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The Role of the Nucleosome Remodeling and Histone Deacetylase (NuRD) Complex in Fetal γ-Globin Expression

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Dedication

I would like to dedicate this thesis to my family because they give me strength and inspire me everyday to be a better person. I'd like to specially dedicate this thesis to my grandmother who was the most remarkable person I have ever met and who helped me become the person I am today.

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List of Abbreviations and Symbols

5-aza……5-azacytidine

α……Alpha

β……..Beta

βmaj……Beta Major

βmin……Beta Minor

βYAC……β-globin Locus Yeast Artificial Chromosome

γ……Gamma

δ……Delta

ε……..Epsilon

μ……Micro

Alad…… δ-Aminolevulinic Acid Dehydratase

bp……Base Pair

BCL11A……B-cell lymphoma/leukemia 11A

BERK……...Berkley Mice

C……Celsius

CBC……..Complete Blood Count

CD……Cluster of Differentiation

CHD……..Chromodomain

CHD3…….....Chromodomain-helicase-DNA-binding protein 3

CHD4……...Chromodomain-helicase-DNA-binding protein 4

ChIP……...Chromatin Immunoprecipitation

CID……..Chemical Inducer of Dimerization

CpG…….Cytosine-Guanine Dinucleotide

DEPC……..Diethylpyrocarbonate

DM……...Differentiation Medium

DMSO……Dimethyl Sulfoxide

DNA…….Deoxyribonucleic Acid

DNMT…….DNA Methyltransferase

EE…….Extramedullary Erythropoiesis

eNOS…..…….Endothelial Nitric Oxide Synthase

EPO…….Erythropoietin

FACS……...Fluorescence-Activated Cell Sorting

FBS……Fetal Bovine Serum

FOG-1……..Friend of GATA-1

G418……...Geneticin

G6PD…….Glucose-6-Phosphate Dehydrogenase

GM……..Growth Medium

GFP……..Green Fluorescent Protein

GWAS……..Genome-Wide Association Study

H3……Histone H3

H&E…….Hematoxylin and Eosin

Hba…… Murine Alpha Hemoglobin

HbA……Hemoglobin A

HbA2…….Hemoglobin A2

Hbb…….Murine beta hemoglobin

HbF……...Fetal Hemoglobin

HbS……..Sickle Cell Hemoglobin

HDAC…..……..Histone Deacetylase

HPFH……...Hereditary Persistence of Fetal Hemoglobin

HPLC……High Performance Liquid Chromatography

IgG……...Immunoglobulin G

IMDM…….....Iscove's Modified Dulbecco's Medium

IP……..Immunoprecipitation

KD……..Knockdown

KO……..Knockout

LCR……Locus Control Region

LSD1……..……..Lysine-Specific Demethylase 1

KLF1……...Krüppel-like Factor 1

KLF2……...Krüppel-like Factor 2

M……Molar

MBD2……Methyl-CpG-Binding Domain Protein 2

MBD3……Methyl-CpG-Binding Domain Protein 3

MTA……...Metastasis-Associated

MYB……..Myeloblastosis

NF-E……Nuclear Factor, Erythroid Derived 2

NO……..Nitric Oxide

NOD/SCID……Nonobese Diabetic/Severe Combined Immune Deficiency

EDTA……..Ethylenediaminetetraacetic acid

FVB……Friend Virus B-type

NuRD……...Nucleosome Remodelling and Histone Deacetylase

OE……Over-expression

PBS……..Phosphate Buffered Saline

PCR……Polymerase Chain Reaction

PHD……...Plant Homeodomain

qPCR……...Quantitative Real Time Polymerase Chain Reaction

RNA……Ribonucleic acid

RbAp……..Retinoblastoma Associated Protein

SCD…….Sickle Cell Disease

SCR……..Scrambled Control

SDS…….Sodium Dodecyl Sulfate

SFEM……..Serum-Free Expansion Medium

shRNA……..short hairpin RNA

siRNA……small interfering RNA

SNP……...Single Nucleotide Polymorphism

Sox6…….Sry-related HMG box 6

SUMOylation…….Small Ubiquitin-like Modifier

Tg……..Transgene

Th…….T Helper Cell

TR2……..Testicular Receptor 2

TR4……..Testicular Receptor 4

Ugt8…….UDP glycosyltransferase 8

Uros……..Uroporphyrinogen III Synthase

WT……Wild Type

Abstract

By Maria Laura Amaya, B.S,

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2013

Major Director: Gordon D. Ginder
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An understanding of the human fetal to adult hemoglobin switch offers the potential to ameliorate β-type globin gene disorders such as sickle cell anemia and β-thalassemia through activation of the fetal γ-globin gene. Chromatin modifying complexes, including MBD2-NuRD and GATA-1/FOG-1/NuRD play a role in γ-globin gene silencing, and Mi2β (CHD4) is a critical component of NuRD complexes. In the studies presented in Chapter 2, we observed that the absence of MBD2 in a sickle cell mouse model leads to a decrease in the number of sickled cells observed in the peripheral blood, and significantly increases survival in these mice. Although further studies will be necessary to fully understand the effect of MBD2 knockout in sickle cell disease mice, absence of MBD2 appears to partially ameliorate the sickle cell anemia phenotype *in vivo*.

In the studies presented in Chapter 3, we observed that knockdown of Mi2β relieves γ-globin gene silencing in β-YAC transgenic murine CID hematopoietic cells and in CD34+ progenitor derived human primary adult erythroid cells. We show that independent of MBD2-

NuRD and GATA-1/FOG-1/NuRD, Mi2β binds directly to and positively regulates both the KLF1 and BCL11A genes, which encode transcription factors critical for γ-globin gene silencing during β-type globin gene switching. Remarkably, less than 50% knockdown of Mi2β is sufficient to significantly induce γ-globin gene expression without disrupting erythroid differentiation of primary human CD34+ progenitors. These results indicate that Mi2β is a potential target for therapeutic induction of fetal hemoglobin.

Chapter 1. Introduction

I. Hemoglobin and the hemoglobin switch

A. Hemoglobin

Hemoglobin is the oxygen carrying metalloprotein in the body and the major component of red blood cells. Its main function is to carry oxygen from the lungs to tissues. Although there are different types of hemoglobin, each of them is a tetramer composed of four subunits including two α-like chains and two β-like globin chains. In humans, the genes coding α-chains are located on chromosome 16. The α-globin locus contains one functional embryonic ζ-globin gene and two adult α-globin genes. The genes coding for the β globin chains are located on chromosome 11, and they determine the type of hemoglobin produced at different stages of life. The β-type globin genes include ε- (embryonic) γ- (fetal) δ- and β-globin (adult), each expressed at their corresponding stage as named. The ε-globin gene is the most upstream gene in the β-globin locus and the first one expressed during embryogenesis. The γ-chains come from two genes located in tandem, and their protein products only differ by one amino acid at position γ136, where the upstream gene contains a glycine and the downstream gene contains an alanine, $^{G}\gamma^{A}\gamma$. The γ-globin genes are predominantly expressed in the fetal stages of development. The δ-globin gene is a result of a duplication of the β-globin gene and its expression remains low throughout postnatal life. The β-globin gene is the most downstream gene of the β-locus and β-

globin chain synthesis predominates during adulthood (Nathan et al. 2003). All globin chains are relatively small peptides of roughly 150 amino acids (~16kDa).

In human adults, the predominant hemoglobin (HbA) is composed two α- and two β-globin chains, and it comprises ~96-98% of total hemoglobin. HbA2, composed of two α- and two δ-globin chains, comprises ~2.4% of total hemoglobin in adults. Fetal hemoglobin (HbF), composed of two α- and two γ-globin chains, comprises <2% of total hemoglobin in adults (Nathan et al. 2003).

B. The β-globin locus and the hemoglobin switch

In humans, the fetal γ-globin gene is located on the β-globin locus on chromosome 11. As described in section A, this locus consists of a family of genes placed in the sequence they are expressed during gestation and adulthood, and it is preceded by a locus control region (LCR-ε-$\gamma^G\gamma^A$-δ-β) (Stamatoyannopoulos 2005; Sankaran, Xu & Orkin 2010) (Figure 1). During the first 6 to 8 weeks of gestation or embryonic stage, ε-globin is highly expressed in the yolk sac and paired with ζ-globin chains. This is followed by high expression of γ-globin in the liver during most of gestation allowing 2 γ-globin chains to pair with 2 α-globin chains to form fetal hemoglobin (HbF). At birth, γ-globin expression begins to decline as the expression of β-globin increases in the bone marrow (Boyer et al. 1975; Peschle et al. 1985; Ley et al. 1989) (Figure 1). At this stage, 2 β-globin chains pair with 2 α-globin chains to form adult hemoglobin (HbA). This process if often referred to as the "hemoglobin switch" since it describes the "switch" that occurs between fetal γ-globin and adult β-globin expression.

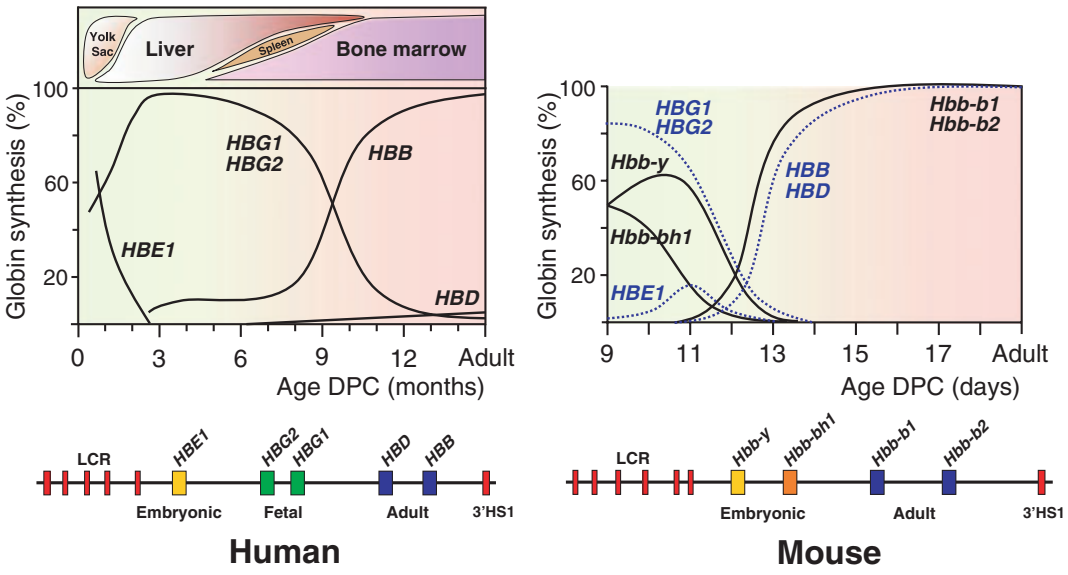


Figure 1. The hemoglobin switch. The human globin locus on chromosome 11 is represented on the left. This locus is preceded by a locus control region (LCR), and the β-type globin genes are positioned in the order in which they are expressed during gestation and after birth. The top panel represents the body compartments in which each of the genes is expressed. On the right is a cartoon representing the hemoglobin switch in mice. Dotted lines represent the human β-type globin genes in transgenic mice undergoing a switch in a similar manner than do murine endogenous embryonic to adult globin genes. Adapted from: Sankaran, Xu & Orkin 2010.

The mouse globin locus greatly resembles the human β-locus. Mice have two embryonic globin genes (εy and βh1) and two adult globin genes (βmaj and βmin), but unlike humans they do not have fetal globin genes (Figure 1). Embryonic genes (εy and βh1) are mostly expressed in primitive (embryonic) erythroid cells and βmaj and βmin are strictly expressed in definitive (adult) erythroid cells (Trimborn et al. 1999). A transition begins at around day E11.5 in gestation when the definitive cells are first detected in the peripheral blood (Popp, D'Surney & Wawrzyniak 1987). In mice, the hemoglobin switch therefore occurs at the embryonic stage as depicted in Figure 1. In transgenic mice containing the human β-globin locus, the human fetal globin genes ($^{A}\gamma$ and $^{G}\gamma$) assume the regulation of the endogenous murine embryonic genes and the hemoglobin switch occurs during gestation as depicted with dotted lines in Figure 1.

II. Erythrocytes and Erythropoiesis

A. Erythrocytes

Red blood cells, or erythrocytes, are oval cells characterized by being highly flexible, and hemoglobin makes up the majority of their protein content. Unlike other cells of the human body, erythrocytes lack a nucleus in order to accommodate a large amount of hemoglobin molecules. Their life span is about 120 days (Alison 1960).

B. Erythropoiesis

Erythropoiesis is the process by which erythroid precursors differentiate to give rise to mature erythroid cells. In humans there are two types of erythropoiesis, primitive (embryonic) erythropoiesis, and definitive (adult) erythropoiesis. Early in ontogeny primitive erythropoiesis

occurs in the yolk sac, where immature erythroid precursors are released into the bloodstream where they mature and enucleate (Palis 2008). Definitive, or adult erythropoiesis occurs at later stages of gestation in the fetal liver. Towards the last three months of gestation, definitive (adult) erythroipoiesis moves to the bone marrow, where erythropoiesis continues through adulthood (Palis 2008; Tsiftsoglou et al. 2009). Unlike primitive erythropoiesis where erythroid cells mature in the bloodstream, definitive erythroipoiesis is characterized by "blood islands" in which erythroid precursors are surrounded by macrophages and they enucleate before entering the bloodstream (Palis 2008).

Erythrocytes differentiate through a series of stages in the bone marrow. Hematopoietic stem cells differentiate into a common myeloid progenitor followed by early erythroid progenitors (burst forming unit-erythroid and colony forming unit-erythroid). These give rise to proerythroblasts, followed by differentiation of these cells in the following order: basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast, polychromatic erythrocyte (or reticulocyte) to finally a mature erythrocyte (Figure 2). Although this terminology refers to their appearance on a light microscope after Wright staining, each of these stages represents a different step in differentiation and is marked by expression of different genes and cell surface markers. During mammalian erythropoiesis, there is a significant decrease in cell size and condensation of the nucleus until enucleation occurs at the orthochromatic erythroblast stage. Reticulocytes are then released into the circulation (Handlin, Lux & Stossel 2002). Reticulocytes contain abundant amount of RNA capable of specifying synthesis of hemoglobin. They comprise ~ 0.5-1.5% of erythrocytes and circulate in the bloodstream for about 1 day before fully maturing into erythrocytes (Skadberg, Brun & Sandberg 2003).

Erythropoiesis is a highly regulated process in terms of cell proliferation, and preventing apoptosis and cell cycle arrest while approaching terminal differentiation. Changes in oxygen tension, iron homeostasis, and stress can affect this process (Tsiftsoglou et al. 2009).

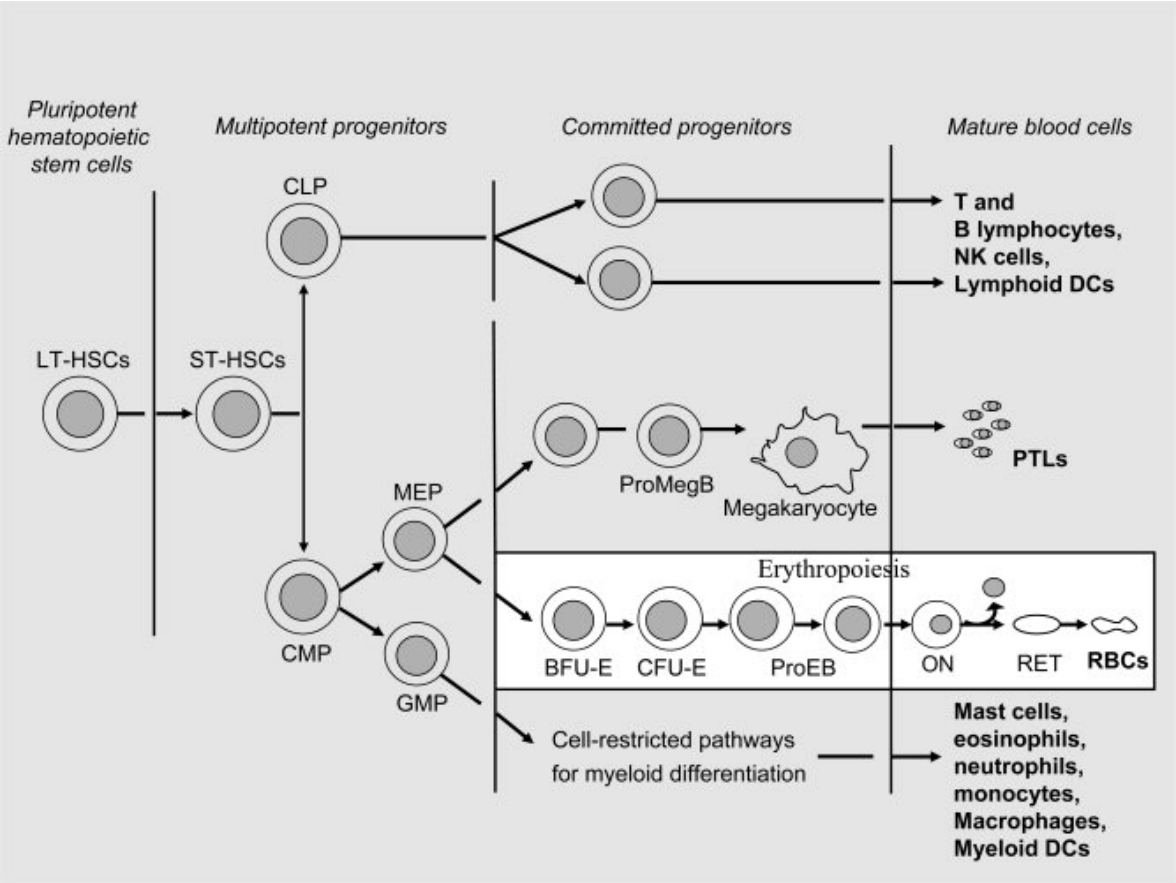


Figure 2. Stages of human erythropoiesis. In the bone marrow, hematopoiesis begins with pluripotent hematopoietic stem cells (HSC). These cells give rise to a common myeloid progenitor (CMP), followed by a megakaryocytic/erythroid pluripotent progenitor (MEP). Erythropoiesis (highlighted in the diagram) occurs in the following order: Burst forming unit-erythroid (BFU-E) gives rise to colony forming unit-erythroid (CFU-E), followed by intermediate forms for Proerythroblasts (ProEB), which give rise to orthrochromatic normoblast (ON). Enucleation occurs at the normoblast stage and reticulocytes (RET) are released into the bloodstream where they eventually become mature red blood cells (RBCs). Adapted from Tsiftsoglou, Vizirianakis & Strouboulis 2009.

III. Hemoglobinopathies

Hemoglobinopathies such as sickle cell anemia and β-thalassemia result from among the most common single gene defects worldwide and affect a significant amount of the population in the United Stated. It is estimated that about 300,000 children worldwide are born each year with either sickle cell anemia or thalassemia (Weatherall 2010). These genetic disorders result from mutations that affect hemoglobin.

A. Sickle cell anemia.

Sickle cell anemia is an autosomal recessive disorder. The first sickle cell anemia patient was described by James Herrick in 1910. In his report, he wrote "what especially attracted attention was the large number of thin, elongated, sickle-shaped and crescent-shaped forms" when describing a case of a patient with severe anemia (Herrik 1910)**.** Sickle cell anemia was first described as a molecular disease in 1949 (Pauling & Itano 1949), and it results from a point mutation in the in the 6^{th} position of the beta globin gene (A-to-T) that replaces a glutamic acid for a valine (Ingram 1956). This recessive disorder is most prevalent in some regions of Africa, where more than a quarter of the population carry the trait. The β-globin mutation became more prevalent in parts of the world where it conferred a survival advantage against malaria caused by Plasmodium falciparum, a parasite that infects erythrocytes (Livingstone 1958). It is also prevalent in parts of Saudi Arabia, Greece, India and Brazil, and about 8% of African Americans are also carriers (Steinberg 2008).

i. Pathophysiology.

In sickle cell anemia, a point mutation in the β-globin gene causes a change from glutamic acid to valine. This valine, a hydrophobic amino acid, is exposed when hemoglobin assumes its deoxy conformation (under low oxygen conditions), and it clusters with hydrophobic pockets of other β-chains causing polymerization of hemoglobin molecules as shown in Figure 3B (Nathan et al. 2003). Polymers accumulate and contribute to an abnormally elongated shape in erythroid cells, preventing their deformability. Although polymerization is a reversible process, the shape change of erythroid cells is not always reversible. The lack of deformability of sickle-shaped cells makes their passage through the microvasculature difficult, therefore causing vaso-occlusion (Figure 3C). Adhesion of sickle cell anemia erythrocytes to the vascular endothelium is also common, further contributing to vaso-occlusion (Ballas & Mohandas 1996; Nathan et al. 2003).

