads to reduced protein expression levels of RPS17 and RPS19. Western blotting was used to detect RPS3A, RPS17 and RPS19 protein expression in RPS3A knockdown K562 cells 4 days post shRNA transduction. β-actin was used as a loading control.

B) RPS17 mRNA transcript expression is maintained in RPS3A knocked down K562 cells. qRT-PCR using RPS17 primers targeting exon 1 of RPS17 was used to determine total RPS17 mRNA transcript expression. Data (normalized to β-actin) represents mean ± SE of 3 independent experiments.

C) RPS19 mRNA transcript expression is increased in shRPS3A-2 K562 cells and significantly increased in shRPS3A-3 K562 cells. RPS19 primers targeting exon 1 of RPS19 was used to determine total RPS19 mRNA transcript expression. Data (normalized to β-actin) represents the mean ± SE of 6 independent experiments in shRPS3A-2 K562 cells and 3 independent experiments in shRPS3A-3 K562 cells. Statistical significance was determined using Welch’s t-test, as marked with an asterisk, p≤0.0006.
3.3 Study of Shwachman Diamond syndrome (SDS), another ribosomal disorder, reveals that impaired ribosome biogenesis is not responsible for aberrant FLVCR1 splicing and disrupted erythropoiesis

Shwachman-Diamond Syndrome (SDS) and DBA are both categorized as ribosomal disorders. DBA patients have mutations in genes encoding for ribosomal proteins such as RPS19 and are primarily characterized by anemia. However, 90% of SDS patients have mutations in the SBDS gene (Boocock et al., 2003; Ellis & Massey, 2006), and are primarily characterized by neutropenia and pancreatic insufficiency (Bodian et al., 1964; Shwachman et al., 1964). Down regulation of RPS19 disrupts the 40S ribosomal subunit maturation and global protein synthesis (Flygare & Karlsson, 2007; Idol et al., 2007). In comparison, SBDS disruption (associated with SDS) leads to reduced 60S ribosomal subunit maturation and reduced global protein synthesis (Ball et al., 2009; Campagnoli et al., 2008; Choesmel et al., 2008; Flygare & Karlsson, 2007; Idol et al., 2007; Krogan et al., 2006; Menne et al., 2007; Savchenko et al., 2005). Thus, both mutations in either RPS19 or SBDS can disrupt components of the ribosome and translation, but result in different phenotypes.

In order to study the effect of SBDS on FLVCR1 splicing and erythropoiesis, K562 cells disrupted in SBDS protein expression was used as a cell culture model of SDS. Western blotting confirmed SBDS protein reduction in stable SBDS shRNA expressing K562 cells, SBDS S1 and SBDS S3, relative to scrambled shRNA control, SBDS SCR (obtained from Dr. Yigal Dror) (Figure 3-13a). In contrast to RPS19 reduced K562 cells which were disrupted in erythropoiesis, SBDS reduced K562 cells did not show disrupted erythroid differentiation when induced with hemin as determined by similar levels of benzidine positive differentiated cells (Figure 3-13b).
I next determined expression levels of total FLVCR1 transcript and of FLVCR1 transcript containing E2 and E3, using primers described previously, to quantify the degree of FLVCR1 E2/E3 splicing (Figure 2-5b). SBDS reduced K562 cells maintained total FLVCR1 mRNA transcript expression and did not lead to aberrant FLVCR1 splicing of E2 and E3 (Figure 3-14).

As previously shown in Figure 3-10a, RPS19 reduction in K562 cells led to reduced protein expression of RPS17 and RPS3A. Interestingly, SBDS reduced K562 cells did not show disrupted protein expression of RPS19, RPS17 and RPS3A despite SDS being characterized as a ribosomal disorder (Figure 3-15) (Aidoo, F., M.Sc. thesis, 2012). To assess whether RPS19 knockdown disrupts expression of SBDS protein, I analyzed SBDS protein expression in RPS19 shRNA expressing cells. Interestingly, SBDS protein expression was not disrupted in RPS19 shRNA expressing cells relative to expression in shLuc cells (Figure 3-16). Thus, based on my results, down-regulation of RPS19 in K562 cells disrupts expression of specific proteins.

Interestingly, analysis of mRNA expression in SBDS reduced K562 cells showed an increase in RPS19 mRNA expression with no change in expression of RPS17 and RPS3A transcript expression, when compared to the respective mRNA expression in control scrambled shRNA cells, SBDS SCR (Figure 3-17).

In summary, SBDS reduced cells were not disrupted in erythropoiesis, in splicing of FLVCR1 transcripts and in expression of RPS19, RPS17 and RPS3A. Moreover, similar to my findings in figures 3-11b and 3-11c, there was a correlation between increased RPS19 mRNA transcript expression, normal FLVCR1 splicing and normal erythropoiesis.
Figure 3-13: SBDS knockdown in human K562 cells leads to normal erythropoiesis.

A) Western blotting confirmed SBDS knockdown in stably transduced human K562 cells. β-actin was used as a loading control.

B) SBDS knocked down K562 cells were induced to differentiate using 30µm hemin. Benzidine staining was used to detect hemoglobinized cells. Data (normalized to β-actin) represents the mean ± SE of 4 independent experiments in SBDS S1 K562 cells and 3 independent experiments in SBDS S3 K562 cells.
Figure 3-14: SBDS knockdown in human K562 cells leads to normal FLVCR1 splicing of exons 2 and 3.

A) Total FLVCR1 transcript expression (Exon 1-1) was quantified by qRT-PCR using primers targeting exon 1. FLVCR1 exons 2 and 3 expression (Exon 2-3) was quantified by qRT-PCR using primers targeting the junction of exons 2 and 3 of FLVCR1. Data (normalized to β-actin) represents the mean ± SE of 3 independent experiments.