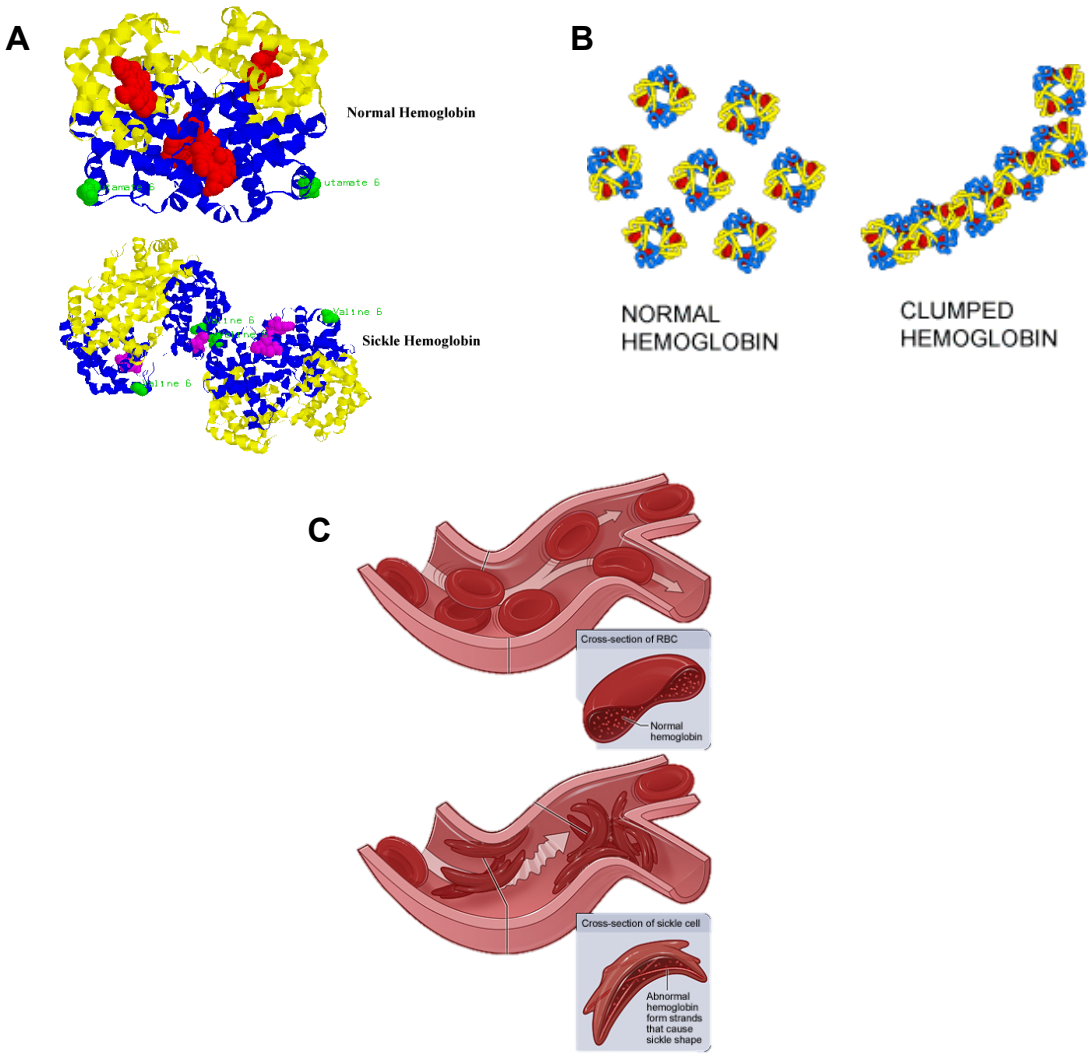


Figure 3. Pathophysiology of sickle cell anemia. (A) The glutamic acid-to-valine mutation causes valine hydrophobic residues to become exposed and cause a conformational change in the hemoglobin tetramer under low oxygen conditions. (B) Hydrophobic interactions allow hemoglobin molecules to form polymers. (C) Polymers lead to a shape change of red blood cells that resembles a sickle. Sickle shaped erythrocytes cause occlusion in small vessels, resulting in ischemia and pain crises. Adapted from carnegiescience.edu (A) evolution.berkeley.edu (B) and www.nhlbi.nih.gov (C).

ii. Clinical manifestations of sickle cell anemia.

Since polymer formation in carriers is very rare, heterozygote patients (also known as sickle cell trait) do not usually show any symptoms. Nonetheless, clinical findings such as urinary tract infections, hematuria and splenic infarct during strenuous exercise and low oxygen conditions are possible (Sears 1978; Kark & Ward 1994).

Sickle cell anemia is largely characterized by chronic anemia. Vaso-occlusion caused by abnormally sickle shaped erythrocytes obstructing small vessels is responsible for most of the complications of this condition. Such complications include acute chest syndrome, strokes, retinopathy, priapism, and chronic nephropathy. Hemolysis (lysis of erythrocytes) not only contributes to severe anemia, but also to vaso-occlusive crisis through reduction of nitric oxide availability (Nathan 2003).

Clinical manifestations can vary depending of the stage of life. In the early years of life, the most common manifestations include painful episodes, acute chest syndrome and stroke. Painful episodes are caused by vaso-occlusion and present as painful swelling of the hands and feet. Acute chest syndrome is characterized by fever, chest pain, wheezing, cough, hypoxia, and lung infiltrate. Later in life, chronic organ hypoxia leads to organ damage and failure. Renal disease oftentimes leads to renal failure in older patients (Nathan et al. 2003; Steinberg 2008). Loss of splenic function makes these patients more susceptible to bacterial infections, in particular pneumococci. Pneumococcal vaccines are therefore standard prevention for patients with sickle cell anemia. Acute chest syndrome is a common cause of death in adults with sickle cell anemia (Nathan et al. 2003).

B. β-thalassemia.

Severe β-thalassemia was first described by Thomas Cooley in 1925, when studying Italian and Greek children with severe anemia. Aside from severe anemia, these children exhibited hepatosplenomegaly, growth retardation and bone deformities. β-thalassemia major is also termed Cooley's anemia (Cooley & Lee 1925).

There are about 23,000 children born with β-thalassemia each year and it is most prevalent in Mediterranean countries, the Middle East, India, North Africa and Central and Southeast Asia (Weatherall 2010).

i. Pathophysiology

The β-thalassemias are a group of disorders that result form either absence or a reduction in the expression of β-globin gene. Point mutations are the most common cause, although deletions can also result in absence of β-globin gene expression (Higgs, Thein & Woods 2001). Reduction or absence of β-globin chain synthesis results in an imbalance of the normal α-to-β-globin chain ratio, leading to accumulation of excess α chains. This abnormal ratio results in ineffective erythropoiesis in the bone marrow (Figure 4) as well as hemolysis in the peripheral blood. In the case of ineffective erythropoiesis, apoptosis of erythroid precursors (Yuan et al. 1993) is triggered by formation of hemichromes (α-globin chains/heme aggregates) (Figure 4). In mature red blood cells, intravascular hemolysis is also caused by α-globin chains' inability to form tetramers and stay in solution. Excess α-globin chains therefore precipitate and the hemichromes interact with cell membrane proteins such as spectrin, causing cell damage and

hemolysis (Shinar , Rachmilewitz & Lux 1989).

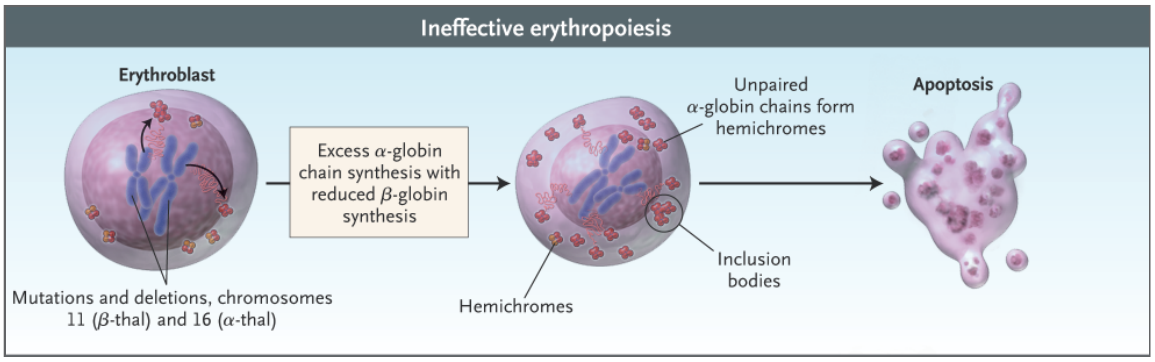


Figure 4. Ineffective erythropoiesis in β-thalassemia. In the bone marrow, excess α-chain deposition (hemichromes) leads to apoptosis of erythroid precursor cells. Adapted from Rund & Rachmilewitz 2005.

ii. Clinical Manifestations of β-thalassemia.

The clinical presentation of β-thalassemia patients can vary from no overt symptoms to severe anemia depending on the type of mutation and the level of β-globin gene expression. β-thalassemias can therefore be classified into 1) β-thalassemia trait in which only one copy of the gene carries a mutation and patients show mild or no anemia. 2) β-thalassemia intermedia in which both copies of the gene carry a mutation but at least one mutation is mild and results in expression of β-globin gene. Patients show mild to moderate anemia and splenomegaly. 3) β-thalassemia major in which both genes carry a severe mutation resulting in little or no expression of the β-globin gene. Patients show severe anemia and are transfusion dependent (Rund & Rachmilewitz 2005).

Aside from severe anemia, β-thalassemia patients can also present with hypercoagulability (Eldor & Rachmilewitz 2001), pulmonary hypertension (Morris & Vichinsky

2011), bone expansion and extramedullary hematopoiesis. Patients who are transfusion dependent are prone to suffer from further complications due to excess iron deposit in organs. This is, in fact, the cause of most complications in patients with β-thalassemia. Although chelating therapy has improved the negative effects of excess iron, its side effects result in poor compliance. Most of the iron deposit occurs in the liver, heart and in endocrine glands. Chronic anemia and iron overload in these patients often results in endocrinopathies and impaired growth. Hypogonadism is also common (Cunningham et al. 2004; De Sanctis 2002; Raiola et al. 2003) and it is treated with hormonal therapy. In older patients, bone disease (including osteopenia and osteoporosis) is caused by bone expansion due to defective erythropoiesis as well as endocrine deficiencies. Although iron deposit affects various organs including the liver resulting in cirrhosis, and the pancreas resulting in diabetes mellitus, it has the most negative impact in the heart, as cardiac events are the most common cause of death in these patients (Rund & Rachmilewitz 2005).

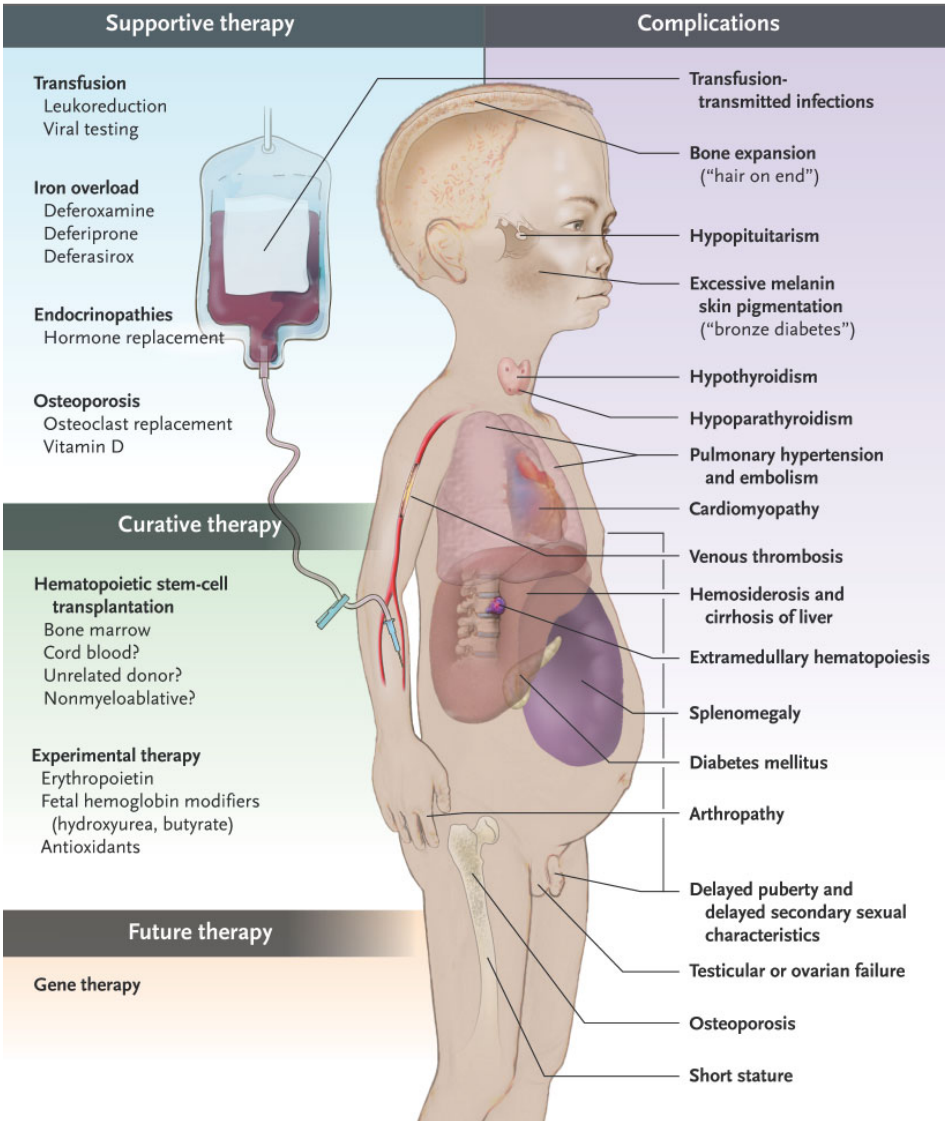


Figure 5. Clinical manifestations of β-thalassemia. Depicted above are some of the clinical manifestations of β-thalassemia patients, which include bone expansion and organ damage due to iron deposit. Organ damage leads to cirrhosis, diabetes mellitus, and cardiomyopathy. Current therapy is mostly supportive, consisting of transfusions along with chelating agents, hormone replacement and vitamin D. The only curative therapy is bone marrow transplantation. Adapted from Rund & Rachmilewitz 2005.

C. The role of fetal hemoglobin on β-type globin disorders

It was first observed that infants with sickle cell anemia did not develop symptoms until several months after birth due to higher levels of fetal hemoglobin (HbF) during this period (Watson 1948). The idea of high HbF conferring protection against sickle cell anemia was later reinforced by the observation that West African sickle cell patients who co-inherited a condition known as hereditary persistence of fetal hemoglobin (HPFH) were highly asymptomatic (Edington & Lehmann 1955). HPFH is a benign condition in which high levels of HbF are caused by either deletions or mutations. Thalassemia patients who co-inherited HPFH were also noted to be asymptomatic (Jacob & Raper 1958).

The mechanism behind HbF's protective role in sickle cell anemia became apparent years later when HbF was shown to prevent polymer formation. In sickle cell anemia, the tetramer $\alpha 2\beta^S 2$ forms polymers in its deoxygenated state. However, a hemoglobin molecule composed of the hybrid tetramer $\alpha 2\beta^S\beta^A$ (one normal β-chain and one mutated β-chain) has only half the probability of polymerizing. Introducing one γ-globin chain forming a hybrid tetramer ($\alpha 2\beta^S\gamma$) does not allow hemoglobin polymerization and therefore prevents sickling (Steinberg 2009). It was later demonstrated than an increase in HbF decreases mortality in sickle cell patients (Platt et al.1994). Of note, the hybrid ($\alpha 2\beta^S\delta$) has also been shown to decrease the polymerization in a similar manner as $\alpha 2\beta^S\gamma$ (Nagel et al.1979).

D. The need for new treatments for sickle cell anemia and β-thalassemia patients

Early studies showed methylation plays an important role in the regulation of embryonic and fetal β-type globin expression and therefore HbF production (Mavilio et al. 1983; McGhee & Ginder 1979; Shen & Maniatis 1980; van der Ploeg & Flavell 1980). In attempts to find agents that would lead to an increase in HbF in patients with sickle cell anemia, studies were conducted using the hypomethylating agent, 5-azacytidine. Treatment of baboons, sickle cell anemia patients (De Simone et al. 1982; Charache et al. 1983) and β-thalassemia patients (Ley et al.1982) with 5-azacytidine successfully showed increases in HbF. Cytotoxicity and myelosuppression, however, were a great concern as potential side effects when using this agent (Ley et al. 1983). Decitabine, another demethylating agent, was also tried with better results and less cytotoxicity (Musallam et al. 2013). Long-term trials testing this agent remain to be conducted.

Paradoxically, it was later inferred that some of the positive effects of increased HbF seen with the use of 5-azacytidine were due to its cytotoxicity. Hydroxyurea, another drug conferring similar S-phase specific cytotoxicity was tested for HbF induction in monkeys with success (Letvin et al. 1984). Hydroxyurea, a ribonucleotide reductase inhibitor, proved to be a successful agent and is now the current treatment for sickle cell anemia. Although its therapeutic effect is not entirely understood, a possible mechanism of action is the induction of stress erythropoiesis (Mabaera et al. 2008). Despite hydroxyurea being a known inducer of HbF in patients, it has variable effects and a large number of patients do not respond to hydroxyurea treatments (Charache, et al. 1992; Steinberg et al. 1997; Ware et al. 2002). Another disadvantage of hydroxyurea use is its inability to induce HbF in a pancellular manner (Platt et al. 1984). Not all

erythrocytes, therefore, are spared of polymer formation. This drug is also completely ineffective in the treatment of β-thalassemia.

Since hydroxyurea has variable effects on sickle cell patients, attempts to find other therapeutic drugs have been made with varying degrees of success. Short chain fatty acids, including butyrate, have been shown to increase HbF expression in animal systems as well as in patients with sickle cell anemia and β-thalassemia (Ginder et al. 1989; Perrine et al. 1993; Weinberg et al. 2005). Although butyrate has antiproliferative effects, intermittent dosage is well tolerated and shows sustained HbF induction (Atweh et al. 1999). Small molecules such as vanillin were developed to inhibit HbS polymerization and appeared promising (Abraham et al. 1991), but later proved to be difficult to achieve high concentrations with low toxicity.

The standard treatment for β-thalassemia major is life-long transfusions. Continued transfusions results in iron overload and iron deposit leading to organ damage. Chelation therapy with parenteral deferroxamine has proved to reduce tissue iron deposit, therefore diminishing the probability of iron overload (Giardina & Grady 2001). Parenteral administration of deferroxamine and its potential side effects, however, have resulted in lower compliance (Olivieri 1999). Transfusions also carry the risk of transfusion-transmitted infections.

Stem cell transplantation is the only curable treatment for both sickle cell anemia and β-thalassemia. A 5-10% mortality rate, high cost, and the difficulty in finding suitable donors makes this option difficult for some patients. Furthermore, stem cell transplantation has not been successful in patients with extensive disease from β-thalassemia. β-thalassemia patients with significant liver damage from iron overload usually experience higher rates of graft rejection (up to 30%) (Lucarelli et al. 1996).

The lack of effective treatments for these conditions has sparked efforts to find new ways to treat these conditions. In order to find more effective and potentially less toxic targeted strategies to induce HbF production, it is important to fully understand the molecular basis of developmental repression of the fetal γ-globin gene.

IV. Regulation of γ-globin gene expression

A. The Locus Control Region (LCR)

There are numerous sequence elements that play a role in the regulation of β-type globin genes, including cis-acting factors as well as trans-acting factors (Stamatoyannopoulos, 2005; Sankaran, Xu & Orkin 2010; Ginder, Gnanapragasam & Mian 2008). An important cis-acting factor that regulates the expression of β-globin genes is an upstream locus control region (LCR) (Tuan et al. 1985, Forrester et al. 1986, Grosveld et al. 1987). The locus control region is an enhancer-like region upstream of the β-locus. It consists of five DNase I hypersensitive sites that are located 6-20kb upstream of the embryonic ε-globin gene. It was originally discovered by a series of experiments searching for the presence of DNase I hypersensitive sites that were necessary for the expression of β-locus genes (Tuan et al. 1985; Forrester et al. 1986). The importance of this region was further confirmed by showing that only in the presence of the LCR, transgenic mice bearing the human β-globin locus were able to express human β-type globin genes at high levels (Grosveld et al. 1987). The DNase I hypersensitive sites found in the LCR have different functions. Some act as insulators (Bell, West & Felsenfeld 2001; Li et al. 2001), while others act as activators (Navas et al. 1998).

Earlier studies focusing on the role of the LCR on globin gene expression, led to two non-exclusive theories about regulation of the genes located in the β-locus. The first was a competition model suggesting expression of globin genes is determined by which gene is closer to the LCR (chromosome looping). The second is an autonomous control model suggesting regulation of gene expression is mostly through sequences located in each globin gene promoter region as well as trans-acting factors regulating each promoter. The competition model was determined by developing transgenic mice with β-type globin genes placed at different distances from the LCR. When placed closer to the LCR relative to embryonic and fetal β-type globin genes, the β-globin gene is activated at earlier stages compared to its normal time of activation, while the γ-globin gene is silenced earlier in development when placed farther from the LCR (Hanscombe et al. 1991). Overall, the ε-globin gene appears to be highly regulated by autonomous control in definitive erythropoiesis (Raich et al. 1990, Dillon & Grosveld 1991), β-globin gene appears to be largely regulated by chromosome looping during definitive erythropoiesis, and the γ-globin gene is regulated by both types of control (Tanimoto et al. 1999). Aside from distance, copy number and orientation are also important in the regulation of β-type globin genes by the LCR (Tanimoto et al. 1999).

B. BCL11A and Sox6

BCL11A (B cell lymphoma/leukemia 11A) is a zinc finger transcription factor that was first identified as being a common retroviral integration site in murine myeloid leukemia leading to high expression of this protein (Nakamura et al. 2000). Genome wide association studies (GWAS) searching for correlations between single nucleotide polymorphism and increased HbF levels first led to the discovery of BCL11A in relation to γ-globin regulation (Menzel et al.

2007). Later studies confirmed BCL11A plays an essential role in hemoglobin switching, as BCL11A conditional knockout mice crossed with transgenic mice bearing the human β-locus show a delay in γ-to-β-globin gene switch between E14.5 and E18.5 in fetal liver cells (Sankaran et al. 2009). Absence of BCL11A also shows amelioration of the sickle cell phenotype in young adult mice (Xu et al. 2012). Despite the silencing delay of γ-globin expression during early life in BCL11A KO mice, γ-globin gene expression drastically diminishes between E18.5 and 4-5 weeks of age (Esteghamat et al. 2013), suggesting BCL11A plays an important role in the hemoglobin switch during ontogeny but a less prominent role during adult regulation of γ-globin expression.

In order to understand the mechanism by which BCL11A regulates γ-globin gene expression, BCL11A has been screened for potential binding partners. BCL11A interacts with many proteins including MBD3/NuRD complex components, GATA-1, FOG-1 and LSD-1 (Sankaran et al. 2008; Xu et al. 2013). BCL11A binds to the β-globin locus at two sites, the hypersensitive site HS3 and the intragenic region between γ- and δ-globin genes (Sankaran et al. 2008). With the help of Sox6, BCL11A also contributes to the β-locus chromosome looping necessary for γ-globin gene silencing as shown by 3C assays (Xu et al. 2010). Although a potential candidate as a target in the re-expression of γ-globin, absence of BCL11A has devastating effects on other cell populations such as B-lymphocytes (Liu et al. 2003).

Sox6 is a member of the Sry-related high-mobility group (HMG) box transcription factors. The sox family of transcription factors binds to the minor groove of the DNA and leads to DNA looping (Ferrari et al. 1992; Connor et al. 1994). Sox6 deficient mice show prolonged expression of murine εy-globin gene beyond the hemoglobin switch, since part of its function is to bind to and negatively regulate the εy-globin promoter (Yi et al. 2006). In the human β-locus,

Sox6 physically interacts with BCL11A and binds to several regions of the β-locus, most likely contributing to chromosome looping (Xu et al. 2010) (Figure 6).