B) Bars represent percent (%) of FLVCR1 mRNA transcripts containing exons 2 and 3 relative to shLuc. Data (normalized to β-actin) represents the mean ± SE of 3 independent experiments.
Figure 3-15: SBDS knockdown in human K562 cells does not affect protein expression levels of RPS19, RPS17 and RPS3A.
Western blotting was used to detect RPS17, RPS19 and RPS3A protein expression in stable SBDS knocked down K562 cells. β-actin was used as a loading control.

Figure 3-16: RPS19 knockdown in human K562 cells does not affect SBDS protein expression level (Figure from Aidoo, F., M.Sc. thesis, 2012).
Western blotting was used to detect β-actin, RPS19 and SBDS protein expression in RPS19 reduced K562 cells. β-actin was used as a loading control.
Figure 3-17: RPS19, RPS17 and RPS3A mRNA transcript levels are maintained in SBDS knockdown K562 cells.

A) RPS19 mRNA transcript expression is slightly increased in SBDS knocked down K562 cells, although not significantly. Data (normalized to β-actin) represents the mean ± SE of 3 independent experiments.

B) RPS17 mRNA transcript expression appears reduced in SBDS knocked down K562 cells, although not significantly. Data (normalized to β-actin) represents the mean ± SE of 3 independent experiments.

C) RPS3A mRNA transcript expression appears reduced in SBDS knocked down K562 cells, although not significantly. Data (normalized to β-actin) represents the mean ± SE of 3 independent experiments.
3.4 Does FLVCR1 over expression rescue defective erythropoiesis in RPS19 reduced K562 cells?

My findings in this study show a strong correlation between reduced RPS19 transcript expression, enhanced FLVCR1 aberrant splicing and disrupted erythropoiesis. To further understand the significance of FLVCR1 aberrant splicing in defective erythropoiesis, I over expressed full-length FLVCR1 cDNA in RPS19 reduced K562 cells and assessed its ability to rescue erythroid differentiation of the target cells. FLVCR1 containing a hemagglutinin (HA) epitope was over expressed using two different retroviral vectors: pFBneo (G418-resistant) and pMSCV (blasticidin-resistant) (Figure 2-1).

FLVCR1 HA was efficiently expressed in the pFBneo-FLVCR1 HA K562 cells (Figure 3-18a). Knockdown of RPS19 using shRPS19-2 in FLVCR1 over expressed K562 cells, was successful at the protein level (Figure 3-18a), but not at the mRNA transcript level (Figure 3-18b). Attempting to achieve knockdown of RPS19 mRNA transcript levels at earlier time points was unsuccessful. Benzidine staining, which indicates erythroid differentiation (see Methods section 2.7) revealed extreme cell death of FLVCR1 over expressed RPS19 reduced K562 cells, and control (shLuc) cells. However, one replicate did result in approximately 20% rescue of erythroid differentiation in shRPS19-2 K562 cells in response to FLVCR1 over expression (Figure 3-19). However, this was only a single replicate.

The pMSCV blasticidin- resistance retroviral vector was used due for a shorter selection time (~3 days) compared to the pFBneo G418-resistant retroviral vector (~2 weeks). The stable and transient methods of pMSCV- FLVCR1 HA and pMSCV- THTR1 HA infections in RPS19 reduced K562 cells resulted in complete cell death. Similar results
were observed in shLuc reduced K562 cells (control). Therefore, erythroid differentiation could not be assessed. The loss of cell viability in pMSCV- FLVCR1 HA rescued RPS19 knocked down K562 cells was potentially due to the lack of exogenous FLVCR1 HA expression (Figure 3-20). However, these pMSCV- FLVCR1 HA K562 cells survived under blasticidin selection. Additionally, pMSCV- THTR1 HA cells expressed exogenous THTR1 (Figure 3-19), however led to cell death when infected with shRNA targeting luciferase (control) and RPS19. Moreover, pMSCV- FLVCR1 HA and pMSCV- THTR1 HA infection in human HeLa and TE672 cells also led to cell death indicating that toxicity was not specific to human K562 cells.
Figure 3-18: Reduced RPS19 protein expression and increased RPS19 mRNA transcript expression in FLVCR1 over expressed K562 cells (pFBneo-FLVCR1 HA).

A) Anti- HA and anti- RPS19 antibodies were used to detect HA- tagged exogenous FLVCR1 and RPS19 protein expression, respectively.

B) RPS19 mRNA transcript expression was determined by qRT-PCR using primers targeting RPS19 exon 1 (n=1)
Figure 3-19: Erythroid differentiation in RPS19 reduced K562 cells alone, and with over expressed FLVCR1.

RPS19 reduced, FLVCR1 over expressed K562 cells were induced to differentiate with 30µm hemin. Benzidine was then applied to these cells to stain for the differentiated hemoglobinized cells. The proportion of benzidine stained cells is indicative of erythroid differentiation. Grey bars represent erythroid differentiation in shLuc and shRPS19-2 K562 cells alone (n=3). The grey bars represent the mean ± SE of 8 independent experiments. Statistical significance was determined using the Welch’s t-test as marked with an asterisk, (p≤0.0002). Black bars represent erythroid differentiation of shLuc and shRPS19-2 K562 cells in response to FLVCR1 over expression using the pFBneo retroviral expression construct (n=1).
Figure 3-20: FLVCR1 and THTR1 protein expression in pMSCV- retroviral generated over expressed FLVCR1 and THTR1 K562 cells.