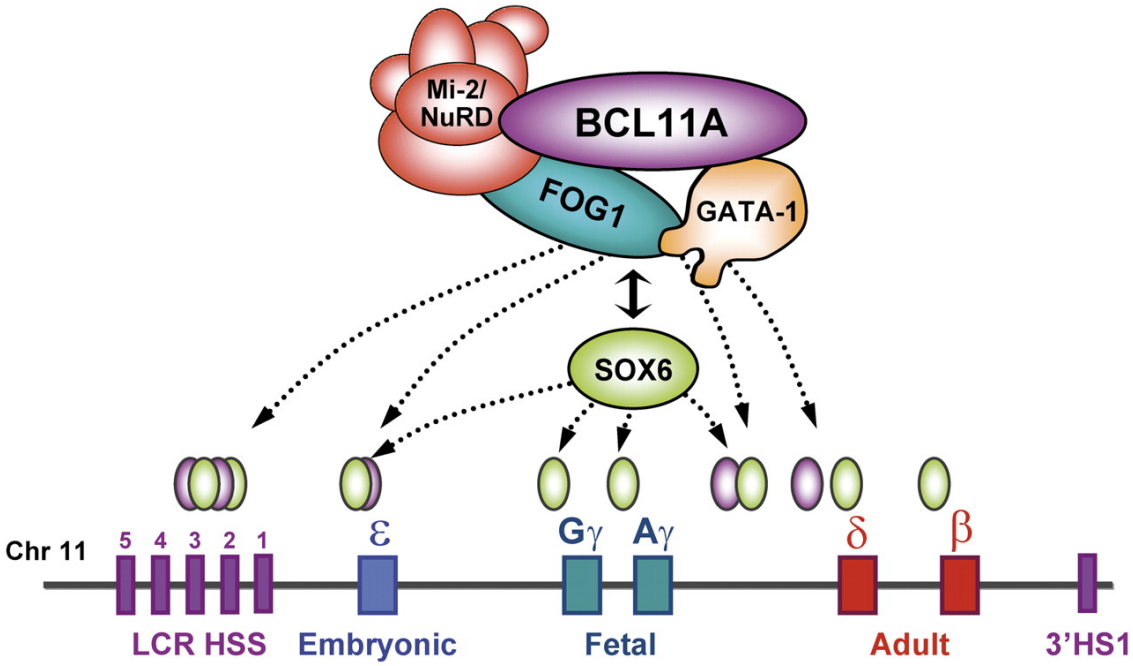


Figure 6. BCL11A regulates γ-globin expression through its interaction with the NuRD complex and contributes to long-range chromosomal interactions through Sox6. BCL11A interacts with Mi2/NuRD complex components as well as GATA-1 and FOG-1. Green dots represent Sox6 binding sites within the globin locus, while purple dots represent BCL11A binding sites. Adapted from Xu et al. 2010.

C. The KLFs

The Krüppel-like family of transcription factors (KLF) also plays an important role in the regulation of mouse and human globin genes. Krüppel-like factors are a family of DNA binding proteins, characterized by three Cys2 His2 zinc fingers, which bind to the consensus CACCC motifs (Bieker 2001). KLF1 (formerly known is EKLF) was the first Krüppel-like factor to be identified in erythroid cells (Miller & Bieker 1993). KLF1 plays a crucial role in erythroid development, and knockout mice die at day E14.5 of severe anemia due to failed fetal liver erythropoiesis (Perkins, Sharpe & Orkin et al. 1995; Perkins, Gaensler & Orkin et al. 1996). KLF1 binds to the CACCC motif of the β-globin gene where it acts as an activator, and mutating this motif results in β-thalassemia (Feng, Southwood & Bieker 1994). Recent studies have discovered yet a new mechanism by which KLF1 regulates the globin locus. KLF1 binds to and activates the BCL11A gene (Borg et al. 2010; Zhou et al. 2010), thereby indirectly inhibiting γ-globin gene expression. Although mutations in the KLF1 DNA binding domain causing KLF1 haploinsufficiency have been associated with HPFH (Borg et al. 2010), KLF1 also regulates other non-globin erythroid-specific genes. Thus, it is not surprising that other erythroid conditions have been associated with mutations of this transcription factor. A dominant negative mutation in the zinc finger domain of KLF1 has been associated with hereditary spherocytosis and hemolytic anemia (Heruth et al. 2010; Siatecka et al. 2010).

KLF1 is a very dynamic protein and is able to act as a transcriptional activator as well as a repressor (Bieker 2001). The different roles of KLF1 are tightly regulated by post-translational modifications and interactions with different cofactors. One of these post-translational modifications, SUMOylation at lysine 74, allows KLF1 to interact with Mi2β and act as a transcriptional repressor in order to inhibit megakaryopoiesis (Siatecka et al. 2007).

Given the high degree of homology in the KLF family of proteins, the study of KLF1 led to the screen of more Krüppel-like factors for a potential role in the γ-globin gene regulation. KLF2, 3, 4, 5, 8, 11, 12 and 13 are all expressed in erythroid cells (Zhang et al. 2005). Of these factors, KLF2 plays an important activating role in mouse embryonic εy- and βh1-globin genes as well as human embryonic genes (Basu et al. 2005). KLF1, in conjunction with KLF2, regulates the expression of human embryonic ε- and human fetal γ-globin genes by binding to their promoter regions in transgenic mice during embryogenesis (Alhashem et al. 2011).

D. TR2TR4 and LSD1

A Direct Repeat Erythroid Definitive (DRED) complex was originally proposed to regulate the expression of human ε- γ-globin genes and murine εy- and βh1-globin genes through binding to direct repeats in their respective proximal promoter regions (Tanimoto et al. 2000). The direct repeats are analogous to binding sites for nonsteroidal nuclear receptors. DRED was later characterized as a large complex containing orphan nuclear receptors TR2 and TR4. Although mutations in the binding sites for this complex in βYAC transgenic mice caused a clear de-repression of embryonic and fetal globin genes (Omori et al. 2005; Tanimoto et al. 2000), studies attempted at understanding the mechanism of action of TR2/TR4 appeared paradoxical. TR2/TR4 null mice crossed with mice bearing the human β-locus showed delayed silencing of ε- and γ-globin genes in fetal livers, but forced expression of these receptors also led to an increase in γ-globin expression in definitive erythroid cells (Tanabe et al. 2007).

TR2/TR4 was found to interact with co-repressors such as DNA methyltransferase 1 (DNMT1), lysine-specific histone demethylase 1 (LSD1), as well as components of the nucleosome remodeling and histone deacetylase (NuRD) complex Mi2 and HDAC1/2 (Cui et al.

2011). In order to dissect the role of other proteins belonging to the DRED complex, recent studies have focused on LSD1 and its role in globin gene regulation. LSD1, a flavin-dependent monoamine oxidase, can demethylate mono- and di-methylated lysines, especially H3K4 (Shi et al. 2004). Recent reports have shown a knockdown of LSD1 increases fetal hemoglobin in human primary erythroid cells (Shi et al. 2013). Because nonselective monoamine oxidase (MAO) inhibitors have been shown to inhibit LSD1 (Lee et al. 2006), the use of MAO inhibitors to induce HbF was appealing as these drugs are FDA approved for the treatment of depression. An irreversible monoamine oxidase inhibitor, tranylcypromine, was recently shown to induce HbF expression on human primary erythroid cells (Shi et al. 2013). Although an FDA approved drug, tranylcypromine it is not widely used due to its significant side effects. In 1964 it was withdrawn from the market due to alarming side effects such as paradoxical hypertension and intracranial bleeding, resulting in the death of several patients. It was later reintroduced in the market with greater limitations for its use (Atchley 1964). Considering tranylcypromine only induces HbF by a 2-fold, its positive effect may not compensate for the drug's potential negative side effects. Furthermore, recent reports have also shown that inhibiting LSD1 blocks erythroid differentiation, which may inacuratly appear as increased levels of HbF in adult human primary erythroid cells (Xu et al. 2013).

E. HBS1L and MYB

Genome wide association studies (GWAS) comparing populations with high vs. low HbF also identified SNPs in the region between HBS1L and MYB genes located on chromosome 6 (Menzel et al. 2007; Thein et al. 2007; Lettre et al. 2008; Uda et al. 2008). Patients with elevated HbF showed a decrease in the expression of both HBS1L and MYB, but only forced expression

of MYB resulted in an inhibition of γ-globin gene expression in the human myelogenous leukemia line K562 (Jiang et al. 2006). An older observation that patients with partial trisomy 13 show a delay in the hemoglobin switch (Huehns et al. 1964; Sankaran & Sapp 2012) was recently connected to the regulation of MYB. High expression of two microRNAs located on chromosome 13 (miR15a and miR16-1) is thought to be responsible for this mechanism (Sankaran et al. 2011). miR15a and miR16-1 target MYB, and forced expression of these two microRNAs have been shown to increase γ-globin gene expression (Sankaran et el 2011). Recently, MYB was also shown to positively regulate BCL11A and KLF1 in human primary erythroid cells (Suzuki et al. 2013).

F. GATA-1 and FOG-1

GATA-1 was one of the first factors identified as a regulator of globin gene expression. It is a zinc finger protein involved in the regulation of many erythroid specific genes (Evans, Reitman & Felsenfeld 1988; Pevny et al. 1991; Simon et al. 1992; Weiss, Keller & Orkin 1994; Fujiwara et al. 1996). It binds to the β-globin locus (Evans & Felsenfeld 1989; Martin, Tsai & Orkin 1989; Tsai et al. 1989), and facilitates chromosome looping of the β-locus (Vakoc et al. 2005). Furthermore, GATA-1 binds to the distal promoter region of the γ-globin gene (Harju-Baker et al. 2008), allowing recruitment of the nucleosome remodeling and histone deacetylase (NuRD) complex in a FOG-1 dependent manner (Fox et al. 1998; Hong et al. 2005). This contributes to γ-globin silencing in definitive erythropoiesis. Friend of GATA-1 (FOG-1) is a hematopoietic specific zinc finger protein and an important co-factor of GATA-1. In fact, most of the functions of GATA-1 depend on binding to FOG-1 (Tsang et al. 1997). Importantly, FOG-1 acts as a cofactor for GATA-1 in both its activating and repressive roles (Letting et al. 2004).

V. Epigenetics

Epigenetics involves the study of changes in gene expression that are not the result of a change in the DNA sequence (Berger et al. 2009). Epigenetic changes include DNA methylation and histone modifications, and they play an important role in developmental proceses such as inactive X chromosome, genetic imprinting, silencing of certain developmental genes and cell differentiation.

A. DNA methylation and hydroxymethylation

DNA methylation is a major form of epigenetic regulation in which cytosine residues are covalently modified at palindromic CpGs at their 5-position carbon (Figure 7). This modification is regulated by a family of DNA methyltransferase enzymes (DNMTs) which include DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L (Bestor et al. 1988; Cheng & Blumenthal 2008; Yoder & Bestor 1998; Okano, Xie & Li 1998; Bestor 2000). DNMT1, the maintenance DNMT, methylates hemimethylated DNA, and is therefore in charge of methylating daughter strands during replication (Probst, Dunleavy & Almouzni 2009). DNMT3a and DNMT3b show a preference for unmethylated DNA and perform de novo methylation (Okano et al. 1999). CpGs throughout the genome are methylated at at rate of 60%-90% depending on the tissue (Ehrlich et al. 1982), yet clusters of CpGs (CpG islands) found in promoter regions are usually unmethylated. Methylation of CpG islands plays a regulatory role in a handful of genes during development, and aberrant methylation of CpG islands of tumor suppressor genes is known to contribute to cancer development (Gopalakrishnan, Van Emburgh & Robertson 2008).

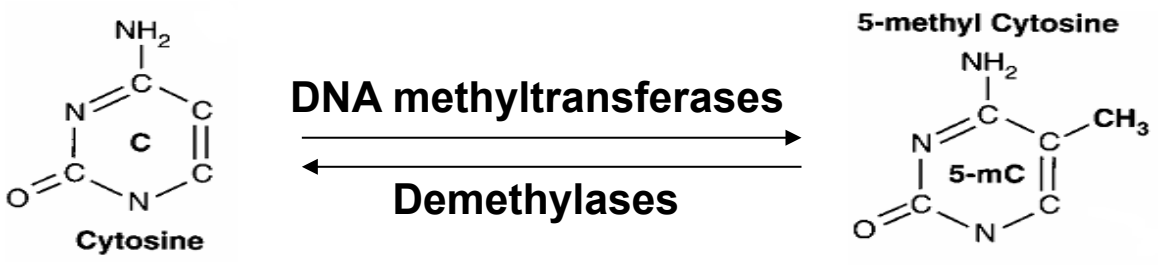


Figure 7. DNA methylation in humans. DNA methyltransferases covalently modify the 5-C of cytosine by adding a methyl group. Adapted from Ginder, Gnanapragasam & Mian 2008.

Although originally discovered in 1972 (Penn et al. 1972), 5-hydroxymethylation was recently recognized as another epigenetic mark (Kriaucionis & Heintz 2009; Tahiliani et al. 2009). The Tet family of proteins are 2-oxoglutarate (2OG)-dependent and Fe(II)-dependent dioxygenases responsible for this DNA modification. Tet proteins can carry out oxidation reactions requiring Fe(II) and 2 (OG) to convert 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) (Ito et al. 2010). 5hmC is thought to be an intermediate for 5mC demethylation (He et al. 2011; Ito et al. 2011; Guo et al. 2011; Inoue & Zhang 2011; Wossidlo et al. 2011; Iqbal et al. 2011), but its ability to hinder binding of 5mC binding proteins (Valinluck et al. 2004), and a plausible 5hmC binding protein (MBD3) make this a potential epigenetic mark. Based on recent publications, however, it is debatable whether MBD3 has higher affinity for 5hmC (Yildirim et al. 2011; Baubec et al. 2013).

B. Methyl binding domain proteins

A largely conserved family of nuclear proteins, the methyl CpG binding domain (MBD) proteins, are known to be "readers" of DNA methylation. The role of MBD proteins is to translate DNA methylation into histone modifications by the recruitment of silencing complexes (Figure 8) (Jaenisch & Bird, 2003; Wade 2001). This family of proteins is composed of 5 known proteins, MeCP2, and methyl-binding domain proteins (MBD) 1-4. MeCP2 was the first protein to be identified, followed by a complex originally named MeCP1 (Meehan et al. 1989; Lewis et al. 1992). MeCP1 was later discovered to be either MBD2 or MBD3 along with a multisubunit complex (Ng et al. 1999; Cross et al. 1997; Feng & Zhang 2001; Feng et al. 2002). Subsequently, other MBD proteins were identified based on their common MBD domain (Hendrich & Bird 1998; Hendrich et al. 1999a). MeCP2 as well as MBD1-3 act as transcriptional repressors, whereas MBD4 acts as a thymine DNA glycosylase (Hendrich et al. 1999b).

C. MBD2 and MBD3, similar proteins?

Aside from the structural resemblance of their methyl cytosine binding domains (MBD), members of the MBD family of proteins differ significantly in their structures. MBD2 and MBD3 are the exception, sharing ~71% homology (Hendrich & Bird 1998). Despite their structural similarities, however, functional differences between MBD2 and MBD3 have become apparent. Expression of MBD3 appears to be ubiquitous, as it is found in almost every mouse tissue including embryonic stem cells. MBD2, on the other hand, is selectively expressed in certain tissues such as spleen and testes (Hendrich & Bird 1998). The specificity of MBD2 binding to its target genes comes not only from its tissue-specific expression but also adjacent base pairs surrounding the CpGs (Scarsdale et al. 2011). In contrast to MBD2 knockout mice,

which are healthy and viable, MBD3 knockout mice are embryonic lethal (Hendrich, et al. 2001). Although all MBD family members bind to methylated DNA, MBD3 appears to be the only member that non-specifically binds to methylated or unmethylated DNA (Hendrich & Bird 1998; Fraga et al. 2003). Recent studies have suggested a higher affinity of MBD3 for hydroxymethylated DNA, although further data supporting this view will be necessary to draw firm conclusions (Yildirim et al. 2011; Baubec et al. 2013).

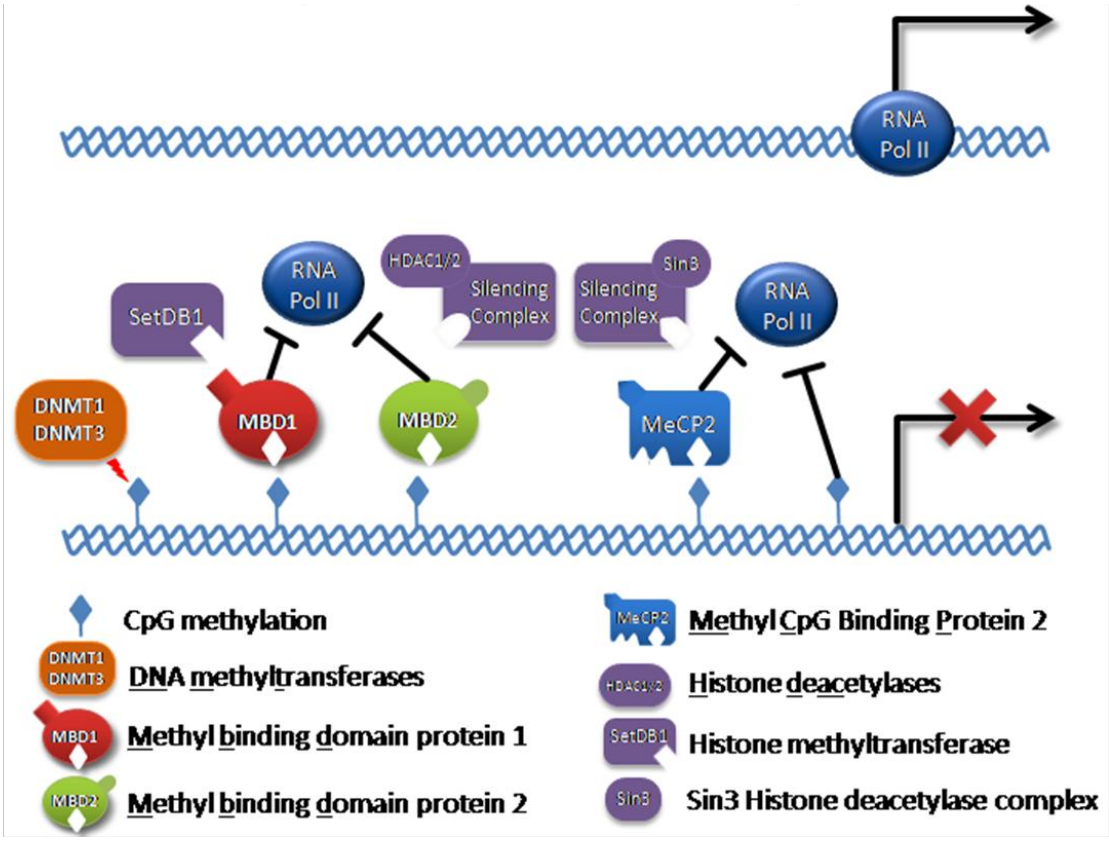


Figure 8. The role of DNA methylation in gene silencing. During development, a number of CpG rich promoter regions are methylated by DNMT enzymes. DNA methylation hampers RNA Pol II from advancing. DNA methylation also recuirts methyl-binding domain proteins such as MBD2, and silencing complexes such as the NuRD complex, which in turn alter histone modification patterns further preventing gene expression. Adapted from Ginder, Gnanapragasam & Mian 2008.

D. Histone modifications

Histone proteins (H1, H2A, H2B and H3 and H4) function to package eukaryotic DNA into nucleosomes (Luger & Richmond 1998; Kornberg & Lorch 1999). Histone modifications are a dynamic system that allows for regulation of transcription. Histones undergo post-translational modifications, which serve as a regulatory function to influence chromatin structure and accessibility of DNA to transcription factors and other transciptional regulators (Li, Carey & Workman 2007). N-tails from histones H3 and H4 protrude from the nucleosome, and can undergo covalent post-translational modifications such as methylation, phosphorylation, ubiquitination, sumoylation and acetylation (Strahl & Allis 2000). Of these modifications, histone acetylation and methylation are perhaps the best studied. Trimethylation of H3K4, H3K36 and H3K79 are associated with euchromatin and characteristic of transcriptional activation. This is also true of histone acetylation. On the other hand, trimethylation of H3K9, H3K27, and H4K20 have been associated with silenced genes. Histone deacetylation is also associated with gene silencing. (Strahl & Allis 2000; Bartova et al. 2008).

This "histone code" of posttranslational modifications is then "read" by complexes which affect gene regulation. This process is complex and involves a large number of proteins/complexes involved in recognizing histone marks. (Yun et al. 2011). Histone binding proteins can help translate this "histone code" by exerting different effects on transcription, DNA repair and other processes (Figure 9).

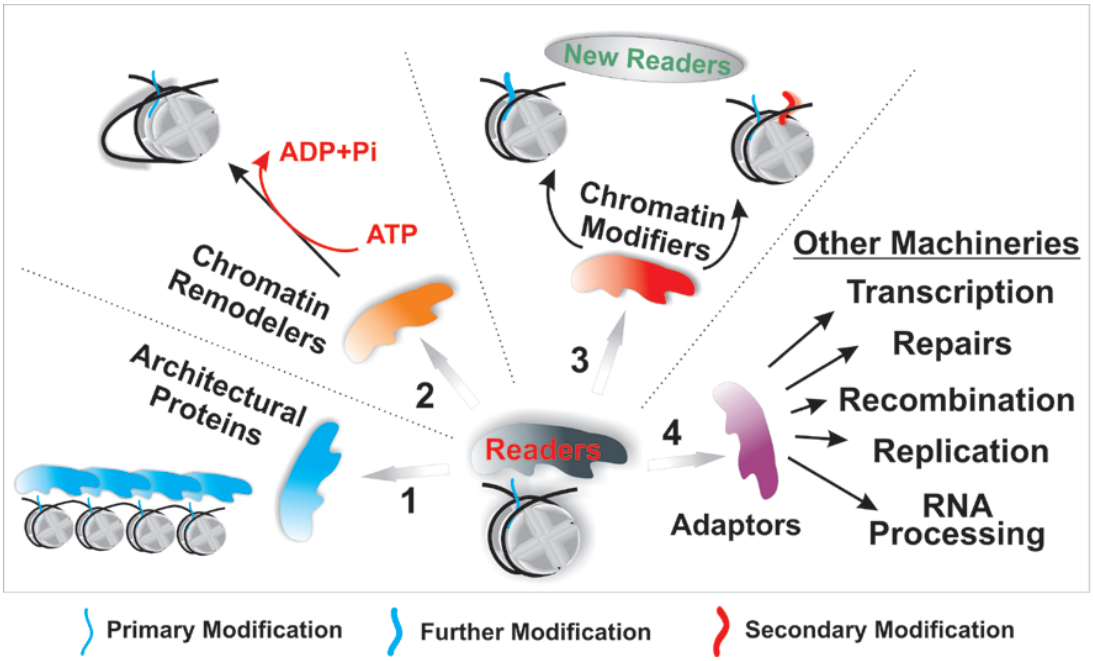


Figure 9. Histone post-translational modifications result in different readouts by recruiting proteins or complexes. 1) Architectural proteins can recognize histone marks and spread through a region leading to chromatin condensation. 2) Through its ATPase function, chromatin remodelers can mobilize nucleosomes to make DNA more/less accessible. 3) Certain histone post-translational modifications recruit modifiers and their sole function is to further recruit secondary modifiers. 4) Histone post-translational modifications can also serve to recruit other machineries such as transcription factors, DNA repair machineries among others. Adapted from Yun et al. 2011.

E. Epigenetics and globin gene regulation

Epigenetic mechanisms including DNA methylation and histone modifications play an important role in developmental γ-globin gene silencing (Singal et al. 1997; Hsu et al. 2007; Mabaera et al. 2007; Forsberg et al. 2000; Gribnau et al. 2000). DNA methylation was first shown to play a role in globin regulation by several studies in different species, where an inverse correlation between DNA methylation and gene expression was found (Mavilio et al. 1983; McGhee & Ginder 1979; Shen & Maniatis 1980; van der Ploeg & Flavell 1980). Further studies demonstrated that treating both primates and patients with the demethylating agent 5-azacytidine resulted in increased expression of embryonic and fetal hemoglobins (DeSimone et al. 1982; Charache et al. 1983; Ley et al. 1982). Whether or not hydroxymethyl DNA plays a role in the regulation of these genes remains to be determined.

F. The role of the MBD2/NuRD complex in globin gene switching

MBD2 is known to bind to densely methylated CpGs (Meehan et al. 1989) and mediate the recruitment of a silencing complex (Nucleosome Remodeling and Histone Deacetylase Complex, NuRD). NuRD complexes includes at least one copy of each of the proteins Mi2α and –β, HDAC-1 and -2, MTA-1 and -2, RbAp46/48, and p66α and –β (Hendrich & Bird 1998; Hendrich & Tweedie 2003; Feng & Zhang 2001) as depicted in Figure 10.

The first association between MBD2 and globin gene regulation came from studies in the avian systems, where DNA methylation was shown to have an inverse correlation to gene transcription (Burns, Glauber & Ginder 1988). Using nuclear extracts from avian primary erythroid cells, studies showed the MBD2/NuRD complex binds to the methylated DNA proximally transcribed region of the ρ-globin gene (the avian embryonic globin gene) (Singal et

al. 2002). Further purification and characterization of this complex showed MBD2 but not MBD3 was found in this complex (Kransdorf et al. 2006). These studies led to the first direct evidence of the MBD2/NuRD complex binding to, and regulating β-type globin genes during development.

Studies crossing MBD2 knockout mice with transgenic mice bearing a yeast artificial chromosome with the human β-locus (βYAC) led to a ~20- to 40% increase in γ-globin gene expression (Rupon et al. 2006). Likely due to the lack of CpG rich nature of the human β-locus, MBD2 does not interact directly with the promoter region of the γ-globin gene, suggesting its regulatory function on this locus is via an indirect mechanism (Rupon et al. 2006). MBD2 is also an important regulator of ε-globin gene expression (Rupon et al. 2011).

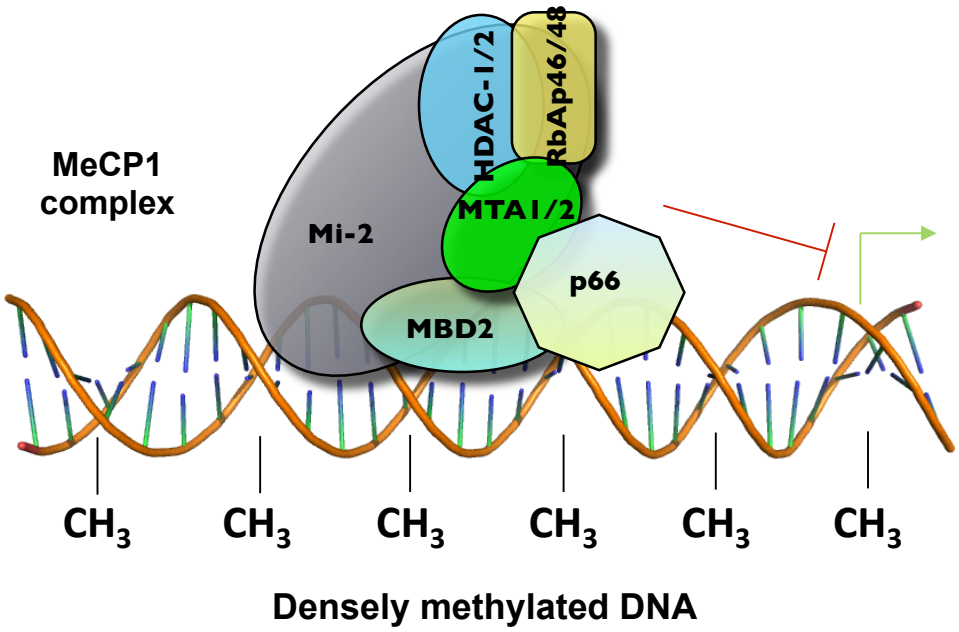


Figure 10. The MBD2/NuRD complex. Densely methylated DNA is able to recruit methyl-binding domain protein 2 (MBD2) as well as the NuRD (MeCP1) complex. Mi2α/β confers the chromatin remodeling function of the complex. MTA1/2 and p66α/β act as transcriptional repressors. The histone deacetylase activity is mediated by HDAC1/2, and RbAp46/48 are histone-binding proteins.

The potential of MBD2 as a therapeutic target for β-thalassemia and sickle cell anemia has led to many studies investigating its structure, ability to recognize methylated DNA, and potential ways of disrupting its association with the NuRD complex. The use of a small peptide that interferes with the coiled coil interaction between MBD2 and p66α leading to displacement of p66α and Mi2β from the NuRD complex, results in a significant increase in γ-globin gene expression in mouse erythroid cells bearing the human β-locus (CID cells) (Figure 11) (Gnanapragasam et al. 2011).

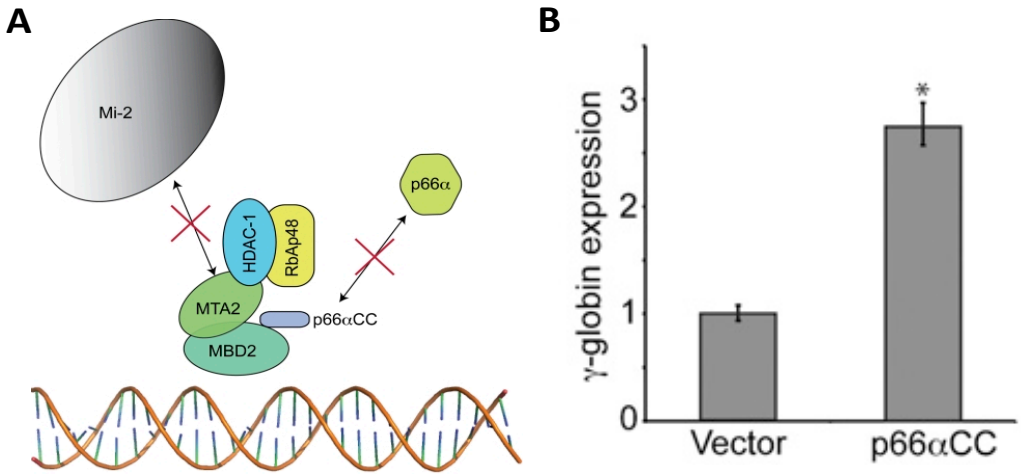


Figure 11. A small peptide is able to target the MBD2/NuRD complex and relieve γ-globin gene silencing. (A) A small peptide comprising the p66α coiled coil domain is able to interact with MBD2 and displace key components of the NuRD complex (Mi2β and p66α), partially inhibiting its function and relieving γ-globin gene silencing. (B) Forced expression of p66α coiled coil domain leads to an ~3-fold increase in γ-globin expression in CID cells. Adapted from Gnanapragasam et al. 2011.

These studies suggest small peptides are a feasible approach when targeting epigenetic complexes. Drug delivery and bioavailability of such peptides in an *in vivo* model remain to be determined.

G. The role of the MBD3/NuRD complex in globin gene switching

Methyl-CpG binding domain protein 3 (MBD3), in contrast to other members of the MBD family, does not show a significantly higher binding affinity for methylated DNA (Hendrich & Bird 1998; Fraga et al. 2003). Similarly to MBD2, MBD3 is associated with a NuRD complex, although the MBD3/NuRD and MBD2/NuRD complexes have independent, non-overlapping functions (Le Guezennec et al. 2006; Hendrich et al. 2001). With respect to hemoglobin switching, the MBD3/NuRD complex has been shown to interact with and negatively regulate the globin locus through the association with two important transcription factors, GATA-1 and Friend of GATA-1 (FOG-1) (Rodriguez et al. 2005; Hong et al. 2005; Harju-Baker et al. 2008). The GATA-1/FOG-1/NuRD complex negatively regulates γ-globin gene expression in βYAC transgenic mice by binding to its distal promoter (Harju-Baker et al. 2008). Of note, FOG-1 has also been shown to co-localize with GATA-1 in genes that are both positively as well as negatively regulated by GATA-1 (Wang et al. 2002; Letting et al. 2004; Pal et al. 2004; Jing et al. 2008), suggesting that GATA-1/FOG-1/NuRD complexes can be context dependent activators or repressors. Although the GATA-1/FOG-1/NuRD interactions have been well characterized in the mouse system, they remain to be elucidated in humans. The majority of the MBD3/NuRD complex components also associate with BCL11A in mouse erythroleukemia cells, suggesting a link between the regulatory function of BCL11A and the MBD3/NuRD complex (Xu et al. 2013).

H. The complexity of the hemoglobin switch

The developmental switch of fetal γ-globin to adult β-globin gene expression is characterized by several layers of regulation, ranging from cis-acting regulatory factors such as

the LCR to trans-acting factors such as transcription factors and epigenetics. The hemoglobin switch appears to have several axes of regulation and some of the connecting links between different regulatory branches, if any, remain to be elucidated (Figure 12). Of note, the mechanisms behind γ-globin gene silencing during development may not completely parallel the mechanisms behind γ-globin gene silencing during adulthood.

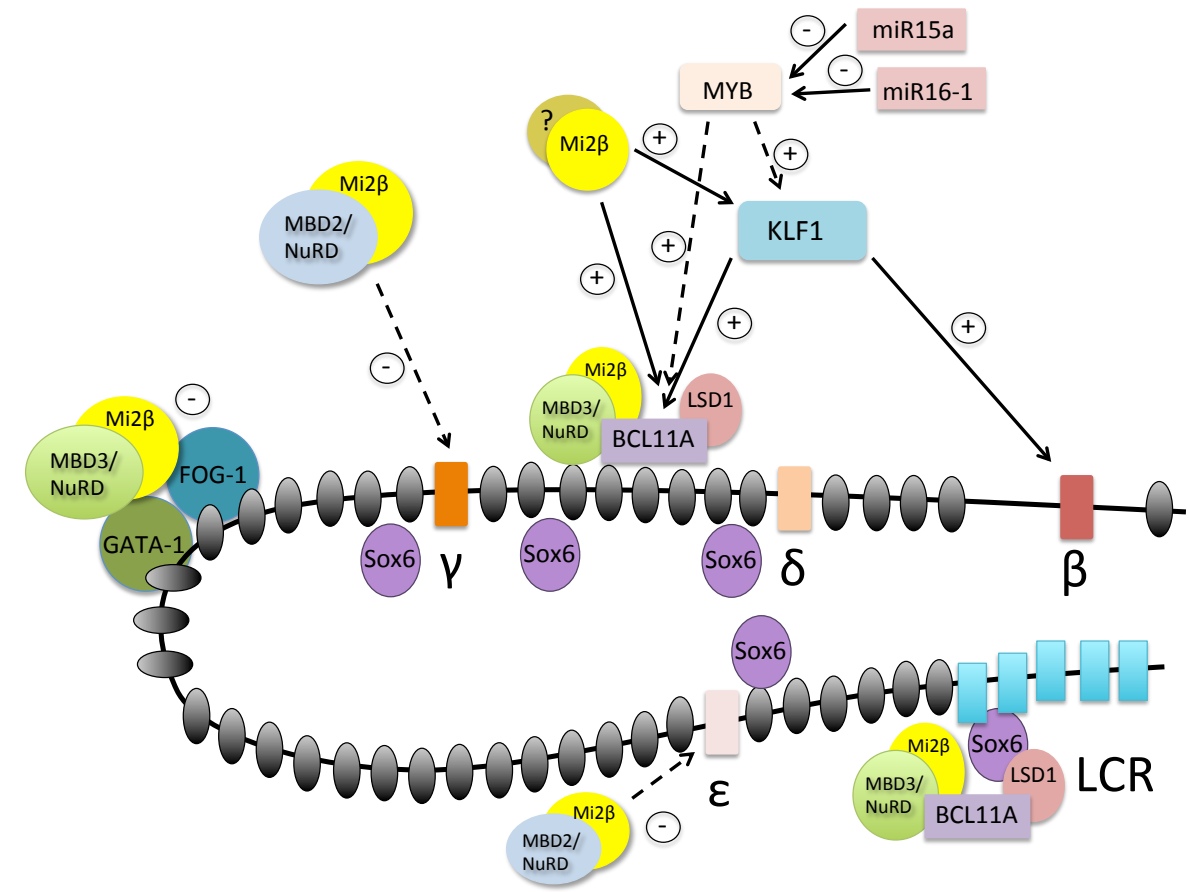


Figure 12. Factors contributing to the hemoglobin switch. Depicted above are some of the factors that contribute to γ-globin gene silencing in adult erythroid cells. Chromosome looping allows the locus control region (LCR) to remain closer to β-globin gene. Sox6 along with BCL11A contribute to chromatin looping. KLF1 positively regulates the β-globin gene as well as BCL11A. MYB positively regulates KLF1 and BCL11A. The MBD3/NuRD complex interacts with BCL11A and it also binds to the distal promoter region of γ-globin via its interaction with GATA-1 and FOG-1. The MBD2/NuRD complex negatively regulates the γ-globin in an indirect fashion. Solid arrows indicate direct interactions, whereas dotted arrows indicate indirect interactions.

VI. Advances in techniques and animal models for the study of hemoglobin switching

In humans, the hemoglobin switch begins during development, and soon after birth γ-globin chain synthesis is replaced by β-globin chain synthesis. In the adult bone marrow, erythroid precursors also show elevated γ-globin expression that declines as cells differentiate (Papayannopoulou, Brice & Stamatoyannopoulos 1976). Over the years, many systems have been developed in order to study this complex process *in vivo* during development, and *in vitro* by culturing hematopoietic progenitor cells. Each method has provided insights into this highly regulated process, although proper regulation of globin genes has posed a challenge in all of the systems.

A. Cell lines

Although a number of erythroleukemia cell lines have been useful in the study of erythroid differentiation and γ-globin gene silencing, these lines have failed to show normal expression of the β-type globin genes. The most commonly used human erythroleukemia cell line is the brc-abl(+) chronic myelogenous leukemia line, K562 (Lozzio & Lozzio 1975). K562 cells are able to be differentiate into erythroid cells using the proper stimuli, but they display high levels of γ-globin expression. The use of this particular cell line to find inducers of γ-globin expression may therefore be misleading since basal γ-globin levels are higher than normal human adult erythroid precursors. An adult mouse erythroleukemia line, MEL, has also been used due to its ability to recapitulate erythroid differentitation (Friend et al. 1971). This cell line expresses exclusively murine adult β-globins (βmaj and βmin) and no embryonic globin genes (βh1 and εy). Different human globin constructs have been stably transfected into these cells in order to study human globin regulation. However, γ-globin expression is not properly regulated

in MEL cells (Skarpidi et al. 1998; Vassilopoulos et al. 1999). Even though erythroleukemia cell lines have served as a good vehicle to study and understand erythroid differentiation, they are not appropriate cell lines for finding targets to relieve γ-globin gene silencing in adult erythroid cells.

A new cell line was created in 2005 that resembles the adult hemoglobin expression pattern. Chemical Inducer of Dimerization (CID) cells are bone marrow cells isolated from mice bearing the human β-globin locus in a yeast artificial chromosome (βYAC). CID cells have been stably transfected with the thrombopoietin receptor which, upon introduction of a commercially available compound, is activated and signals these multipotential progenitor cells to differentiate into megakaryocytic cells, erythroid cells, neutrophils and monocytes (Blau et al. 2005). This cell line expresses high levels of murine alpha globin, which pairs with human β-globin to form adult hemoglobin. γ-globin gene expression is low in CID cells, greatly resembling the pattern of expression in human adult erythroid cells (Figure 13).

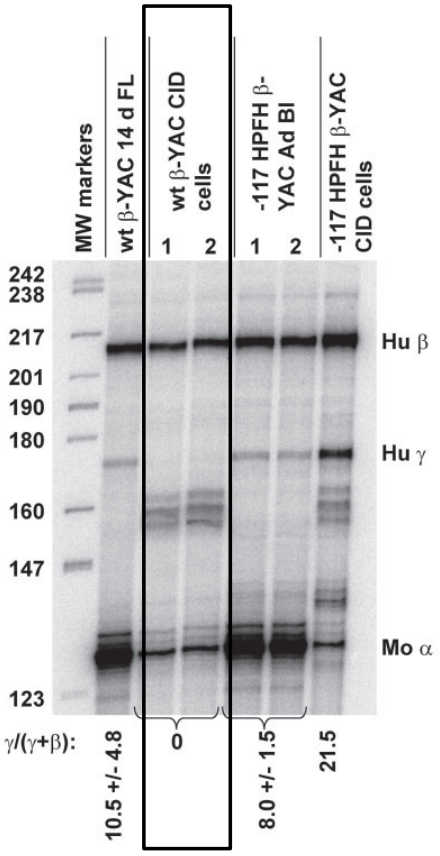


Figure 13. CID cells exhibit a human adult globin expression pattern. RNase protection assay showing WT βYAC CID cells (highlighted by the box) expressing low levels of human γ-globin (Hu γ) gene and high levels of human β-globin (Hu β) and mouse α-globin (Mo α). Adapted from Blau et al. 2005.

B. Stem cells

Mouse embryonic stem (ES) cells have served as a suitable way to study hemoglobin switching. They can differentiate *in vitro* with the use of growth factors into erythroid cells, and primitive cells can be clearly distinguished from definitive erythroid cells (Olsen, Stachuar & Weiss 2006). Because erythropoiesis in the bone marrow takes place in a niche called erythroblastic island, in which erythroblasts surround a single macrophage that contributes to phagocytosis of the nuclei of erythroid cells (Bessis 1958), new culturing conditions have been designed to recapitulate the bone marrow environment. Reports have shown co-culture of ES cells along with stromal cells (OP9) help induce differentiation (Nakano 1996; Kitajima et al. 2003). The use of human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPS) has also been of interest, as these could be used for mass production of hematopoietic progenitors *in vitro* and have also been used to study erythroid differentiation and γ-globin gene regulation. However, generating functional differentiated erythrocytes from hESCs and hiPS cells poses a challenge *in vitro*, as they tend to exhibit embryonic-like behavior (expressing high levels of embryonic globin genes). Co-culturing hESCs and hiPS cells with stromal cells has allowed these cells to properly differentiatiate (Ma et al. 2008).

Attempts to use homogeneous, unilineage cells have led to recent advances by using human progenitor cells (CD34+) from either neonatal blood or adult bone marrow. A two-phase differentiation system allows high proliferation as well as synchronous differentiation of these cells (Migliaccio et al. 2002). The use CD34+ cells has become standard in the studies of hemoglobin switching. The major disadvantage of using stem cells for the study of globin gene regulation is their low viability in culture. These cells are not viable past their differentiation period.

C. Animal models

In an attempt to understand human β-type globin gene regulation *in vivo*, several transgenic mouse models were created. Failed attempts to achieve high expression of human globin genes in transgenic mice (Costantini et al. 1985; Townes et al. 1985; Kollias et al. 1986; Chada, Magram & Costantini 1986) led to the discovery that flanking regions, particularly the 5' DNase I hypersensitive sites, in the β-globin locus are important for the regulation of β-type genes. The development of transgenic mice with the entire globin locus including its flanking regions was then achieved (Behringer et al. 1990; Enver et al. 1990, Gaensler, Kitamura & Kan 1993; Peterson et al. 1993), and these mice successfully recapitulated the hemoglobin switch. Because the mouse β-locus lacks fetal globin genes (it only contains embryonic and adult globin genes), human γ-globin assumes the embryonic pattern of gene regulation in transgenic mice as shown with dotted lines in Figure 1. The transgenic mouse model bearing the human β-locus and its flanking regions in a yeast artificial chromosome (βYAC) has become the most widely used among these mouse models (Peterson et al. 1993).

Meanwhile, several attempts were made at creating a sickle cell anemia mouse model by using transgenic mice with human globin gene sequences including the β-globin mutation (Ryan et al. 1990; Greaves et al. 1990; Trudel et al. 1991; Rubin et al. 1991; Fabry et al. 1992). Several groups were successful at achieving cell sickling in these mice, but failed to recapitulate all of the pathophysiology seen in sickle cell patients including organ damage. Further studies using mice with additional mutations in the β-locus meant to increase polymerization showed sickling, spleen and lung disease but also failed to recapitulate all of the clinical manifestations seen in sickle cell patients (Trudel et al. 1994; Fabry et al. 1995). Thus, it became apparent that endogenous murine globin genes were preventing the sickling required to mimic a full sickle cell

disease phenotype. A successful sickle cell anemia mouse model was finally developed in 1997 (Paszty et al. 1997). Three DNA fragments containing a miniLCR, the human α1-globin gene, and the human fetal and adult globin genes including mutated β-globin in place of wild type β-globin ($^{G}\gamma^{A}\gamma,\delta,\beta^{S}$) were coinjected into a fertilized egg to create the transgenic model. These mice were then bred with murine α-globin knockout mice (Paszty et al. 1995) and murine β-globin (β_{maj} and β_{min}) knockout mice (Ciavatta et al. 1995). Sickle cell disease mice express exclusively human α- and human β^{S}-globin genes and produce low HbF ($\alpha2\gamma2$) during early life (Figure 14). This mouse model became known as the BERK mouse since it originated at the University of California, **Berke**ley.