Western blotting with anti- HA antibody was used to detect exogenous HA- tagged FLVCR1 (lane 1) and HA- tagged THTR1 (lane 2) in 30ug of protein from stable pMSCV- derived FLVCR1 and THTR1 human K562 cells, respectively. K562 wild-type cells (lane 3) were used as a negative control.
CHAPTER 4: DISCUSSION

In this study, the roles of ribosomal proteins (RP) S19, S17 and S3A on FLVCR1 aberrant splicing and erythroid differentiation were investigated. This was done using a DBA cell culture model generated by shRNA-mediated knockdown of each RP in human K562 cells, which can be induced to differentiate into the erythroid lineage. Previous studies have established the role of RPS19 (mutated in DBA) in maintaining erythropoiesis. However, the role of RPS17 (associated with DBA) on erythropoiesis has never been studied. Using human K562 cells as a DBA cell culture model, I have shown for the first time that RPS17 knockdown does not disrupt FLVCR1 splicing and erythropoiesis, which would suggest that RPS17 is not involved in DBA pathogenesis, despite being found mutated in <1% of DBA patients. However, future studies in human CD34 positive bone marrow progenitor cells would be more conclusive. I also found that RPS3A knockdown does not disrupt erythropoiesis or FLVCR1 splicing, which is consistent with the lack of RPS3A mutations being associated with DBA. Our lab’s findings reveal a strong correlation between aberrant FLVCR1 splicing and disrupted erythropoiesis. These defects were specific to RPS19 reduced K562 cells, which were representative of DBA when compared to SDS; another ribosomal disorder that was studied using SBDS reduced K562 cells. Furthermore, upon examination of the protein and mRNA expression of RPS19, RPS17 and RPS3A in RP- and SBDS-reduced K562 cells, we found an interesting correlation between reduced RPS19 mRNA expression, enhanced FLVCR1 aberrant splicing, and disrupted erythropoiesis. This study therefore helps to further understand the mechanism underlying defective erythroid differentiation in DBA.
4.1 RPS17 knockdown does not disrupt erythropoiesis in human K562 cells

A defining characteristic of DBA is a block in development of early erythroid progenitors cells (Nathan et al., 1978). Ribosomal protein S19 (RPS19) is mutated in 25% of DBA patients, and has been shown to have a major role in maintaining erythropoiesis both \textit{in vitro} and \textit{in vivo}. \textit{In vitro} studies have shown that down-regulation of RPS19 leads to disrupted erythropoiesis, which can be rescued by over expressing RPS19 (Hamaguchi et al., 2002), thereby indicating that it has a causal role on erythropoiesis. RPS19 reduced K562 cells in my study have recapitulated the defective erythroid differentiation seen in DBA and therefore served as a good positive control.

Ribosomal protein S3A (RPS3A) has found to not be mutated in DBA patients to date. This conclusion was based on a study from the Czech National DBA Registry of 14 DBA patients, 5 of which had mutations in RPS19 (Cmejla et al., 2001). However, this study also claimed that RPS24 was not involved in DBA pathogenesis since no mutations in this gene were found in any of the 14 DBA patients. Since then, RPS24 has been found mutated in approximately 2.4% of DBA patients (Choesmel et al., 2008). Therefore, it was entirely possible that even though Cmejla and colleagues (Cmejla et al., 2001) found that RPS3A was not mutated in their study, it may indeed contribute to DBA pathogenesis. I found that RPS3A reduction in human K562 cells did not disrupt erythropoiesis. Under the context of my cell culture model, this suggests that RPS3A is not associated with DBA and hence, serves as an appropriate control in my study.

Similar to RPS19, ribosomal protein S17 (RPS17) is also found mutated in DBA patients. However, unlike RPS19, the role of RPS17 on erythropoiesis has yet to be studied \textit{in vitro} or \textit{in vivo}. I have shown for the first time that reduction of RPS17 does not disrupt
erythropoiesis despite this RP being mutated in DBA patients. While RPS17 protein expression is expected to be reduced in RPS17 mutated DBA patients, it was not examined. This raises the possibility that RPS17 reduced K562 cells in this study may not completely represent RPS17 mutations in DBA patients. Another hypothesis of normal erythropoiesis is that RPS17 may be associated with a non-anemic characteristic in these DBA patients, similar to RPL5 and RPL11 mutations are that associated with cleft palate and thumb abnormalities in DBA patients (Gazda et al., 2008). The first RPS17 mutated DBA patient had a deletion of GA residues at position 200 and 201 that was predicted to create a frameshift at codon 67 and a stop codon at position 86; this patient did not have any malformations (Gazda et al., 2008). The other two patients had a mutation in their initiation codon, which was predicted to create a short 4 amino acid peptide as a result of a downstream start codon. One of these patients displayed growth retardation (Song et al., 2010) while the other had short stature, in addition to facial abnormalities such as flat thenar and facial dysmorphism (Cmejla et al., 2007). Other than anemia associated with de novo RPS17 mutations, there were no other similarities in all three RPS17 DBA patients; therefore, it is unlikely that RPS17 was associated with any other characteristics.

Unlike RPS19, which has been directly linked to DBA and demonstrated to play a causal role on erythropoiesis, RPS17 has been associated with DBA based on the three DBA patients found to each harbour a RPS17 mutation. It is therefore plausible that the RPS17 mutations in DBA are rare polymorphisms or rare non-pathogenic genetic variants. Hence, RPS17 may not be involved in erythroid failure, a defining characteristic of DBA, and maybe therefore not be a candidate DBA gene. In order to confirm whether RPS17 was responsible
for the severe anemia observed in the three RPS17 DBA patients, erythroid differentiation should be examined in response to RPS17 over expression in their erythroid cells.