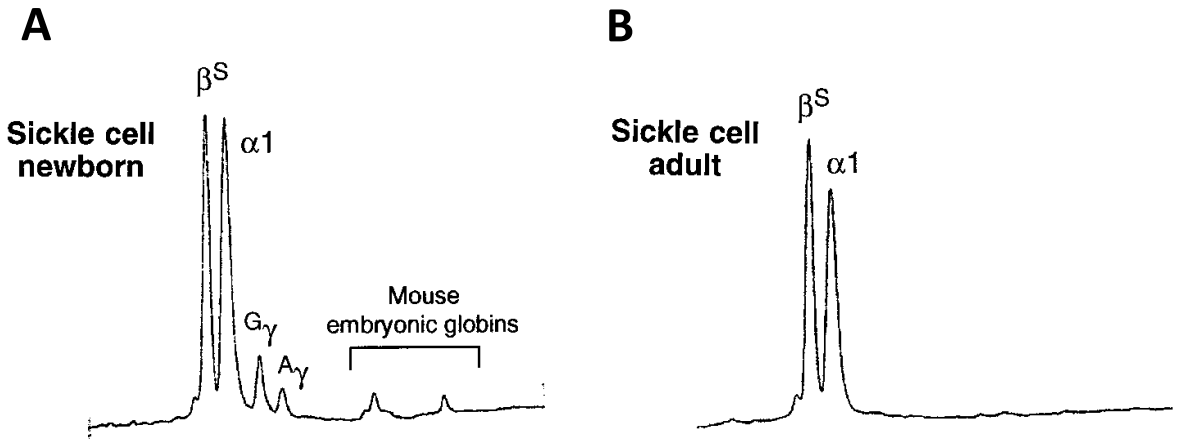


Figure 14. Sickle cell anemia mice express exclusively human sickle hemoglobin (HbS) in adult life. (A) High performance liquid chromatography (HPLC) showing globin chains from peripheral blood at the time of birth. These mice express mostly HbS ($\alpha2\beta^{S}2$) and a small percentage of HbF ($\alpha2\gamma2$). (B) HPLC showing adult BERK mice globin chain composition. BERK mice express exclusively HbS ($\alpha2\beta^{S}2$). Adapted from Paszty et al. 1997

BERK sickle cell disease mice show features similar to patients with sickle cell anemia such as low hemoglobin, low hematocrit, and a high reticulocyte count (Paszty et al. 1997). Some of the characteristic pathophysiology of sickle cell anemia such as large necrotic spleens, microinfarcts in the liver and kidneys are also present in this mouse model.

A similar sickle cell disease mouse model was developed the same year using a construct containing the LCR, the γ- and the mutated β-globin genes linked to a fragment containing the α-1 globin gene (Ryan, Ciavatta & Townes 1997). This transgenic mouse model was crossed with murine α- and β- knockout mice (Paszty et al. 1995; Ciavatta et al. 1995) in the same manner as the BERK mice. Similar to the BERK mouse model, they express exclusively α- and β^S- chains as adults. This mouse model was also successful in recapitulating the sickle cell phenotype, showing sickled cells in the peripheral blood, spleens characterized by vascular occlusions and thrombosis, focal necrosis in the liver, and vascular occlusions in the kidney (Ryan, Ciavatta & Townes 1997).

Although sickle cell anemia mice have offered a great model to study this condition *in vivo*, they have one disadvantage. They express very low expression of HbF as adults (<1%), which is lower than human adults with sickle cell anemia (2-8% HbF). Hence, in order to reach the minimum HbF threshold necessary to observe amelioration of the sickle cell disease phenotype, a higher level of γ-globin induction is necessary.

The use of baboons to study HbF synthesis began in the 1970s, when it was demonstrated that baboons exposed to acute hemolytic stress as well as hypoxia showed an increase HbF production (DeSimone, Biel & Heller 1978; DeSimone, Heller & Adams 1979). Baboons also served as a good model to study the effects of 5-aza on HbF production (DeSimone et al. 1982).

VII. Scope of this thesis

Our laboratory has previously shown the importance of the MBD2/NuRD complex in the regulation of the β-globin locus genes. The work presented in Chapter 2 of this thesis details the study of the *in vivo* effect of absence of MBD2 in the context of a humanized sickle cell anemia mouse model. Importantly, the role of MBD2 in fetal hemoglobin regulation and its potential as a target in sickle cell anemia is assessed in this model.

Two components of the MBD2/NuRD complex, MBD2 and p66α, play important roles in the regulation of γ-globin gene expression (Rupon et al. 2006; Gnanapragasam et al. 2011). In an attempt to explore the functions of other MBD2/NuRD components in γ-globin gene silencing, we have investigated the role of Mi2β in the regulation of β-type globin genes in Chapter 3. The independent function of Mi2β from the MBD2/NuRD complex and its dual function as a repressor and an activator are explored in this chapter.

An insight into possible new therapeutic targets for hemoglobinopathies is also reviewed in Chapter 4. Recent discoveries about γ-globin gene regulation have led to new potential ways to treat β-type globin disorders such as sickle cell anemia and β-thalassemia. However, drug development and delivery methods pose a challenge and will need to be accounted for when considering such targets.

VIII. Significance

Many factors contribute to the regulation of the developmental switch of fetal γ-globin expression to the adult β-globin gene expression, including a locus control region, transcription factors and epigenetics. The level of complexity of this process is augmented by the fact that γ-globin gene regulation during development may not be completely analogous to the mechanism involved in suppression of this gene during adulthood. Advances in understanding the regulation of γ-globin gene expression will prove to be of upmost importance for patients with sickle cell anemia and β-thalassemia, where an increase in HbF has clinical benefit. Given the complexity and many pathways involved in the mechanisms behind silencing this gene, it is important to find efficient ways to relieve γ-globin silencing in adult erythroid cells. Considering these disorders are most prevalent in the developing world, the feasibility of drug delivery and affordability of treatment should be considered when attempting to find ways to re-express γ-globin expression as a potential therapy.

Chapter 2: Absence of MBD2 ameliorates the phenotype of sickle cell mice.

I. Introduction

Sickle cell anemia is one of the most common single gene disorders worldwide, although it is most prevalent in Africa, South America, the Caribbean, and the Mediterranean countries. It is caused by a point mutation in the 6th position of the beta globin gene (A-to-T) that replaces a glutamic acid for a valine (Ingram 1956). This mutation leads to reversible polymerization of the affected hemoglobin (HbS) in deoxygenated states. These polymers injure the erythrocytes by damaging the membrane cytoskeleton causing hemolytic anemia (Lux, John & Karnovsky 1976). Furthermore, sickle-shaped erythrocytes cause a reduction in tissue perfusion by obstructing capillaries and further adhesion to the vascular endothelium (Hebbel, Osarogiagbon & Kaul 2004).

Sickle cell disease is characterized by extravascular hemolysis (uptake and degradation of erythrocytes by macrophages subsequently taken to the spleen), but a considerable amount of intravascular hemolysis (rupture of cells within the circulation) also occurs. Free plasma hemoglobin and arginase in the circulation affects the nitric oxide (NO) availability (Figure 15), which reduces the vessels' ability to vasodilate as well as to inhibit platelet activation (Steinberg 2009). It is well known that an increase in fetal hemoglobin (HbF) can ameliorate the phenotype of sickle cell anemia by decreasing the amount of fiber formation (Nathan et al. 2003). Although hydroxyurea, the current treatment for sickle cell anemia, is able to induce HbF, it has variable

effects on patients (Charache et al. 1992; Steimberg et al. 1997; Ware et al. 2002). Current efforts are focused on finding new, more efficient targets to increase fetal hemoglobin in these patients. In order to conduct these studies, several transgenic mouse models that closely recapitulate sickle cell anemia have been established (Ryan et al.1990; Greaves et al. 1990; Trudel et al. 1991; Rubin et al. 1991; Fabry et al. 1992; Trudel et al. 1994; and Fabry et al. 1995).

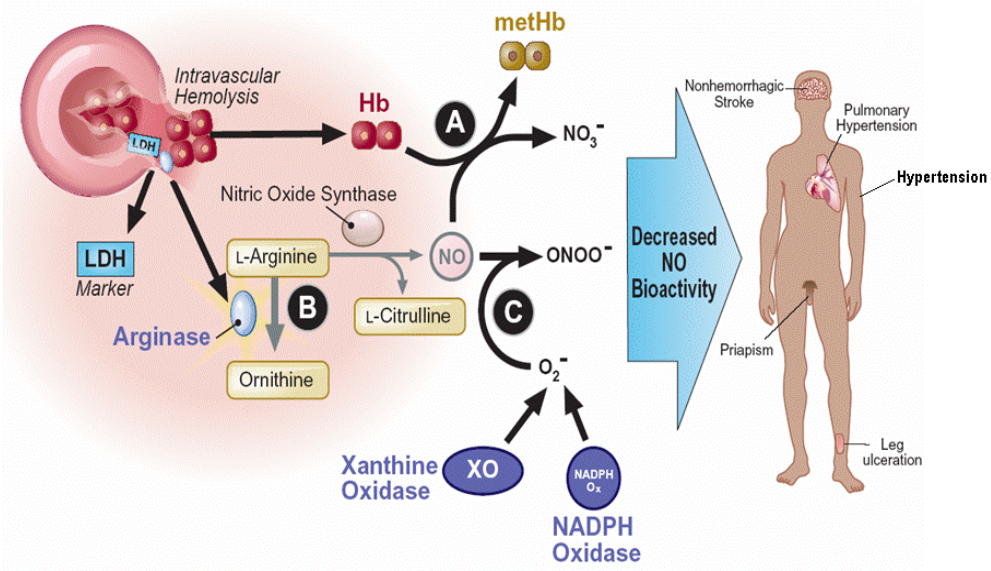


Figure 15. Intravascular hemolysis in sickle cell anemia leads to decreased nitric oxide (NO) availability. Intravascular hemolysis leads to release of arginase and hemoglobin. (A) On one end, hemoglobin inactivates NO, turning it into methemoglobin (metHb) and nitrate (NO_3^-). (B) The released arginase also consumes L-Arginine, a substrate converted to NO by nitric oxide synthase (NOS). (C) NO availability is also decreased by reactions with reactive oxygen species. Decreased NO availability leads to pulmonary hypertension, leg ulcers, hypertension, priapism and strokes in patients with sickle cell disease. Adapted from Kato et al. 2007.

The BERK mouse model is the most widely studied mouse model for sickle cell anemia (Paszty et al. 1997). These mice contain three DNA fragments: a 6.5-kb miniLCR, a 1.5-kb fragment containing the human α1-globin gene, and a 39-kb fragment containing the human fetal and adult globin genes including the mutated sickle β-globin in place of wild type β-globin ($^{G}\gamma^{A}\gamma,\delta,\beta^{S}$). They also lack endogenous murine α-globin as well as endogenous adult β-globin genes (β_{maj} and β_{min}). BERK mice express exclusively the human α and human β^{S} genes with minimal expression of HbF during adulthood (Paszty et al. 1997). The pathophysiology of the BERK mice greatly resembles that of sickle cell anemia patients. It is characterized by hemolytic anemia, low hematocrit and high reticulocyte counts. BERK mice also exhibit large necrotic spleens (~13-fold larger than WT mice) and micro-infarcts in the liver and kidneys.

In humans, the gene responsible for producing the β-subunits of fetal hemoglobin, γ-globin, is located on the β-globin locus on chromosome 11. This locus consists of a locus control region and a tandem array of genes placed in the order they are expressed during gestation and adulthood (LCR-ε-$\gamma^{G}\gamma^{A}$-δ-β) (Stamatoyannopoulos 2005; Sankaran, Xu & Orkin 2010). In addition to the locus control region, other cis-acting factors, and trans-acting factors including epigenetics play an important role in the regulation of the genes encompassing the β-globin locus.

Regarding the role of epigenetics in β-type globin gene regulation, DNA methylation has been inversely correlated with globin gene expression (Mavilio, et al. 1983; McGhee et al. 1979; Shen, et al. 1980; van der Ploeg Cell & Flavell, 1980). The methyl-binding domain protein 2 (MBD2) is known to bind to densely methylated DNA and negatively regulate genes through its interaction with the nucleosome remodeling and histone deacetylase (NuRD) complex (Meehan et al. 1989; Hendrich & Bird 1998; Hendrich & Tweedie 2003; Feng & Zhang 2001). MBD2

plays a significant role in the regulation of human fetal γ-globin gene expression (Kransdorf et al. 2006; Rupon et al. 2006, Gnanapragasam et al. 2001). Transgenic mice bearing the human beta locus (βYAC) crossed with MBD2 knockout mice show ~20-fold increase in γ-globin gene expression (Rupon et al. 2006), making MBD2 a potential therapeutic target in sickle cell anemia (Figure 16).

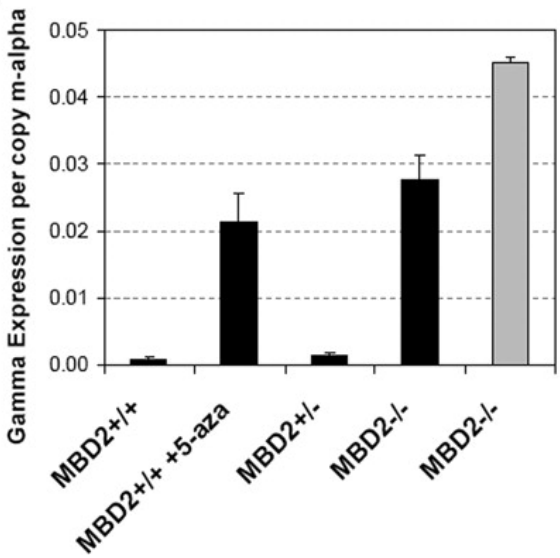


Figure 16. Absence of MBD2 leads to an increase in γ-globin gene expression in transgenic mice bearing a human β-globin gene locus. (A) RNase protection assay showing a significant increase in γ-globin expression in MBD2 knockout mice crossed with βYAC mice. Two lines of βYAC mice (depicted in black and gray) were used showing similar results. WT mice treated with 5-azacytidine also lead to a similar increase in γ-globin expression suggesting MBD2 acts via DNA methylation. Adapted from Rupon, et al. 2006

In addition to its role in γ-globin gene regulation, MBD2 binds to and silences methylated promoter regions of the endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor receptor 2 (VEGF-R2) genes. Likely due to this function, MBD2 knockout mice are protected from hind-limb ischemia and endothelial cells are also protected from $H_2O_2^-$ induced apoptosis (Rao et al. 2011). MBD2 could therefore play an important role in the pathophysiology of sickle cell anemia via its regulation of endothelial genes involved in ischemic injury. In this study, we sought out to determine whether absence of MBD2 could confer amelioration of the phenotype seen in sickle cell disease (SCD) mice.

II. Methods

Mouse strains:

MBD2 knockout mice were a kind gift from Dr. Adrian Bird. These mice are currently in a mixed genetic background (FVB/C57BL/6 BALB/C). MBD2 knockout mice are viable, and overall healthy. The only phenotype reported is a mildly abnormal nurturing behavior in mothers (Hendrich, et al. 2001). BERK sickle cell disease (SCD) mice were a kind gift from Dr. Ivo Torres. This strain, termed *Hbatm1Paz Hbbtm1Tow* Tg(HBA-HBBs)41Paz/J, is available from Jackson laboratories, and it has been well characterized and described in the literature (Paszty et al. 1997). BERK mice are transgenic for a human β-locus containing three constructs: a 6.5-kb miniLCR, a 1.5-kb human α1-globin gene, and 39-kb encompasing the human fetal and adult globin genes including the mutated sickle β-globin in place of wild type β-globin ($^{G}\gamma^{A}\gamma,\delta,\beta^{S}$). Endogenous mouse adult globin genes have been knocked out. These mice recapitulate to a great extent the phenotype seen in sickle cell anemia patients.

Mouse breeding and maintenance:

Sickle cell disease (SCD) mice were mated and checked for vaginal plugs after 24 hours. Mice were weaned 20 days to 1 month after birth. At this time, mice were ear punched and the tissue was incubated for 4-5 hours at 55°C in the following digestion buffer: 50mM KCl, 10mM Tris at a pH of 8.5, 4mM $MgCl_2$, 0.45% NP-40, and 0.45% Tween-20. Samples were boiled twice for 10 minutes, followed each time by a rapid spin in a mini centrifuge. Digested samples were then stored at -20°C until PCR reactions were carried out. SCD mice were fed a custom

made diet high in folic acid (Harlan, cat # TD. 120486, which consists of diet 7012 + 22mg Folic Acid)

Breeding Genotypes and abbreviations:

Genotype	Abbreviation
Wild Type Mice	WT
MBD2 knockout mice	MBD2-/-
Sickle cell disease (SCD) BERK mice (contains wildtype MBD2)	SCDMBD2+/+ or SCD control mice
Sickle cell disease (SCD) BERK mice crossed with heterozygote MBD2 knockout mice	SCDMBD2+/-
Sickle cell disease (SCD) BERK mice crossed with homozygote MBD2 knockout mice	SCDMBD2-/-

Table 1. List of abbreviations used in figure legends throughout chapter 2

Experiment	Genotypes Used	Note
Survival Rate	SCDMBD2+/+ SCDMBD2+/- SCDMBD2-/-	
Globin RNA expression and hemoglobin chain synthesis	SCDMBD2+/+ SCDMBD2+/- SCDMBD2-/-	WT or MBD2-/- mice do not synthesize human globin genes and therefore could not be tested
Blood Smears	SCDMBD2+/+ SCDMBD2-/-	
Spleen size, H&E stains and histological grade scoring	WT MBD2-/- SCDMBD2+/+ SCDMBD2+/- SCDMBD2-/-	Organs from WT and MBD2-/- mice were collected and considered normal controls for comparison

Table 2. Mouse genotypes used for each experiment

Mice Screening:

Polymerase chain reaction samples included 0.5μM of each primer, 200μM dNTPs, 1 unit of Taq polymerase, >1μg template DNA, 2.5μL of 10X reaction buffer (Invitrogen), and nuclease-free water totaling a 25μL reaction. The reaction was carried as follows: 94°C (3min), 40 cycles of 94°C (30s), 58°C (30s), 72°C (1min) according to Paszty et al. 1998. Primers used are included in Table 3.

Complete blood count and high performance liquid chromatography (HPLC):

Prior to each experiment, a fresh solution of the anesthetic Avertin was prepared by adding 10mL of tert-amyl alcohol (Fisher) to 10g of 2-2-2 Tribromoethanol (Sigma). A working solution was then made by mixing 2.5mL of the above Avertin solution with 97.5mL of saline. Mice were anesthetized with an intraperitoneal injection of Avertin at a concentration of 0.015ml/gm of body, and cardiac punctures were performed in order to collect ~500μL of blood. The blood was transferred to 1mL dipotassium EDTA blood collection tubes (BD Microtainer) and a drop was used for blood smears. Slides and blood samples were then taken to VCU hematology for further Giemsa staining and HPLC hemoglobin fractionation.

Organ Collection:

Mice spleen, liver and kidneys were collected from sacrificed mice. Organs were first rinsed in 1X PBS. Spleen weights were measured and all organs were placed on a 10% formaldehyde/1XPBS solution. Samples were taken to the VCU Department of Pathology for hematoxylin and eosin (H&E) staining.

Organ pathology scoring:

Following H&E staining for each organ collected, Dr. David Williams (a certified pathologist) blindly scored each slide. The scores range on a scale of 0-3, with 0 representing no disease observed and 3 representing the highest grade of disease.

Primer Table

	Forward	Reverse
Murine MBD2	CCTCAGCTGGCAAGATACCT	GGGGGTCATTCCGGAGTCT
β-Geo Cassette	GGTCAGGTCATGGATGAGC AGA	CGCGGATCATCGGTCAGACGATT
Murine α-globin (Hba)	AGTGGGCAGCTTCTAACTAT GC	GTCCCAGCGCATACCTTG
Murine α-globin deletion	ATAGATGGGTAGCCATTTAG ATTCC	CCGGGTTATAATTACCTCAGGTC
Murine β-globin genes	TTAGGTGGTCTTAAAACTTT TGTGG	ACTGGCACAGAGCATTGTTATG
Murine β-globin genes deletion	AGATGTTTTTTTCACATTCTT GAGC	AATGCCTGCTCTTTACTGAAGG
Human β-globin transgene	GTATGGGAGAGGCTCCAACT C	TCTGCCCAAATCTTAGACAAAAC

Table 3. The table above incudes all the PCR primers used for mice screening.

III. Results

A. Absence of MBD2 in sickle cell disease mice increases survival compared to control sickle cell disease mice

The first difference noted while breeding MBD2 knockout mice with sickle cell disease mice was the survival rate between knockouts and control groups. Sickle cell disease mice (SCD) were noted to have small litters and females either took longer to impregnate or lost more litters compared to WT mice. Additionally, sickle cell disease pregnant females often delivered stillborn pups. In contrast to sickle cell disease mice (SCDMBD2+/+), SCD mice crossed with MBD2 heterozygote or homozygote knockouts (SCDMBD2-/- and SCDMBD2+/-) appeared to have an improvement in mating efficiency and delivered larger litters. In order to quantify our observation, we calculated the survival rates of the groups in question by comparing observed vs. expected values per group. To do this, we first calculated the observed number of desired genotypes obtained (SCDMBD2+/+, SCDMBD2+/- or SCDMBD2/-) per total number of mice in the litter (observed value). Only the mice that survived until at least 1 month of age, at the time of screening, were included in the total number. This was followed by calculating the probability of obtaining the desired genotype for each group (expected value). Comparing the "observed" category of mice to the "expected" category for each group (SCDMBD2+/+, SCDMBD2+/- and SCDMBD2-/-) allowed us to quantitatively compare experimental groups to the control. In normal survival rates, the observed/expected ratio should approach 1. As expected, table 4 shows that the only group that displayed a significant decrease in survival was the sickle cell disease control group (SCDMBD2+/+). Although not statistically significant, SCDMBD2+/- mice also show a slight decrease in survival rate with a 0.89 observed/expected

ratio, suggesting 50% deficiency of MBD2 does not fully rescue sickle cell disease mice. Overall, SCDMBD2-/- showed the greatest improvement in survival which was 5-fold higher than SCDMBD2+/+, suggesting this genotype confers a survival advantage to this group.