4.2 The role of the FLVCR1 heme exporter in erythropoiesis

FLVCR1, a retroviral receptor and heme exporter (Quigley et al., 2000; Tailor et al., 1999), has been found to play a crucial role in erythropoiesis as well as in DBA both in vitro and in vivo. Disruption of this receptor in human hematopoietic stem cells directly mimicked the defective erythropoiesis observed in DBA patients (Rey et al., 2008). Interestingly, Rey and colleagues (Rey et al., 2008) found enhanced aberrant splicing of FLVCR1 in DBA patient erythroid cells negative for RPS19 mutations, but not in normal patient erythroid cells (Rey et al., 2008). RPS19 reduced K562 cells also exhibited enhanced aberrant splicing of FLVCR1, specifically of exons 2 and 3, which are important in cell surface protein expression, receptor function and heme export (Keel et al., 2008; Rey et al., 2008). I therefore hypothesized that aberrant splicing of FLVCR1 transcripts leads to defective erythropoiesis in DBA.

RPS19 reduced K562 cells used in my study recapitulated the enhanced aberrant splicing of FLVCR1 E2 and E3 observed by Rey and colleagues, which strongly correlated with disrupted erythropoiesis. As previously described (in Discussion section 4.1), reduction of RPS3A (not associated with DBA) or RPS17 (associated with DBA) did not lead to a disruption of erythroid differentiation. Interestingly, I found that RPS3A or RPS17 reduction also did not lead to aberrant FLVCR1 splicing of E2 and E3. Therefore, there is a strong correlation between disrupted erythropoiesis and aberrant FLVCR1 splicing. My results further support the role of FLVCR1 splicing in erythropoiesis.
The observation of FLVCR1 splicing in this study was solely in the context of exon 2 and exon 3. It was previously shown that FLVCR1 E2 was aberrantly spliced in all 5 DBA patient erythroid cells (Rey et al., 2008). Although the effect of FLVCR1 E2 was not studied, it was hypothesized to be non-functional since it resulted in a premature termination codon (PTC). Transcripts with PTC signals are degraded through the nonsense-mediated decay pathway (Chang et al., 2007). Furthermore, FLVCR1 E3 was aberrantly spliced in one DBA patient and was found to be important in maintaining surface expression and heme export function (Keel et al., 2008; Rey et al., 2008). Therefore, it is conceivable that aberrant FLVCR1 splicing of E2 and E3 (seen in RPS19 knocked down K562 cells) disrupted erythroid differentiation by reducing the surface expression and function of FLVCR1 protein. Although not tested, I hypothesize that this in turn led to the accumulation of heme, which is highly produced in early erythroid progenitor cells and human K562 cells. Consequently, the build-up of heme within cells is toxic and ultimately led to cell death. Since RPS19 reduced K562 cells are a cell culture model of RPS19 mutated DBA patients, this may explain the pathway through which early erythroid progenitors are reduced in DBA patients.

While there is no aberrant FLVCR1 splicing in RPS17 and RPS3A reduced K562 cells, the splicing of the other 8 exons of FLVCR1 were not tested. Another potential aberrantly spliced FLVCR1 exon is exon 6 which was found to be aberrantly spliced in DBA patients (Rey et al., 2008). However, I primarily focused on exons 2 and 3 of FLVCR1 in this study due to their known roles in affecting the function of FLVCR1. So while no aberrant FLVCR1 splicing was observed in RPS17 and RPS3A reduced K562 cells in the context of E2 and E3, it is possible that other exons of FLVCR1 may be spliced. This possibility should be addressed in the future.
Interestingly, RPS17 and RPS3A reduced K562 cells led to normal FLVCR1 splicing and normal erythropoiesis, but also led to significantly increased levels of total FLVCR1 mRNA transcript expression, compared to the control (shLuc). It is possible that down regulation of RPS17 and RPS3A may have directly or indirectly increased FLVCR1 mRNA expression through a pathway or a factor in an unknown mechanism. Due to unavailability of an efficient anti-FLVCR1 antibody, I was unable to detect whether FLVCR1 protein expression was increased in correlation to FLVCR1 mRNA levels. However, I postulate that the normal erythropoiesis observed in RPS17 and RPS3A reduced K562 cells was attributed to increased FLVCR1 protein expression which has been shown by others to be important in maintaining erythroid differentiation.

Similar to a previous study (Rey et al., 2008), this study shows that that RPS19 reduced K562 cells exhibited enhanced aberrant FLVCR1 splicing but maintained total FLVCR1 mRNA transcript expression. This is in contrast to DBA patient erythroid cells that are significantly down regulated in FLVCR1 mRNA transcript levels (Rey et al., 2008). It is possible that in DBA patients, there are additional factors associated with FLVCR1 total mRNA transcript expression regulation that may be disrupted, and this may not be occurring in human K562 cells. However, one DBA patient did have enhanced aberrant FLVCR1 splicing while maintaining the same level of total FLVCR1 mRNA transcript expression as a normal patient (Rey et al., 2008).

Furthermore, Rey and colleagues found that RPS19 reduced K562 cells maintained total FLVCR1 mRNA transcript expression, but had decreased FLVCR1 protein expression (Rey et al., 2008). I hypothesize that this is because of alternatively spliced transcript variants that were stable, but translated unstable proteins that were subsequently degraded. I
was unable to examine FLVCR1 protein expression for reasons mentioned earlier, but it is very likely that FLVCR1 protein expression was also reduced in RPS19 reduced K562 cells used in this study. Therefore, total FLVCR1 mRNA transcript levels do not necessarily dictate erythropoietic potential in RP reduced K562 cells.

According to the results obtained in this study, there is a very strong correlation between aberrant FLVCR1 splicing and defective erythropoiesis, further supporting the role of FLVCR1 in maintaining erythropoiesis.

4.3 The role of RPS19 and erythropoiesis

It has been widely postulated that RPS19 protein haplo-insufficiency is underlying DBA pathogenesis. (Ebert et al., 2005; Flygare et al., 2005; Gazda et al., 2004; Miyake et al., 2005; Miyake et al., 2008). This is because RPS19 down regulation led to disrupted erythropoiesis, which was rescued by RPS19 over expression.

I have shown that RPS19 reduction in human K562 cells disrupts erythropoiesis. But more interestingly, I found that RPS19 protein down regulation to similar levels in RPS17 and RPS3A reduced K562 cells that did not disrupt erythroid differentiation. This suggests that reduced RPS19 protein levels are not responsible for defective erythropoiesis in human K562 cells.

I did however find a strong correlation between reduced RPS19 mRNA transcript levels and disrupted erythropoiesis in RPS19 reduced K562 cells. Interestingly, RPS17 and RPS3A reduced K562 cells had increased RPS19 mRNA levels. These correlative results suggest that RPS19 mRNA transcript levels are important in maintaining differentiation of erythroid progenitors (represented by human K562 cells). This could be attributed to the fact
that RPS19 mRNA transcript is highly expressed in early erythroid progenitors, and this expression decreases as these cells mature (Da Costa et al., 2003; Ebert et al., 2005; Hamaguchi et al., 2002). It is therefore possible that high RPS19 mRNA transcript levels play an important role in the differentiation of early erythroid progenitors through extra-ribosomal function(s). The RPS5 mRNA transcript and protein expression was found to play a role on delaying the onset of erythropoiesis and cell entry in murine erythroleukemia (MEL) cells (Matragkou et al., 2008). Furthermore, increased mRNA levels of certain ribosomal proteins such as RPS3, RPL15, RPL19, RPL7A and RPL37 have been found in diseases including colorectal, gastric and prostate cancers (Kasai et al., 2003; Pogue-Geile et al., 1991; Vaarala et al., 1998), therefore raising the possibility that RPs transcripts may have extra-ribosomal functions. It is therefore possible that RPS19 mRNA transcript levels may be important for differentiating erythroid progenitors and low levels of RPS19 mRNA, as seen in RPS19 reduced K562 cells, may be deleterious through as yet unidentified extra-ribosomal function.

It is important to note however, that RPS19 reduced K562 cells dramatically reduced protein levels of RPS17 by 93-99% and RPS3A by 89.6-100%. This is in contrast to RPS17 reduced K562 cells which maintained 15-30% of RPS17 protein expression, and RPS3A reduced K562 cells which maintained 21% of RPS3A protein expression. Both RPS17 and RPS3A reduced K562 cells led to normal erythropoiesis, whereas RPS19 reduced K562 cells disrupted erythropoiesis. These results raise the possibility of a certain threshold of RPS17 and RPS3A protein levels that may be responsible for maintaining normal FLVCR1 splicing and erythropoiesis in RPS17 and RPS3A reduced K562 cells. It should also be noted that the expression of mutated RPs in DBA patients are never completely abolished, therefore, the
depleted RPS17 and RPS3A protein expression in RPS19 reduced K562 cells is not representative of RP levels in DBA patients. While further studies of RPS17 and RPS3A over expression in RPS19 reduced K562 cells should be conducted to determine whether they have a role in erythropoiesis, I hypothesize that this is most likely not the case. Previous studies have shown that free RPS3A protein interacts with CHOP, a transcription factor that is up-regulated in erythropoiesis. This interaction somehow prevents the functional role of CHOP, and inhibits erythroid differentiation. Therefore, increased RPS3A protein levels were associated with disrupted erythroid differentiation (Cui et al., 2000). This suggests that abolished RPS3A protein levels seen in RPS19 reduced K562 cells are most likely not involved in disrupting erythropoiesis.

It is however possible that RPS17 protein may directly or indirectly affect erythropoiesis through a transcription factor or another protein responsible for regulating erythropoiesis. While no extra-ribosomal functions of RPS17 have been identified, there is evidence of RPs that are able to influence the expression of other protein. An example of this is human RPL26 which binds to the 5’UTR of p53 mRNA in response to DNA damage, thereby increasing p53 translation, which influences apoptosis and cell cycle regulation (Takagi et al., 2005). Moreover, upon interferon-gamma activation, human RPL13a is released from the 60S ribosomal subunit, phosphorylated and binds to the 3’UTR of ceruloplasmin (Cp) mRNA to inhibit translation of the encoded blood plasma protein (Mazumder et al., 2003). Therefore, it is possible that RPS17 protein may affect the expression of proteins or factors required for erythropoiesis.
4.3.1 Expression profile of ribosomal proteins

While studying the protein and mRNA transcript expression of RPS19, RPS17 and RPS3A in the RP reduced K562 cells, I observed coordinated reduction of RPs at the protein level but not at the mRNA transcript level. Reduced mRNA transcript expression was only specific to the shRNA-targeted gene. For instance, RPS19 knockdown K562 cells reduced protein expression of RPS3A, while RPS3A mRNA levels were unaffected. But a knockdown of RPS17 also led to reduced RPS3A protein levels. This suggests that reduced protein expression of RPS3A in both RPS19 and RPS17 reduced K562 cells was not due to reduced RPS3A mRNA transcript levels or to knockdown of RPS19 and RPS17 specifically. Choesmel et al. (2008) observed similar results where RPS15 and RPS24 knockdown HeLa cells reduced RPS19 protein levels, but left RPS19 mRNA levels unaffected (Choesmel et al., 2008). The effect of one reduced ribosomal protein on another at the protein level was also evident in DBA patients. RPS24 mutated DBA patients had reduced RPS19 protein levels compared to normal patients (Gazda et al., 2006). Similarly, Robledo et al. (2008) demonstrated that depleted 40S subunit ribosomal proteins in HeLa cells did not affect mRNA transcript expression of the other 40S subunit ribosomal proteins (Robledo et al., 2008). It is well established that the reduction of one 40S subunit ribosomal protein (for example, RPS19) leads to a reduction of the other 40S subunit ribosomal proteins (including RPS19, RPS17, RPS24, RPS16, RPS12 and RPS6) at the protein level (Badhai et al., 2009; Badhai et al., 2009; Idol et al., 2007; Robledo et al., 2008).