Genotype	Total # of mice obtained per group	Observed/Expected
SCDMBD2+/+	**5**	**0.27***
SCDMBD2+/-	**14**	**0.89**
SCDMBD2-/-	**25**	**1.37**

Table 4. SCDMBD2-/- mice exhibit a higher survival rate at 1 month of age compared to control sickle cell mice (SCDMBD2+/+). A Z-Test was performed after comparing the ratio of observed number of desired mice (SCDMBD2+/+, SCDMBD2+/-, SCDMBD2-/-) in each litter to the probability of obtaining the desired mice (SCDMBD2+/+, SCDMBD2+/-, SCDMBD2-/-) for each mating. Observed mice only included mice of at least 1 month of age at the time of screening, and the total number of mice obtained per group is listed. A minimum of 40 matings per group were included in the study. The Z-Test was performed making the null hypothesis H_0: proportion = expected against an alternative hypothesis H_A: proportion < expected. The null hypothesis was rejected for SCDMBD2+/+. * signifies a p value < 0.001.

Genotype	# of mice alive vs. total number of mice (alive/total)	% of genotyped mice surviving from 1 month of age until 3 months of age
SCDMBD2+/+	**5/7**	**71.4%**
SCDMBD2+/-	**12/14**	**85.7%**
SCDMBD2-/-	**22/24**	**91.7%**

Table 5. A greater number of SCDMBD2-/- mice survive to adulthood compared to SCDMBD2+/- and SCDMBD2+/+ mice. Shown above are the percentage of mice that survived beyond 1 month of age until the time of sacrifice for experimentation (3 to 5 months of age). Although not significantly different by a chi square test, SCDMBD2-/- mice have the highest (91.7%) survival compared to SCDMBD2+/- (85.7%) and SCDMBD2+/+ (71.4%). At least 7 mice per group were included in this study.

Even though the critical survival stage for sickle cell mice has been reported to be within the first few days of life (Ryan et al. 1997), when the lungs begin to supply oxygen to the body, adult sickle cell mice are still at risk of dying from sickle cell disease-related complications. We therefore calculated the percentage of mice that survived between 1 month of age and the time of sacrifice for experimentation (2 to 5 months of age). SCDMBD2-/- mice show a 91.7% survival compared to SCDMBD2+/- (85.7%) and SCDMBD2+/+ (71.4%) (Table 5).

B. Absence of MBD2 is able to induce γ-globin gene expression and results in higher HbF production

We hypothesized that the increased survival of SCDMBD2-/- mice was due to increased γ-globin gene expression leading to an increase in HbF. We tested the γ-globin mRNA amounts in blood taken from 2-5 month old SCDMBD2+/+, SCDMBD2+/- and SCDMBD2-/- mice. SCDMBD2-/- mice express ~15-20% γ-globin mRNA calculated as γ/(γ+β)-globin mRNA ratio, compared to SCDMBD2+/+ mice which express ~0.8% γ/(γ+β)-globin (Figure 17A). SCDMBD2+/- mice also show a slight increase in γ/(γ+β)-globin mRNA (1.71%), correlating with the increased survival rate of this group (Figure 17A). In order to detect hemoglobin protein levels, blood was analyzed by high performance liquid chromatography (HPLC). The HbF percentage levels of both SCDMBD2+/+ and SCDMBD2+/- were found to be undetected, whereas SCDMBD2-/- showed a ~4% HbF (Figure 17B). The discrepancy between γ/(γ+β) values detected by qPCR vs. protein levels detected by HPLC could be a result of the high sensitivity of qPCR and low sensitivity of HPLC (since low amounts of mRNA are detected by qPCR but appear undetected by HPLC in Figures 17A vs. 17B).

Interestingly, HbA2, which is composed of 2 δ-globin chains and 2 α-globin chains, is significantly elevated in the SCDMBD2+/+ control mice compared to normal values seen in SCDMBD2-/- mice (normal range = 1.2-3%) (Figure 17C). Since HbA2 is also known to be protective against sickle cell anemia (Nagel et al.1979) this could be attributed to a selective advantage, where only SCD control mice that express higher levels of HbA2 survive. Hence, we are only able to test control SCD mice with elevated HbA2 levels. A slight elevation of HbA2 in SCDMBD2+/- in combination with a slight increase in γ-globin gene expression (Figures 17A & 17C) most likely accounts for the increase in survival in this group.

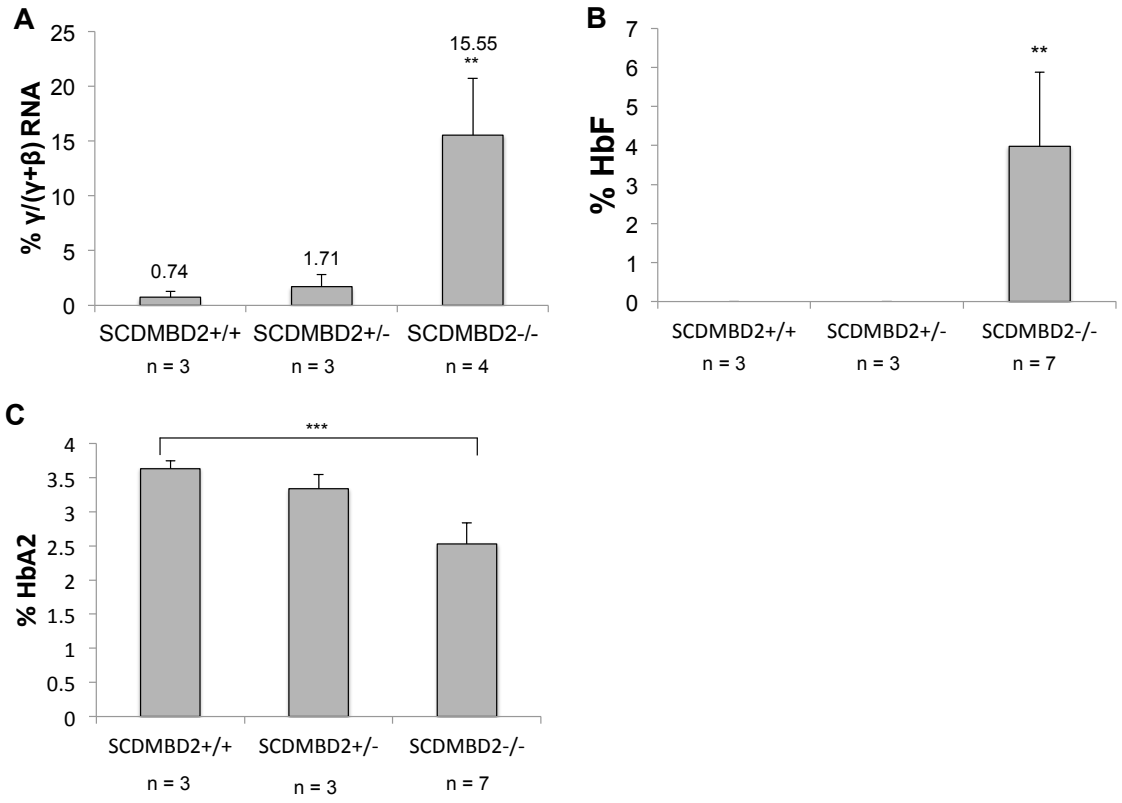


Figure 17. Absence of MBD2 results in an increase in γ-globin gene expression and HbF but no increase in HbA2. (A) qPCR showing SCD mice crossed with MBD2KO mice express 15.55% γ/(γ+β)-globin mRNA compared to 0.74% γ/(γ+β)-globin mRNA expression in control SCD mice. Heterozygote SCDMBD2+/- mice also show a slightly elevated γ/(γ+β)-globin ratio (1.71) (B) HPLC showing elevated HbF levels (~4.5%) in SCDMBD2-/- mice. SCDMBD2+/- and SCDMBD2+/+ show undetected HbF levels by HPLC. (C) HPLC showing hemoglobin HbA2 was significantly elevated in SCDMBD2+/+ control mice compared to SCDMBD2-/- mice. **signifies $p < 0.02$ and *** signifies $p < 0.001$ according to the students t-test.

C. Absence of MBD2 reduces the number of sickle cells in the peripheral blood of sickle cell mice

A representative blood smear taken from peripheral blood of adult SCD control mice shows sickle cells admixed with target red blood cells and a high amount of polychromatic cells (Figure 18B). Although a representative blood smear taken from a SCDMBD2-/- mouse shows less sickling (Figure 18C), peripheral blood still shows a significant amount of polychromasia, suggesting a large number of immature red cells are found in the circulation (Figure 18C) compared to WT mice (Figure 18A). In order to quantify the amount of sickling, the number of sickle cells were counted in 10 different fields for each mouse blood smear. On average, WT mice showed 0 sickle cells per field, SCDMBD2+/+ mice showed 1.9 cells per field, and SCDMBD2-/- mice showed 1.1 sickle cells per field. This suggests a decrease in the amount of sickle cells in SCDMBD2-/- mice compared to SCDMBD2+/+ control mice. Since only one mouse per group has been tested thus far, a larger number of mice will be needed in order determine if this effect is significantly different between SCDMBD2-/- and SCDMBD2+/+ control mice.

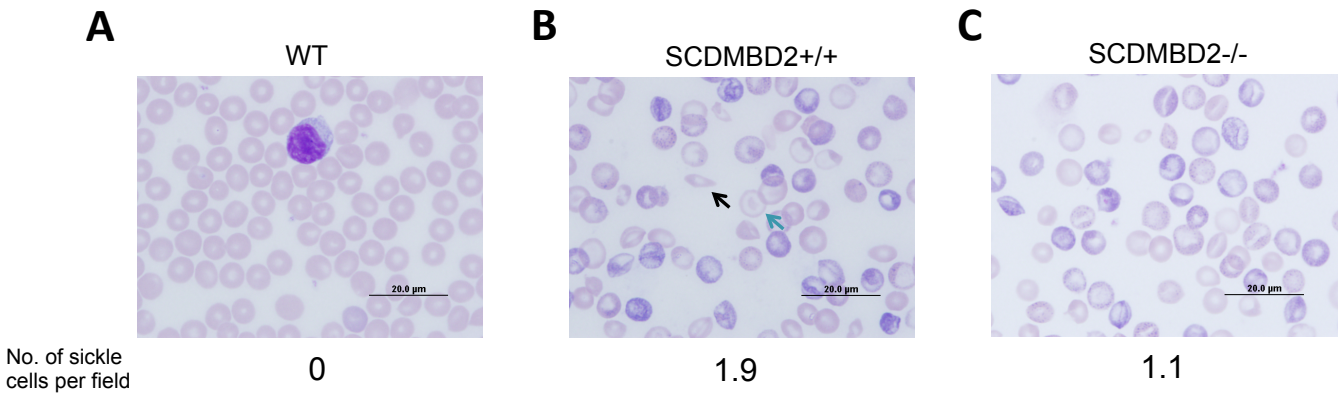


Figure 18. Blood smears show a decrease in the number of sickle cells in SCDMBD2-/- mice compared to SCDMBD2+/+ mice. Giemsa stain showing representative blood smears from (A) WT (B) SCDMBD2+/+ and (C) SCDMBD2-/- mice. Arrows show sickle cells (black) and target cells (blue), characteristic of sickle cell anemia (B). The large portion of polychromatophillic cells (purple) is likely due to reticulocytosis (B & C). The numbers below represent number of sickle cells counted per field in a total of 10 fields for each mouse.

D. Absence of MBD2 does not result in a significant improvement of sickle cell disease organ damage

Although SCDMBD2-/- showed a marked improvement in survival over SCD control mice likely due to an increase in HbF, pathophysiology characteristic of sickle cell anemia is still present in SCDMBD2-/- mice. In order to quantify the degree of organ damage observed in the groups tested, spleen weights were measured, and degree of damage was determined by scoring H&E stains from three different organs (spleen, liver and kidney). Due to increased extra-medullary erythropoiesis in the spleen, sickle cell disease mice are characterized by having large necrotic spleens, usually ranging from 10- to 15-fold larger in size than WT mice (Ryan et al. 1997). As shown in Figure 19A, SCDMBD2-/- and SCDMBD2+/- mice displayed large spleens, about 15 fold larger by percentage of body weight than WT and MBD2-/- control mice. This increase in spleen weight was no different than SCDMBD2+/+ control mice, suggesting a decrease in MBD2 expression does not alter the amount of extra-medullary erythropoiesis seen in sickle cell mice.

To further determine whether MBD2 knockout confers protection of organs by decreasing sickling and protecting the endothelium, spleen, kidney and livers were dissected from mice post-mortem and cross sections were stained by hematoxylin and eosin (H&E). Following H&E staining, samples were scored on a histological grade scale ranging from 0-3, where 0 represents normal histology and 3 represents extensive organ damage and necrosis. WT and MBD2ko mice were used as controls and show 0 histological grade in all three organs (Figure 19B). WT and MBD2 knockout animals do show a small amount of extra-medullary erythropoiesis (EE) as marked with a 1 in the histological grading scale (Figure 19B). Regarding organ damage, no significant difference was seen between control SCD mice compared to

SCDMBD2+/- or SCDMBD2-/- mice. The most affected organ in these mice was the spleen, with a histological grade of 3 in all three groups (SCDMBD2+/-, SCDMBD2-/- and SCDMBD2+/+). Although not significantly different, a trend was observed in SCDMBD2-/- mice, in which the kidneys were spared from damage compared to SCDMBD2+/+ kidneys. However, the opposite trend was seen in the liver, where SCDMBD2-/- mice show greater liver necrosis than control mice (Figure 19B). This could be due to the difference in the amount of capillaries found in the kidney and the liver, although further studies will be necessary to determine this.

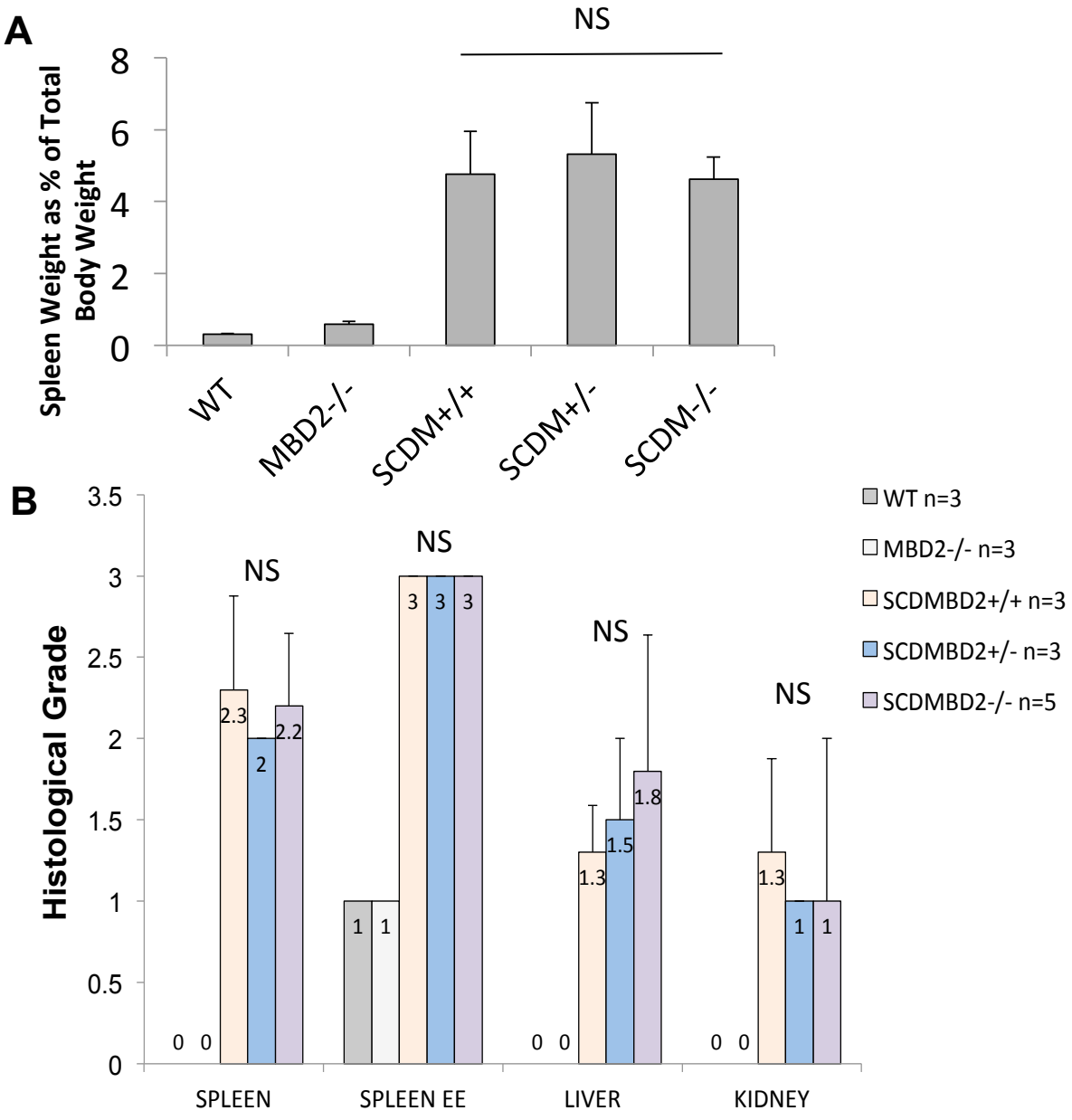


Figure 19. Absence of MBD2 in SCD mice does not result in a significant decrease in organ damage when compared to SCD control mice. (A) Spleen weights were measured as a percentage of body weight. Error bars represent at least three mice. (B) Histological grading scales from 0 to 3 (0 represents no damage or no extra-medullary erythropoiesis (EE), and 3 represents most damage and highest amount of extra-medullary erythropoiesis). Error bars represent at least three mice as shown in the legend. No significant difference was found between the two experimental groups (SCDMBD2+/- SCDMBD2-/-) compared to sickle cell disease control mice (SCDMBD2+/+) in any of the tested parameters. NS = no significant difference between SCDMBD2+/+ control mice and SCDMBD2+/- and SCDMBD2-/- mice.

Representative H&E slides from spleen, kidney and livers dissected from either SCDMBD2+/+, SCDMBD2+/- and SCDMBD2-/- show extensive organ damage compared to WT and MBD2-/- controls (Figure 20). As shown by the arrows in Figure 20, all three groups (SCDMBD2+/+, SCDMBD2+/- and SCDMBD2-/-) show pathology characterized by ischemic infarcts and necrosis in the liver, increased hemorrhage and extra-medullary erythropoiesis in the spleen, and infarcts and coagulative necrosis in the kidneys compared to normal histology seen in WT or MBD2-/- control groups.

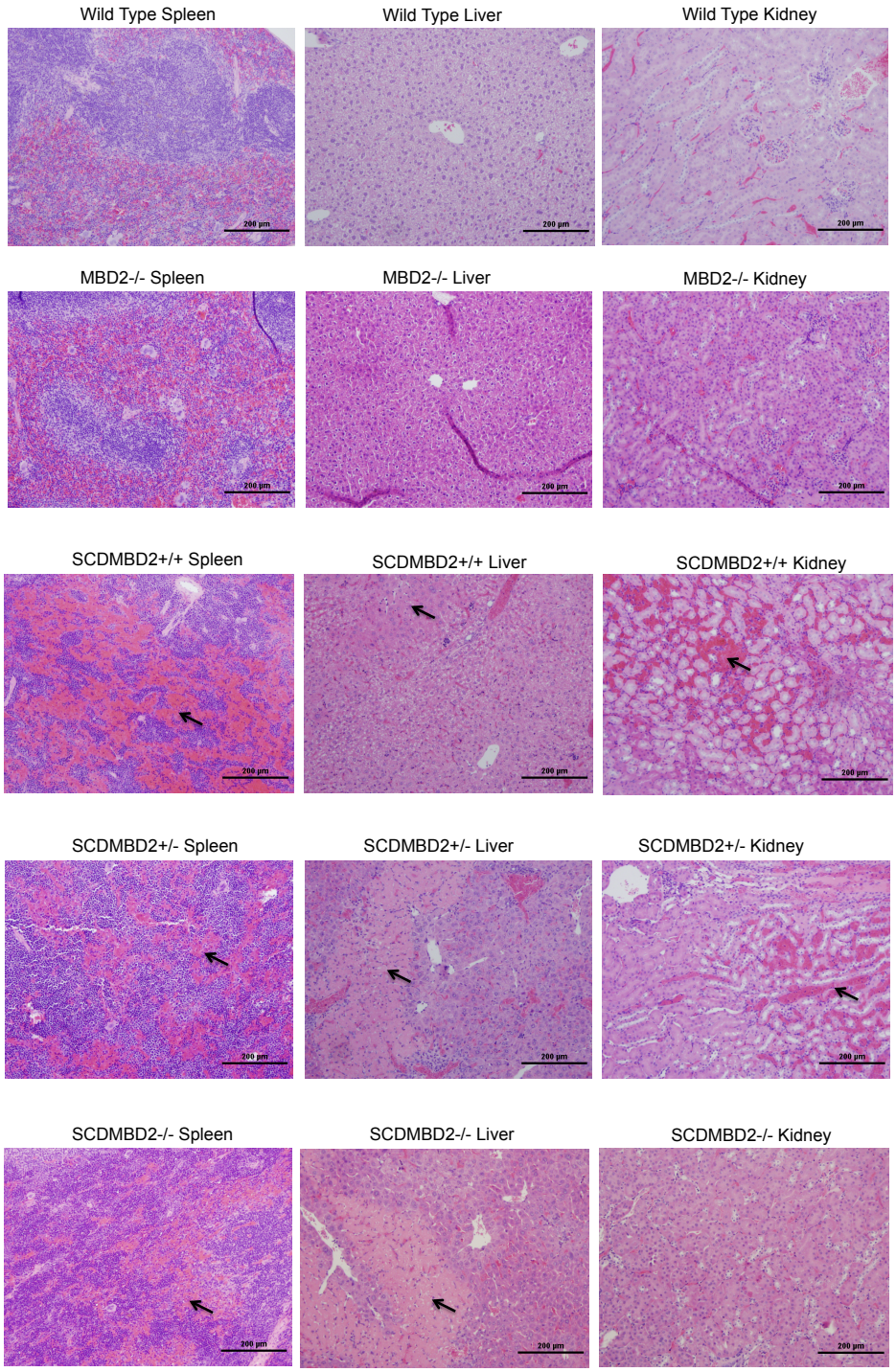


Figure 20 Absence of MBD2 does not fully correct organ disease characteristic of sickle cell anemia. H&E slides showing spleen, liver and kidney taken from either healthy control mice (WT, MBD2-/-), or SCDMBD2+/+, SCDMBD2+/- and SCDMBD2-/- mice. Arrows show organ damage including: increased extra-medullary erythropoiesis, coagulative necrosis and hemorrhage in the spleen, ischemic infarcts and coagulative necrosis in the liver, and infarcts and coagulative necrosis in the kidneys as well as loss of glomeruli.