I hypothesize that reduced protein expression and not mRNA transcript expression of RPS19, RPS17 and RPS3A in the RP reduced DBA cell culture models is due to 40S ribosomal subunit dissociation. That is, RPS19 knockdown in human K562 cells reduces the
levels of RPS19 protein which is a structural part of the 40S ribosomal subunit. This in turn prevents the formation of the 40S subunit, therefore leading to the degradation of free RPS17 and RPS3A which usually incorporate into the 40S ribosomal subunit. This hypothesis can be tested using sucrose gradient density ultracentrifugation in RP reduced K562 cells to examine 40S ribosomal formation. This theory is reasonable since early studies demonstrated that excess ribosomal proteins not structurally incorporated into ribosomes are rapidly degraded (Warner, 1977; Warner, 1979). Alternatively, it is possible that excess RPS17 and RPS3A could associate into a nascent subunit with other 40S ribosomal proteins which disassembles and is subjected to degradation in the absence of stoichiometric conformation due to reduced RPS19 (Moritz et al., 1990). It therefore appears that RPS19, RPS17 and RPS3A are essential for 40S ribosomal subunit formation. In accordance with this, studies show that RPS19 and RPS17 reduction lead to disruption of 40S subunit maturation (Choesmel et al., 2008; Flygare et al., 2007; Idol et al., 2007; Robledo et al., 2008).

4.4 Disrupted ribosome biogenesis is not responsible for defective erythropoiesis

So far, all of the mutated genes identified in DBA patients encode ribosomal proteins which are important in translation. Furthermore, it has been found that DBA patients, regardless of whether they harbour RPS19 mutations, have lower levels of global translation relative to non-DBA patients (Cmejlova et al., 2006). In addition, erythroid precursors which are disrupted in DBA have high rates of ribosome biogenesis to produce increased amounts of globin required for red blood cell development (Flygare & Karlsson, 2007; Rifkind et al., 1964). It has therefore been postulated that erythropoiesis is specifically
affected by mutated ribosomal proteins in DBA patients since early erythroid progenitors require maximal ribosome biogenesis and high rates of proliferation and protein synthesis, thus making them highly sensitive to impaired ribosomal proteins (Ellis & Massey, 2006; Robledo et al., 2008).

I therefore aimed to determine whether disrupted erythropoiesis and aberrant FLVCR1 splicing in RPS19 reduced K562 cells was specific to DBA, or a result of defective ribosome biogenesis. In order to do this, I used human K562 cells reduced in the gene encoding SBDS, which is mutated in patients with Shwachman diamond syndrome (SDS), another ribosomal disorder. In this study, RPS19 reduced K562 cells were representative of DBA whereas SBDS reduced K562 cells were used as a cell culture model to study SDS.

I found that RPS19 knockdown K562 cells disrupted erythropoiesis and aberrant FLVCR1 splicing whereas SBDS knockdown K562 cells led to normal erythropoiesis and constitutive FLVCR1 splicing. This is consistent with a recent study that reported normal erythroid differentiation of SBDS reduced K562 cells (Sen et al., 2011). RPS19 is a structural component required for 40S ribosomal subunit maturation (Idol et al., 2007; Lutsch et al., 1990) whereas SBDS is associated with and required for 60S ribosomal subunit maturation (Menne et al., 2007). This raises the possibility that disrupted 40S maturation could play a role in defective erythropoiesis and FLVCR1 splicing, however, I have shown that a reduction of RPS17, which is also required for 40S maturation (Robledo et al., 2008), does not disrupt erythropoiesis and FLVCR1 splicing. In addition to this, DBA patients have mutations in ribosomal protein genes of both the 40S and the 60S subunit, hence, aberrant FLVCR1 splicing and defective erythropoiesis in RPS19 reduced K562 cells is not due only to disrupted 40S maturation.
Both RPS19 (DBA mutant) and SBDS (SDS mutant) are highly expressed during early stages of erythroid differentiation (Hamaguchi et al., 2002; Sen et al., 2011). Furthermore, both these genes are involved in ribosome biogenesis and protein synthesis (Ball et al., 2009; Campagnoli et al., 2008; Choesmel et al., 2008; Flygare & Karlsson, 2007; Idol et al., 2007; Krogan et al., 2006; Menne et al., 2007; Savchenko et al., 2005), yet lead to different phenotypes. This suggests that impaired ribosome biogenesis and translation was not the main mode through which erythropoiesis and FLVCR1 splicing was disrupted in RPS19 reduced K562 cells. Therefore, the erythropoietic and FLVCR1 splicing defect in RPS19 reduced K562 cells is indeed specific to DBA. However, it should be noted that there are other ribosomal disorders such as dyskeratosis congenital and cartilage-hair hypoplasia (see Table 1) that were not investigated in this study. Therefore, defective erythroid differentiation and aberrant FLVCR1 splicing in this study is specific to DBA only when compared to SDS.

4.5 Potential mechanisms through which FLVCR1 regulates erythroid differentiation

In accordance with previous findings, this study supports the role of aberrant FLVCR splicing on defective erythroid differentiation. While rescue studies need to confirm whether FLVCR1 splicing has a causative role on erythropoiesis, I propose mechanisms through which this may occur.