IV. Discussion

The MBD2/NuRD complex has previously been shown to play an important role in the silencing of γ-globin gene expression. Selectively binding to CpG rich DNA, MBD2 is associated with the NuRD complex and exerts a negative regulatory function (Meehan et al. 1998; Hendrich & Tweedie 2003; Feng & Zhang, 2001). The MBD2/NuRD complex does not bind to the γ-globin promoter region, but rather it indirectly plays a role in silencing this gene (Rupon et al. 2006). MBD2 Knockout mice bearing a yeast artificial chromosome with the human β-globin locus (βYAC) have a ~15-20-fold increase in the γ-globin gene expression (Rupon et al. 2006). Importantly, MBD2 knockout mice have a minimal phenotype affecting only maternal nurturing (Hendrich et al. 2001). This renders MBD2 a great candidate as a target in sickle cell anemia.

The BERK sickle cell disease mouse model has served as a useful way of studying this condition. BERK mice exhibit a typical phenotype characterized by sickle-shaped erythroid cells, severe anemia and organ damage. The severity of their phenotype is perhaps greater than in humans because they express significantly lower amounts of HbF (<1%) and exhibit a greater amount of hemolytic anemia (Paszty et al. 1997).

In our studies we have shown that knocking out MBD2 in sickle cell disease (SCD) mice results in an marked increase in survival rate (~5-fold) compared to control SCD mice. The increase in survival is likely due to an increase in γ-globin gene expression leading to higher HbF synthesis. This finding is consistent with studies reporting a decrease in mortality in sickle cell anemia patients with elevated HbF (Platt et al. 1994).

Likely due to the lower basal expression of HbF in sickle cell mice (<1%), a large fold increase in γ-globin production upon knocking out MBD2 is not sufficient to completely correct

the pathophysiology associated with this condition. Spleen size and organ pathology show no significant difference between SCDMBD2+/+ and SCDMBD2-/- mice. Although it is apparent that absence of MBD2 does not render complete correction of the sickle cell phenotype, the lack of a difference between the pathophysiology of SCDMBD2+/+ and SCDMBD2-/- mice may be due in part to a survival selection in control SCDMBD2+/+ mice. Adult SCDMBD2+/+ mice display increased HbA2, a phenotype that confers protection against sickling (Nagel et al.1979).

The lack of improvement of organ pathology in SCDMBD2-/- compared to SCDMBD2+/+ control mice may be attributed to the lack of a linear relationship between increased HbF and decreased morbidity. Instead, there is a threshold at which morbidity begins to decline (Powars et al. 1984). It is likely that SCDMBD2-/- mice produce sufficient HbF to decrease mortality, but they lack sufficient HbF to decrease complications and fully correct the phenotype of this disease.

Although not statistically significant, a trend was seen in the pathology of the kidney, where both SCDMBD2+/- and SCDMBD2-/- mice showed less severity of kidney disease compared to SCDMBD2+/+ control mice. Because the absence of MBD2 is known to increase expression of eNOS and thus improve reperfusion injuries in mice (Rao et al. 2011), it is possible that reduced expression of MBD2 in sickle cell mice increases NO availability. This effect may reduce damage to the kidney, an organ with an extensive number of capillaries. This effect was not seen in the liver, which may reflect a difference in the amount of capillaries present in these two organs. Further studies such as measuring eNOS levels in vessels from these organs will be necessary to determine whether this is the case.

Another factor that can potentially explain the lack of improvement in morbidity of SCDMBD2-/- mice is the transgene copy number. SCD mice can either have one or two copies

of the transgene. Due to the lack of information shared by the investigators who created the mouse model, the site of insertion is unknown. The lack of information on the transgene's flanking regions has made it difficult to determine whether each mouse contains one or two copies of the transgene. Transgene copy number (1 vs. 2) could therefore play a role in the severity of the phenotype seen in each mouse. An increase in the number of mice tested to account for a possible bias in each group tested will be necessary.

Another possible reason the absence of MBD2 did not result in greater phenotype improvement is the genetic background. Studies have shown genetic modifiers in different genetic backgrounds can account for a difference in phenotype seen in mice. A clear example of this was shown on a diabetes model, where the mutations for obesity (lep^{ob}) and diabetes ($lepr^{db}$) results in obesity and mild diabetes in the C57BL/6 genetic background, whereas the same genotype results in obesity and overt diabetes in the C57BLKS/J genetic background (Coleman & Hummel 1973; Coleman, 1978). Genetic modifiers in different backgrounds must account for different phenotypes. Given that MBD2 knockout mice are in a mixed genetic background (FVB/C57BL/6 BALB/C), genetic modifiers could be contributing to the severity of the phenotype. Other groups have shown a greater improvement in the SCD phenotype with a similar increase in γ-globin expression in C57BL/6 mice (Xu et al. 2011). The mixed genetic background of our mouse model could explain why the large fold induction of γ-globin gene expression is insufficient to prevent disease progression in these mice. Studies on eNOS gene regulation by MBD2 were also conducted in C57BL/6 mice (Rao et al. 2011). Hence, a pure genetic background is needed to determine whether genetic modifiers affect the severity of the SCD phenotype. SCD control mice that have selectively survived show an increased HbA2, which is consistent with our hypothesis that a mixed genetic background has the potential to

affect particular genes. Because the BERK mice are on a mixed genetic background, the higher HbA2 levels could be associated with a particular genetic background.

Although further work will be necessary to determine the level of improvement in the sickle cell phenotype in mice under a pure genetic background, the results obtained so far are promising. Survival of SCD mice increases significantly when MBD2 is knocked out likely due to an increase in HbF, and a decrease in the number of sickle cells seen in the periphery. Furthermore, the limitations when studying sickle cell anemia in transgenic mouse models suggests targeting MBD2 could be more beneficial in sickle cell anemia patients. Compared to sickle cell anemia adult patients, BERK mice express much lower basal levels of HbF. The significant fold induction of HbF seen in the SCDMBD2-/- mice compared to SCD control mice could have a greater impact on the morbidity of sickle cell adult patients compared to transgenic mouse models.

Chapter 3: Mi2β-mediated silencing of the fetal γ-globin gene in adult erythroid cells

I. Introduction

Sickle cell anemia and β-thalassemia are two of the most common single gene disorders worldwide. A need for a more effective treatment for these disorders has led to extensive studies on the regulation of globin genes, since an increase in fetal hemoglobin (HbF) is known to be beneficial for these patients. Hydroxyurea, currently part of the standard treatment for sickle cell anemia, increases HbF levels in sickle cell patients but its effects are variable and it is not effective in the treatment of most cases of β-thalassemia (Charache, et al 1992; Steinberg et al. 1997; Ware et al. 2002).

Development of effective and potentially less toxic targeted strategies to induce HbF production will require full understanding of the molecular basis of developmental repression of the fetal γ-globin gene. The γ-globin gene is located on chromosome 11 within the β-globin gene locus. The β-locus consists of a locus control region followed by a group of five β-type globin genes positioned in the order in which they are expressed during development (5'-LCR-ε-AγGγ-δ-β-3') (Stamatoyannopoulos 2005; Thein & Menzel 2009; Sankaran, Xu & Orkin 2010). During the embryonic stage of development, the ε-globin gene is expressed in the yolk sac, followed by expression of the γ-globin gene in the fetal liver during most of gestation. At birth, γ-globin expression declines as the expression of adult β-globin in bone marrow derived erythroid cells

predominates (Peschle et al. 1985; Ley et al. 1989). There are numerous trans-acting factors and associated complexes involved in γ-globin gene silencing. These include BCL11A, KLF1/EKLF, MBD2/NuRD, TR2/TR4, and GATA-1/FOG-1/NuRD (Sankaran, Xu & Orkin 2010; Ginder, Gnanapragasam & Mian 2008). KLF1 (formerly known as EKLF) is a transcription factor belonging to the family of the krüppel-like factors and it is critical in the expression of many erythroid specific genes (Feng, Southwood & Bieker 1994; Perkins, Sharpe & Orkin 1995; Nuez et al. 1995). It is C2H2 transcription factor that has a highly conserved DNA binding domain (Bierker 2001). KLF1/EKLF binds directly to, and positively regulates the β-globin gene in adult erythroid cells, whereas it negatively regulates the γ-globin gene indirectly through its role in competition between the γ- and β-globin promoters for the LCR and through its binding to and positive regulation of BCL11A, an important γ-globin gene silencer (Zhou et al. 2010; Borg et al. 2010) (Figure 21).

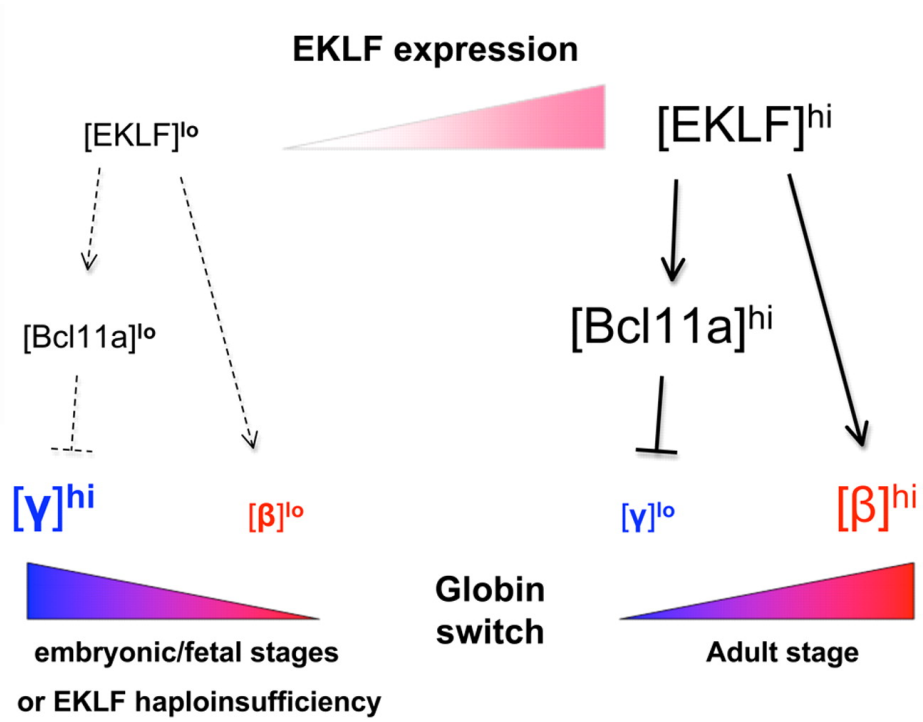


Figure 21. KLF1/EKLF significantly contributes to the globin switch by directly regulating β-globin expression and indirectly regulating γ-globin gene expression via BCL11A. During the embryonic/fetal stages KLF1 expression is low. This leads to lower expression of BCL11A and therefore high expression of γ-globin. Low levels of KLF1 also lead to low expresssion of β-globin. This same effect is seen with KLF1 haploinsufficiency. In adult stages, KLF1 expression levels increase, leading to higher expression levels of BCL11A, and therefore low γ-globin expression. Higher KLF1 levels also lead to higher expression of β-globin gene via its role as an activator. Adapted from Siatecka & Bieker 2011.

Originally identified in a GWAS study (Menzel et al. 2007), BCL11A is a zinc finger transcription factor that acts as a dominant negative regulator of the embryonic to adult hemoglobin switch in murine development (Sankaran et al. 2009). It binds to the locus control region (HS3) as well as an intergenic region of the β locus, between γ-globin and the δ-globin regions (Sankaran et al. 2008). Absence of BCL11A during mice development results in a delay of the switch from embryonic β-type globin genes into adult globin genes (Sankaran et al. 2010). Knockout of BCL11A in humanized sickle cell transgenic mice greatly ameliorates their sickle cell disease pheynotype (Xu et al. 2011). Of note, BCL11A plays an important role in the regulation of B cells (Liu et al. 2003), and homozygous knockout mice are embryonic lethal (Liu et al. 2003).

Epigenetic mechanisms, including DNA methylation and histone modifications, also play an important role in developmental globin gene silencing (Singal et al. 1997; Pikaart, Recillas-Targa & Felsenfeld 1998; Forsberg et al. 2000; Hsu et al. 2007; Mabaera et al. 2007), and inhibitors of DNA methylation induce HbF levels in baboons and in humans (DeSimone et al. 1982; Ley et al. 1982; Charache et al. 1983). The MBD2/NuRD complex, which selectively binds to methylated CpG-rich DNA, has been shown to play an important role in the silencing of the human embryonic ε- and fetal γ-globin genes (Rupon et al. 2006; Gnanapragasam et al. 2011; Rupon et al. 2011). NuRD co-repressor complexes include at least one copy of each of the proteins Mi2α and –β, HDAC-1 and -2, MTA-1 and -2, RbAp46/48, and p66α and p66β (Hendrich & Bird 1998; Feng & Zhang 2001). MBD2/NuRD does not appear to interact directly with promoters of human β-type globin genes, suggesting that its silencing effects occur through an indirect pathway (Rupon et al. 2006). The MBD3/NuRD complex, which is distinct from MBD2/NuRD (Le Guezennec et al. 2006), directly interacts with and regulates genes within the

β-globin locus through its association with the transcription factors GATA-1 and Friend of GATA-1 (FOG-1) (Hong et al. 2005; Rodriguez et al. 2005). In βYAC transgenic mice, the GATA-1/FOG-1/NuRD complex negatively regulates the γ-globin gene by binding to its distal promoter (Harju-Baker et al. 2008). This complex is associated with positive regulation of the adult β-globin gene (Miccio et al. 2010), suggesting that GATA-1/FOG-1/NuRD can act as either an activator or repressor complex.

Mi2β (also known as CHD4) is the largest protein of the NuRD complex. It belongs to a family of proteins called chromatin organization modifier (Chd), a member of the SNF2 family of helicases (Eisen, Sweder & Hanawalt 1995), and confers the chromatin remodeling function of the NuRD complex. Mi2β contains tandem plant zinc finger homeodomains (tPHD) and tandem chromodomains (tCHD) (Woodage et al. 1997), which play an important role in modulating its ATPase activity (Morra et al. 2012; Watson et al. 2012). It was recently shown that the tandem PHDs are important for tCHD binding to dsDNA, and tPHDs also allow binding to nucleosomes (Morra et al. 2012). PHDs specifically recognize histone H3 tails (Musselman et al. 2012) (Figure 22).

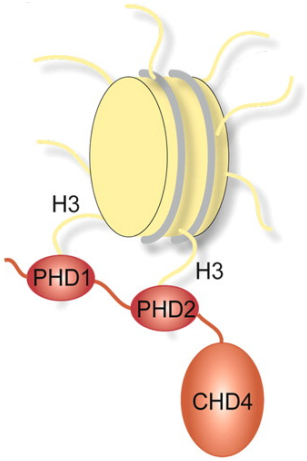


Figure 22. Mi2β (CHD4) binds to histone 3 (H3) tails through interactions with plant homeodomains 1- and 2 (PHD1 and PHD2). Mi2β utilizes both PHD domains to recognize H3 histone tails. Adapted from Musselman et al. 2012

In addition to its negative regulatory role as part of the NuRD complex, Mi2β also acts as a co-activator in lymphocytes (CD4) (Williams et al. 2004) by interacting with p300 and the E box binding protein in the CD4 gene enhancer (Williams et al. 2004). Further evidence of its role as both an activator and a repressor came from studies in T helper (Th2) cells. In these cells Mi2β and GATA-3, can form either a repressive NuRD complex with HDAC activity, or an independent activating complex with p300 (Hosokawa et al. 2013). The mechanism behind Mi2β's switch from a repressor to an activator remains to be elucidated.

Mi2β is highly expressed in tissues such as hematopoietic stem cells and in early lymphoid, myeloid and erythroid precursors (Kim et al. 1999). Mi2β knockout mice are embryonic lethal and conditional Mi2β knockout mice show a block in erythroid differentiation at the proerythroblast stage (Yoshida et al. 2008). However, recent studies with conditional knockout mice under an erythroid specific promoter have reported that a partial Mi2β knockout is able to induce γ-globin gene expression without affecting erythoid differentiation (Costa et al. 2012).

In this study we have explored a novel role of Mi2β in the hemoglobin switch. We show here that Mi2β has an important role in the repression of γ-globin gene expression in mouse hematopoietic cells containing a transgenic human β-globin locus, and in adult human primary erythroid cells. While this repression is mediated in part by known negative regulatory activities of Mi2β-containing NuRD complexes, a major part of the γ-globin gene silencing effect of Mi2β is through direct positive regulation of the genes encoding the transcription factors KLF1/EKLF and BCL11A.

II. Methods

Cell Lines and Growing Conditions:

Chemical Inducer of Dimerization cells were a kind gift from Dr. Kenneth Peterson. Cells were grown on IMDM supplemented with 10% heat inactivated fetal bovine serum (FBS) and 2% Penicillin/Streptomycin. Cells were passaged at a concentration of 3-5 x 10^5 per mL every 2-3 days. The B/B Homodimerizer (AP20187, Clontech) was added at a concentration of 0.1μM, and G418 at a concentration of 100-200μg/ml in every passage.

siRNA knockdown:

For each transfection group, 2.5 million CID cells at a density of 0.5 million cells/mL were plated a day before transfection in a T25 flask. The following day, 5 mL of media were placed on a T25 flask for each transfection group in order for the media to equilibrate. Meanwhile, cells were counted, and 5 x 10^6 cells per group were spun at 100g for 10 minutes at room temperature. Cells were then resuspended in 100μL/group of nucleofector solution (provided by the nucleofector kit) and mixed well by gentle pipetting. 10μL of a 10μM QIAGEN siRNA solution was placed in a sterile tube per group, and 5μg of GFP was used as control. 100μL of the mixture of cells and solution was added to each of the tubes containing the siRNA in order to dilute the siRNA concentration to 1μM. CID cells were transferred to a cuvette supplied by the nucleofector kit (#VCA-1003, Lonza) and pulsed in the D-012 setting of the nucleofector machine (Amaxa, Nucleofector II). The cells were then transferred to the T25 flask with equilibrated media and incubated at 37°C, 5% CO_2. After a 24hr incubation period, the control GFP sample was observed under the microscope to determine the transfection efficiency.

RNA Isolation and qPCR:

RNA was isolated 72 hrs post-transfection by phenol-cholorform extractions (TRIzol, Invitrogen) according to the manufacturer's protocol. Samples were then subjected to DNase I treatment as follows: 20µL reactions were made by adding 10µL of RNA at a concentration of 200ng/µL, 0.5µL of Dnase I (Ambion, at a concentration of 2U/µL), 0.5µL SUPERase Inhibitor (Ambion, at a concentration of 20U/µL), 2µL of 10X Dnase Buffer (Ambion), and 7µL of DEPC treated water. Samples were incubated at 37°C for 30min followed by 97°C for 10 min. 1µg of RNA (5µL of above reaction) were then used for making cDNA in a 10µL reaction per sample also including 0.5µL of RTase, 2µL of RT mix and 2.5µL of DEPC RNase free water from the iScript cDNA synthesis kit (Bio Rad) according to the manufacturer's protocol.

Gene expression was determined by performing qPCR, followed by analysis with the $2^{-\Delta\Delta Ct}$ method. q-PCR was performed using either Power Sybr green PCR master mix (ABI) or Taqman Fast Universal PCR master mix (ABI) in an ABIRT1900 instrument (1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min). Relative quantification was determined using the SDS 1000 software. The primers used for q-PCR, listed in Table 6, were designed to specifically bind to the cDNA of interest and not genomic DNA by crossing exon-exon boundaries.

CD34+ cell isolation:

De-identified CD34+ cells were isolated from 10 mL apharesis packs donated by the VCU bone marrow transplant unit. Cells were thawed quickly at 37°C in a water bath, and then poured on a 50mL tube. Cells were then mixed with 1 volume of 1X PBS containing 2% FBS. ~30mL of volume of cells were added slowly to a 50mL tube containing 15mL of Ficoll-paque-

plus (StemCell Technologies Inc #07907). The mix was spun at 400g for 30 minutes at room temperature with no break. The middle layer containing the mononuclear cells was extracted and placed on a fresh tube, where 40mL of 1X PBS with 2% FBS was subsequently added. Mononuclear cells were then filtered through a 70μM nylon filter (BD Biosciences #352350), and spun at 250g for 6 minutes at room temperature. The pellet was resuspended in 4-5mL of DNase I solution (StemCell Technologies Inc. #07900) and incubated at room temperature for 15 minutes. Following DNase I incubation, 40mL of 1X PBS with 2% FBS and 1mM EDTA were used to dilute the solution, followed by spinning the cells at 250g for 6 minutes at room temperature. The cell pellet was re-suspended in 1X PBS with 2% FBS and 1mM EDTA at a density of 2 x 10^8 cells/ml. EasySep kit (StemCell Technologies) was used for positive selection of CD34+ cells according to the manufacturer's protocol, and cells were subsequentely maintained in growth medium consisting of StemSpan SFEM Medium with 1X CC100 cytokine mix (StemCell Technologies Inc.), 40μg/mL of low density lipoprotein (Sigma) and 2% Penicillin/Streptomycin.