I postulate that RPS19 reduced K562 cells disrupts the expression and/or function of factor(s) that may be involved in regulating FLVCR1 splicing. Interestingly, RPS19 protein has been found to interact with certain splicing factors known as serine-arginine (SR) rich
proteins (Orru et al., 2007) which are important in regulating the splicing of many genes (Sanford et al., 2005) and involved in certain human diseases (Kondo et al., 2004; Watermann et al., 2006). One of these interacting SR proteins is Tra2-β, which was also found to be down-regulated in the conditional RPS19 R62W transgenic DBA mouse model (Devlin et al., 2010) and has putative binding sites on FLVCR1 E3. Interestingly, RPS19 reduced K562 cells leads to reduced Tra2-β protein levels whereas SBDS reduced K562 cells do not (Aidoo, F., M.Sc. thesis, 2012). This correlation suggests that FLVCR1 aberrant splicing of E2 and E3 in RPS19 knocked down K562 cells is caused by reduced expression of SR proteins such as Tra2-β which is important in alternative splicing. However, future studies should focus on determining whether Tra2-β has a causal role on FLVCR1 splicing and erythropoiesis by observing the effects of Tra2-β knockdown in human K562 cells and Tra2-β over expression in RPS19 reduced K562 cells.

In the context of the DBA cell culture model used in this study I have shown that reduced RPS19 protein levels do not necessarily disrupt erythropoiesis. Instead, my findings suggest that reduced mRNA transcript levels of RPS19 may play a role in erythroid differentiation. While the encoded RPS19 protein can be linked to alternative splicing of FLVCR1 through SR proteins such as Tra2-β, it is unknown whether or not RPS19 mRNA transcripts interact with splicing factors that can regulate FLVCR1 splicing. If these SR proteins are found to interact with RPS19 mRNA transcript, then it is possible that RPS19 mRNA may regulate FLVCR1 splicing through the spliceosome similar to how the tRNA interacts with mRNA through the ribosome in order to regulate mRNA translation. There are also examples of non-coding mRNAs such as miRNAs and antisense mRNAs that are known to regulate the expression of other mRNAs. For example, the beta secretase-1
(BACE-1) antisense mRNA which is elevated in Alzheimer’s disease, has been found to increase mRNA translation of BACE-1 sense mRNA (Faghihi et al., 2008) by blocking miR-485-5p-induced translational repression (Faghihi et al., 2010). Similarly, PU.1 antisense mRNA regulates the translation of PU.1 sense mRNA by inhibiting translational elongation factor eEF1A leading to reduced PU.1 mRNA translation (Ebralidze et al., 2008). Therefore it is possible that RPS19 mRNA might interact with FLVCR1 mRNA, either directly or indirectly, and subsequently block the recruitment of splicing factors required for constitutive FLVCR1 splicing.

I hypothesize that FLVCR1, a heme exporter, plays a crucial role in erythroid differentiation because early erythroid progenitors (targeted by DBA) produce high levels of heme (Ponka et al., 1982; Quigley et al., 2004; Wickrema et al., 1992). Heme is required for the synthesis of hemoglobin to produce mature erythrocytes; however, excess levels are toxic due to oxidative cell membrane damage leading to cell death (Ryter & Tyrrell, 2000). Two isoforms of heme-oxygenases, HO-1 and HO-2, are known to regulate the levels of heme by degrading it into carbon monoxide, ferrous iron and biliverdin. HO-1 is induced by stress, such as excess heme itself, whereas HO-2 is constitutively expressed (Maines et al., 1986; Tenhunen et al., 1968; Yoshida et al., 1988). HO has a prolonged half life therefore indicating that may be inappropriate in controlling the heme levels in erythroid cells since they are critical for erythroid progenitor development (Ibrahim et al., 1982). Interestingly, Alves et al. (2011) found that regardless of high heme levels, human bone marrow erythroid precursors and human K562 erythroid cells did not express native HO-1 (Alves et al., 2011). In addition, increased heme exposure reduced HO-2 expression during differentiation of human BM erythroid precursors and K562 cells (Alves et al., 2011).
BM erythroid precursors and K562 cells, increased heme suppresses HO- mediated heme degradation and balanced heme levels are not achieved through HOs. This further supports the role of FLVCR1 in early erythroid progenitors since it is highly expressed on both early BM erythroid progenitor cells and human K562 cells and therefore acts as a safety valve by exporting excess heme out of developing erythroid cells in the absence of HOs (Alves et al., 2011; Quigley et al., 2004).

4.6 Future Directions

4.6.1 Proposed studies to strengthen the results obtained in this study

In order to study the roles of the ribosomal proteins S19 and S17 implicated in DBA, targeted shRNA- mediated knockdown was conducted. However, some RPS19 point mutations known as class II mutations, do not affect protein folding, and are therefore stable. It has been proposed that these function through a dominant negative mechanism. Using a conditional mouse model, Devlin et al (2010) have shown that the RPS19R62W class II mutation is indeed dominant negative. Therefore, it would be useful to investigate whether over expression of common RPS19 mutations such as R56Q, R62W or R62Q in human K562 cells also lead to disrupted erythropoiesis through aberrant FLVCR1 splicing. It would also be interesting to determine the effect of over expressed RPS17 mutations c.2T>G, c.1A>G and deleted GA at position 200 on erythropoiesis in human K562 cells.

While human K562 erythroid progenitor cells are a good model to study DBA because they can be induced to differentiate into the erythroid lineage, other inducible human erythroid leukemic cell lines such as TF-1 and UT-7 cells as well as human CD34+ bone
marrow progenitor cells will further validate the results shown in this study. These additional cell lines would also help determine whether RPS17 is really a candidate DBA gene.

Furthermore, the mRNA transcript and protein expression of other human cell surface proteins such as THTR1 thiamine transporter, a FeLV-A receptor (Mendoza et al., 2006), and the erythropoietin receptor (EPOR), a cell surface protein expressed on erythroid cells (Maouche et al., 1991) should be considered as controls to determine whether the splicing defect is specific to FLVCR1.

One of the limitations of this study was the unavailability of an effective FLVCR1 antibody. It would be extremely useful to detect the protein expression of FLVCR1 in RPS19 reduced K562 cells to determine whether or not a proportion of the maintained FLVCR1 total mRNA transcripts in RPS19 reduced K562 cells are subjected to degradation. Furthermore, it will also help determine whether or not increased FLVCR1 mRNA transcript expression contributes to normal erythropoiesis in RPS17 and RPS3A knocked down K562 cells by increasing FLVCR1 protein levels. In addition to studying the DBA cell culture model, FLVCR1 protein expression should be assessed in DBA patient erythroid cells compared to normal patient erythroid cells.

4.6.2 Confirm the role of FLVCR1 in erythroid differentiation

In order to determine whether FLVCR1 has a causal role on erythropoiesis, I attempted to over express FLVCR1 in RPS19 reduced K562 cells using two retroviral vectors: pFBneo-FLVCR1 HA and pMSCV- FLVCR1 HA (explained in Results section 3.4). The pMSCV blasticidin- resistant retroviral vector was used due for a shorter selection time (~3 days) compared to the pFBneo G418- resistant retroviral vector (~2 weeks).
The pMSCV vector did not efficiently express FLVCR1 but enabled cell growth under selection. In the pMSCV vector, the blasticidin-resistant gene is driven by a phosphoglycerate kinase (PGK) promoter while the insert is driven by the 5’ long terminal repeat (LTR). Therefore it is possible that gene encoding FLVCR1 HA was not expressed due to silencing of the 5’ LTR promoter (Challita & Kohn, 1994; Hoeben et al., 1991). The pMSCV vector expressing THTR1 and the pFBneo vector expressing FLVCR1 both led to cell death upon benzidine staining when infected in luciferase- and RPS19-reduced K562 cells. It is possible that very few K562 cells were expressing both knockdown (of Luc and RPS19) and over expression (of FLVCR1 and THTR1) constructs; therefore making them susceptible to cell death upon selection with double selection. It is also possible that stress on K562 cells upon RPS19 knockdown may be exacerbated with the over-expression of exogenous genes such as FLVCR1 and THTR1, thereby leading to cell death. Moreover, pMSCV-FLVCR1 HA and pMSCV-THTR1 HA infection in human HeLa and TE672 cells also led to cell death indicating that toxicity was not specific to human K562 cells.

Due to the inefficiency of FLVCR1 over-expression in RPS19 reduced K562 cells using both the pFBneo and the pMSCV retroviral vectors, I was unable to determine whether FLVCR1 aberrant splicing has a causal role in disrupting erythropoiesis in DBA.

Retroviral vectors such pFBneo and pMSCV allow the integration of the gene of interest into the chromosome, however are limited by their ability to transduce non-dividing cells only. Therefore, future rescue experiments need to focus on using an efficient vector, such as a lentiviral vector, with a short selection period (~2 days) in order to express FLVCR1 and THTR1 in human K562 erythroid cells. Furthermore, in order to achieve a consistent level of RPS19 knockdown, a stable packaging cell line, such as FLYRD18 cells,
should be used to generate consistently high titres of shLuc, shRPS19-1 and shRPS19-2 retrovirus, for efficient knockdown. A FLVCR1 antibody should also be used to assess endogenous FLVCR1 levels in FLVCR1 over expressed K562 cells since this cannot be detected using an anti-HA antibody.

Further experiments can be done to confirm and solidify the role of FLVCR1 on erythropoiesis. To determine whether the role of FLVCR1 is specific to erythropoiesis, FLVCR1 over expressed RPS19-reduced human K562 cells should also be assessed for megakaryocyte differentiation, which can be induced in human K562 cells upon PMA (phorbol 12-myristate 13-acetate) treatment (Shelly et al., 1998). Moreover, to determine whether or not the erythropoietic defect is specific to FLVCR1 aberrant splicing, erythroid differentiation should be assessed in response to over expression of aberrant FLVCR1 variants such as E2-, E3- and E2-E3- cDNAs in human K562 cells. However, to ultimately confirm whether or not FLVCR1 has a causal role in erythropoiesis, erythroid differentiation in response to FLVCR1 over expression in DBA patient erythroid cells needs to be assessed.

In addition, this study focused on examining the splicing of FLVCR1 exon 2 and exon 3. Future studies however, should also determine whether the other exons of FLVCR1 are being spliced out in the RP- and SBDS-reduced K562 cells. A candidate FLVCR1 exon to study is exon 6, which was also found to be aberrantly spliced out in DBA patient erythroid cells compared to normal patient erythroid cells (Rey et al., 2008).

4.6.3 Determine whether RPS19 mRNA has a role in erythropoiesis

Lastly, I found that decreased RPS19 mRNA transcript may be disrupting erythroid differentiation in RPS19 reduced K562 cells. In order to confirm the role of RPS19 mRNA
transcript on erythropoiesis, RPS19 reduced K562 cells should be transduced with RPS19 cDNA fused with a downstream green fluorescent protein (GFP) tag and separated by an internal ribosomal entry site (IRES). Furthermore, the RPS19 cDNA should have a mutated translational initiation site to prevent the translation of RPS19 protein and mutations to prevent RPS19 shRNA binding and therefore silencing. This should theoretically produce an RPS19 and GFP mRNA transcript, but only a GFP protein, thereby enabling the study of RPS19 mRNA effects on erythropoiesis.

**4.7 Significance**

Ribosomal proteins are the only genes found to be mutated in DBA patients. It is however unclear how mutated ribosomal proteins specifically disrupt erythroid progenitors. This study strengthened the role of aberrant FLVCR1 splicing of E2 and E3 on disrupted erythropoiesis and indicated that low RPS19 mRNA transcript levels may also play a contributing role. Furthermore, this study showed that RPS17 is not involved in erythroid differentiation and may therefore not be a candidate DBA gene. It is vital to establish whether FLVCR1 has a causal role on anemia since it can be utilized as a future diagnostic test and a potential gene therapy treatment for this fatal infant disease.
REFERENCES