Lentiviral Synthesis:

shMBD2 (GGGTAAACCAGACTTGAA) and shMi2β (#1: CGGTGAGATCATCCTGTGTGATA, #2: GGACCTGAATGATGAGAAACAGA) sequences were cloned into a pRRL.H1.shRNA vector. The vectors were then packaged into a lentivirus by calcium phosphate transfections in 293T cells as follows: 6 million cells were evenly plated in 10mL of DMEM (Gibco) containing 20% FBS (Hyclone) media in a 10cm dish and incubated on a 37°C, 5% CO_2 incubator. The following day (at ~80% cell confluency) the medium was removed and 9 mL of fresh media were added and incubated for 1-2 hours. Meanwhile, 3.75μg

of PcmvR DNA (packaging plasmid), 1.5µg of PMD DNA (Envelop plasmid), 5µg of the pRRL.H1 vector, and sterile dH_2O totaling a 437µl solution were mixed in a 2mL tube and vortexed. 63µl of 2M $CaCl_2$ were then added drop wise. This 500µl solution was subsequently added drop wise to 500µl of 2X HBS. The resulting 1mL solution was distributed evenly, and drop-wise over the cells. After 5 minutes of incubation at room tempertature, cells were transferred to a 37°C, 5% CO_2 incubator. Following 16-18 hours of incubation, the media was replaced with 9mL of fresh media and cells were once again placed in a 37°C, 5% CO_2 incubator. After a 24hr incubation period, the media (now containing the virus) was collected in a fresh tube and frozen quickly on dry ice, and later stored in -80°C.

Lentiviral Infection:

Three days following CD34+ isolation, 2 X 10^5 CD34+ cells per well were plated on a 12 well plate with 50µl of growth medium while the frozen virus stored at -80°C was thawed. Once the cells were plated, 4µg/mL of polybrene were mixed with the lentiviral shRNA solution and 500 - 600µl of this solution were immediately added per well. The 12 well plates were incubated at 37°C, 5% CO_2 with slow shaking for 24hr. Growth medium was then added accordingly and the cells were transferred to a regular incubator at 37°C, 5% CO_2. On day 5 of incubation, GFP+ cells were selected by flow cytometry on a BD FACSAria™ II High-Speed Cell Sorter and incubated on IMDM supplemented with 20% fetal bovine serum, 10ng/ml of SCF, 1u/ml EPO, 1ng/ml IL-3, 1µM Dexamethasone, 1µM Estradiol, and 2% Penicillin/Streptomycin for three days. The cells were subsequently changed to a differentiation media consisting of IMDM supplemented with 20% fetal bovine serum, 1u/ml EPO, 10ng/ml Insulin, and 2%

Penicillin/Streptomycin and cultured for a period of 10 days. Cells were spun down and fresh media was added every other day.

Fluorescence Activated Cell Sorting (FACS) on Human Adult Primary Erythroid Cells:

1 x 10^6 cells were washed on 10-12mL of 1XPBS/10%FBS buffer and spun at 300g for 5 minutes at room temperature. The cell pellet was then resuspended in 100µL of the same buffer and incubated with 0.06µg of CD71 and 0.015µg CD235a for 20 minutes. Cells were then washed twice with 1.5mL of buffer by spinning at 300g for 5 minutes and adding fresh buffer each time. The cell pellet was subsequently re-suspended on 400µL of buffer. Samples were then analyzed by flow cytometry in a BD FACSCanto™ II machine. Antibodies used include anti-human CD71 (eBioscience #17-0719-42) and anti-human CD235a (eBioscience #12-9987-82).

CD34+ cell High Performance Liquid Chromatography (HPLC):

10 million of the above sorted differentiated human erythroid cells from either control shSCR group or shMi2β group were spun down at 300g for 5 minutes and cell pellets were taken to VCU hematology for further lysing and HPLC hemoglobin variant testing.

Giemsa Staining Human Adult Primary Erythroid Cells:

FACS sorted differentiated cells were spun in a cytocentrifuge for 10 min at 1000 rpm and subsequently stained with Giemsa.

Chromatin Immunoprecipitation assays (ChIP):

10^7 CID cells or CD34+ hematopoietic progenitor cells, the latter collected at day 2 of

erythroid differentiation, were crosslinked using 2.0 mM ethylene glycol-bis(succinimidyl succinate) (EGS) at room temperature for 30 min followed by formaldehyde for 10 min. ChIP assays were then be performed using the Millipore (EZ-Magna ChIP) kit per manufator's protocol. Antibodies used at the concentration of 7µg include ChIP-grade Mi2β antibody (Abcam ab70469) and Normal Mouse IgG (Millipore #12-371). qPCR was performed as described above and primers used are listed in Table 6.

Co-Immunoprecipitation Assays:

10 million CID cells per group were collected and assays were carried out using Sigma's kit manufacturer's protocol (SIGMA, FLAG Immunoprecitipation kit cat# FLAGIPT1). rProtein G beads were purchased from Invitrogen (#15920-010). Prior to the experiment, the protein G beads were washed 4 times by spinning down at 500g for 30 seconds at 4°C, discarding the supernatant and adding equal volume of 1X washing buffer each time (40µL of beads were used per reaction). 1mL of lysis buffer with protease inhibitors was added to each group, and samples were vortexed and placed on a rotator for 30 minutes at 4°C. The samples were then spun down at 1300g for 10 minutes at 4°C, and the supernatant was transferred to a new tube. Pre-clearing was performed as follows: 40µL of protein-G beads were added to each 1mL of supernatant, and samples were placed on a rotator for 2 hours. Samples were subsequently spun at 5000g for 30 seconds at 4°C. 30µL of supernatant were removed and stored in -80° as the input control. The remaining supernatant was divided into 2 tubes (480µL each). One tube was incubated with 15µg of MBD2 antibody (Santa Cruz) and the other was incubated with 15µg of normal goat IgG, and both samples were placed on a rotator overnight at 4°C. The following morning, 40µL of protein G beads were added to each sample and tubes were placed on a rotator for 2 hours at 4°C.

Following incubation with rProtein G beads, samples were washed 5 times by spinning at 5000g for 30 seconds 4°C, discarding the supernatant and adding 1mL of washing buffer each time. Once the supernatant was discarded from the last wash, gel loading tips were used to remove the excess liquid without disturbing the beads. 40µL of 2x western blot sample buffer was added to each sample and 30µL of buffer was added to the input control. All samples were then boiled for 5 minutes, and subsequently cooled to room temperature. Samples were spun twice at 13000rpm for 30 seconds at room temperature, each time using a gel-loading tip to transfer the supernatant to a new tube to separate the supernatant from the rProtein G beads. The remaining supernatant was then used for western blotting. Antibodies used for western blotting include MBD2 (Santa Cruz sc-1244), p66α (Upstate #07-365), HDAC-2 (Millipore #05-814), RbAp48 (abcam ab79416) and MTA-2 (Santa Cruz sc-28731)

Western Blotting:

Whole cell lysates were made on 4% SDS and briefly sonicated. Samples were then mixed with 2x loading buffer containing beta-mercaptoethanol and 1% bromophenol blue, and subsequently ran on a 10% SDS gel. The protein was transferred onto a PDVF membrane for 1 hour at 100V. The membrane was subsequently blocked for an hour in a blocking buffer consisting of 1X PBST in 5% milk. This was followed by 1-hour incubation with 5-10µg of the antibody of interest in 1X PBST in 5% milk. Three 5-minute washes with 1X PBST were carried out, followed by a 45 minute incubation of secondary antibody in a blocking buffer solution. The secondary antibody was removed by three, 5-minute washes, followed by incubation of the membrane with either West Pico Chemiluminescence substrate or Supersignal West Dura Extended duration substrate (Thermo Scienctific) according to the manufacturer's protocol.

Antibodies used include MBD2 (Santa Cruz sc-1244), CHD4/Mi2β (Abcam ab54603), EKLF/KLF-1 (Abcam ab88417), FOG-1 (Santa Cruz sc-9361) and BCL11A (Abcam ab19487).

Western Blots from Mouse erythrocytes:

Hemolytic anemia was induced in both wild type and MBD2 knockout mice by treatment with two intra-peritonial injections of 1-acetyl-2-phenylhydrazine (10 mg/ml; Sigma) at a dose of 0.4 mg/10g of body weight, 16 hours apart. In order to allow the spleen to become a prominent site of extramedullary erythropoiesis, mice were sacrificed 5 days after the second injection. At the time of sacrifice, the spleens were harvested and single cell suspensions were obtained by gentle brushing. Whole cell lysates were then made according to the Western Blot protocol described above.

	Forward	Reverse	Probe
Human γ-globin	GTG GAA GAT GCT GGA GGA GAA A	TGC CAT GTG CCT TGA CTT TG	FAM/AGG CTC CTG GTT GTC TAC CCA TGG ACC /BHQ
Human β-globin	GCA AGG TGA ACG TGG ATG AAG T	TAA CAG CAT CAG GAG TGG ACA GA	FAM/CA GGC TGC TGG TGG TCT ACC CTT GGA CCC
Murine Alpha Globin	AAT ATG GAG CTG AAG CCC TGG	ACA TCA AAG TGA GGG AAG TAG GTC T	
Murine glycophorin A	GCC GAA TGA CAA AGA AAA GTT CA	TCA ATA GAA CTC AAA GGC ACA CTG T	FAMTTGACATCCAATCTCCTGA GGGTGGTGA /BHQ
Murine MBD2	TTT GAC TTC AGG ACC GGC AAG ATG	ATT GCT CGG GTG GTT CGT GAA TTT	
Murine Mi2β	GAA CCA CAG GGA GTT AAT GAG	CTT ATA GAG GGA GTA GAG GAA GAC	
Murine Mi2α	GAT GAA GAC TTT GAT GAG CGT	ACA CAT AGG CCT TAA ACT CCT	
Murine α-1- spectrin	TTA GCA CCA CAT ACA AAC AC	AAA CAT ATC CTT TCC TCC CTG	
Murine aminolevulinate dehydratase (Alad)	GAG TTC CCA AGG ATG AAC AG	CTC CTC TGC TAG GAA TGC TC	
Murine Ferrochelatase	GCG AGG TGG TCA TTC TGT TT	ACT GGA CCA ACC TTG GAC TG	
Murine erythropoietin (EPO) Receptor	CCC AAG TTT GAG AGC AAA GC	TGC AGG CTA CAT GAC TTT CG	
Murine transferrin Receptor	ATA AGC TTT GGG TGG GAG GC	CTT GCC GAG CAA GGC TAA AC	
Murine GATA-1	CTG GGA TCG CCT ACA ACC TC	CTG CCA CAA GGT CAA GGC TA	
Murine glucose-6-phosphate dehydrogenase (G6PD)	TCG AAA TTG TAG GGG CAG CG	CCA TTT AAC GCA AGA GGG CG	
Murine uroporphyrinogen III synthase	GCT GCC TCT TCT CTT TCC GT	TGC ATG CTT TCC ATG GGG AT	
Murine α-globin	AAT ATG GAG CTG AAG CCC TGG	ACA TCA AAG TGA GGG AAG TAG GTC T	
Murine cyclophilin A	GAG CTG TTT GCA GAC AAA GTT C	CCC TGG CAC ATG AAT CCT GG	
Murine FOG-1	TTC TGG TGG ACT GCT ATA TGT G	AAG ACG TCC TTG TTG ATG AC	
Human KLF1	GCA AGA GCT ACA CCA AGA G	GTG TTT CCG GTA GTG GC	
Human γ promoter	GCC TTG ACC AAT AGC CTT GAC A	GAA ATG ACC CAT GGC GTC TG	
Human Mi2β	ATA AAG ATA AGC CAT TGC CTC C	GAC ATA TGC CTT GAA CTC TTT CTC	
Human BCL11A	AAC CCC AGC ACT TAA GCA AA	GGA GGT CAT GAT CCC CTT CT	
Human BCL11A Promoter	TCC TTC TTT CTA ACC CGG CTC	CTG CGC GCT CTC GTG ATT AT	
Human GAPDH Promoter	TCC CCT TCC TGC AGA CAG CTC C	AGG GAG GGC AGC ATA CCG GG	
Human KLF-1 Promoter	GCC TGG GCC CCC ACC TGA TA	GAC TTG GCA CGA GCT CCC CG	

Table 6. The table above lists the primers used for all the experiments described in the methods section.

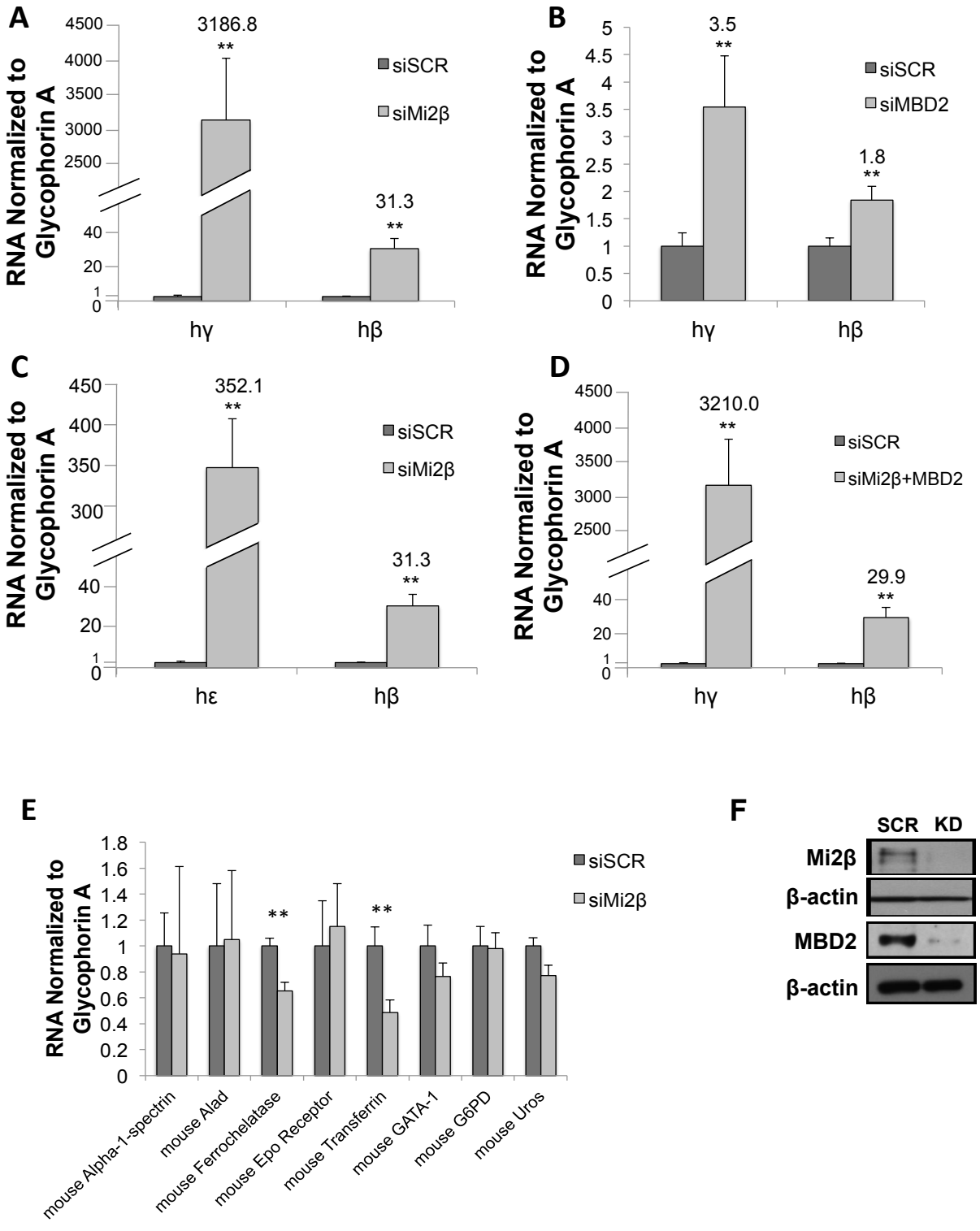
III. Results

A. Mi2β is an important developmental regulator of the human β-type globin genes and acts partially independently from the MBD2/NuRD complex

To study the role of Mi2β in MBD2/NuRD mediated γ-globin gene silencing in adult erythroid cells, gene knockdown was carried out in chemical inducer of dimerization (CID) cells. These cells are hematopoietic precursor cells derived from adult mice bearing a yeast artificial chromosome containing the complete human β-globin gene locus (βYAC). CID cells have been stably transduced with a receptor which, upon introduction of a commercially available chemical ligand, triggers a signal transduction pathway allowing them to differentiate into erythroid cells that do not express the γ-globin gene (Blau et al. 2005). They display characteristics of adult erythroid cells and express mainly the β-globin gene with minimal γ-globin gene expression (Blau et al. 2005).

Transient siRNA-mediated knockdown of Mi2β in CID cells resulted in a very large increase in expression of the γ-globin gene at 72-hours post-transfection (Figure 23A). This effect is ~900-fold greater than with knockdown of MBD2 (Figure 23B) which contrasts with the equivalent induction of γ-globin gene expression after knockdown of Mi2β, MBD2 and p66α for 24-hours (Gnanapragasam et al. 2011). This led to a detailed study of Mi2β and its role in β-type globin gene regulation. Knockdown of Mi2β also resulted in a significant increase in human embryonic ε-globin gene expression in CID cells (Figure 23C), although the increase is ~8-10-fold less than for γ-globin RNA. As shown in Figure 23D, simultaneous knockdown of Mi2β and MBD2 did not result in greater γ-globin gene expression than knockdown of Mi2β alone. Knockdown of MBD3 does not affect the expression of the γ-globin gene in CID cells

(Gnanapragasam et al. 2011). These data suggest that Mi2β silences the γ-globin gene in this cell line partially independently of MBD2/NuRD and MBD3/NuRD complexes. While β-globin gene expression is also induced by 30-fold following Mi2β knockdown, this effect is relatively small compared to the effect seen for the ε- or γ-globin genes, which are induced 350-fold and >3000-fold respectively when normalized to glycophorin A expression (Figures 23C and 23A). In order to determine if Mi2β knockdown induces expression of other erythroid-specific genes, we measured expression of the mouse α-1 spectrin, Ferrochelatase, Epo receptor and Alad genes, as well as the transferrin, GATA-1, G6PD, and Uros genes. There was no significant increase in expression of any of these genes (Figure 23E), nor of the mouse α- globin gene (Figure 24C) after Mi2β knockdown. These results suggest that Mi2β knockdown preferentially induces expression of the human γ- and ε-globin genes rather than promoting further erythroid differentiation of CID cells.


A
RNA Normalized to Glycophorin A
4500
4000
3500
3000
2500
40
20
1
0
3186.8
**
31.3
**
siSCR
siMi2β
hγ
hβ
B
RNA Normalized to Glycophorin A
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
3.5
**
1.8
**
siSCR
siMBD2
hγ
hβ
C
RNA Normalized to Glycophorin A
450
400
350
300
40
20
1
0
352.1
**
31.3
**
siSCR
siMi2β
hε
hβ
D
RNA Normalized to Glycophorin A
4500
4000
3500
3000
2500
40
20
1
0
3210.0
**
29.9
**
siSCR
siMi2β+MBD2
hγ
hβ
E
RNA Normalized to Glycophorin A
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
siSCR
siMi2β
mouse Alpha-1-spectrin
mouse Alad
mouse Ferrochelatase
mouse Epo Receptor
mouse Transferrin
mouse GATA-1
mouse G6PD
mouse Uros
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Mi2β
β-actin
MBD2
β-actin

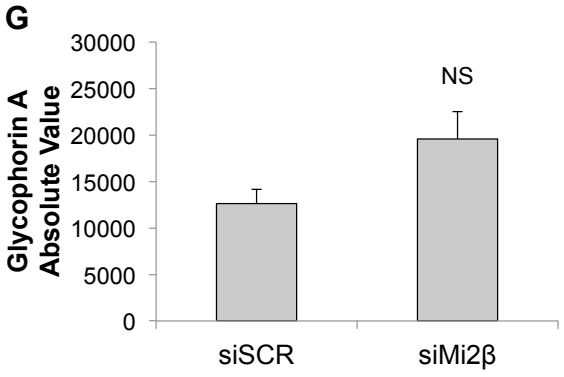

Figure 23 Mi2β preferentially regulates human β-globin locus gene expression in CID cells. CID cells were transiently transfected with siRNA for either Mi2β, MBD2 or scramble control (siSCR) as indicated. (A) Transient knockdown of Mi2β by siRNA leads to a 3186.8-fold increase in the expression of human γ-globin (hγ) gene expression in CID cells, and a 31.3-fold increase in human β-globin (hβ) determined by qPCR. (B) Transient knockdown of MBD2 by siRNA leads to a 3.5-fold induction of γ-globin gene expression in CID cells, and a 1.8-fold induction of β-globin. (C) A 352.1-fold increase is seen in the expression of human ε-globin (hε) upon knocking down Mi2β, and a 31.3-fold increase in β-globin. (D) Combined knockdown of Mi2β and MBD2 leads to a 3210-fold increase in the γ-globin gene expression, and a 29.9-fold increase in β-globin, similar to Mi2β knockdown alone. Data are expressed as human γ-, β-, or ε-globin RNA normalized to Glycophorin A RNA. (E) qPCR analysis showing the expression of six murine genes [alpha-1-spectrin, aminolevulinate dehydratase (Alad), erythropoietin (Epo) receptor, GATA-1, glucose-6-phosphate dehydrogenase (G6PD), and uroporphynogen III synthase (Uros)] was not altered upon Mi2β knockdown, while the mouse ferrochelatase and transferrin genes are slightly significantly down regulated. (F) Western blot showing the degree of Mi2β and MBD2 protein knockdown, respectively in the CID cells, used for the globin gene expression studies shown in both Figure 23 and Figure 24. (G) Absolute values of Glycophorin A/5ng of RNA determined by qPCR show no significant difference between siScramble control and siMi2β samples in CID cells therefore serving as an appropriate normalizing control. Error bars represent the standard deviation of three independent experiments. *signifies $p < 0.05$ and **signifies $p < 0.02$ and *** signifies $p < 0.001$ according to the students t-test.

Expression of the endogenous mouse εy and βh1 globin genes was measured in CID cells after Mi2β knockdown. As shown in Figure 24A & 24B, both genes are significantly upregulated, suggesting that Mi2β plays a silencing role in both the murine and human β-globin loci. Interestingly, upon Mi2β knockdown the murine εy gene is de-repressed to a much greater extent than βh1 (>100- fold versus ~3-fold), an observation similar to the relative effect on the human fetal and embryonic β-type globin genes, in which the γ-globin gene is up-regulated much more than the ε-globin gene (>3000-fold versus ~350-fold). These data are also consistent with the finding that the εy gene is activated later in murine erythroid development than βh1 (Kingsley et al. 2006) and thus is more analogous to the human γ-globin gene than is βh1. The murine α-globin gene is not affected upon Mi2β knockdown, suggesting its effect is confined to the β-globin locus (Figure 24C).

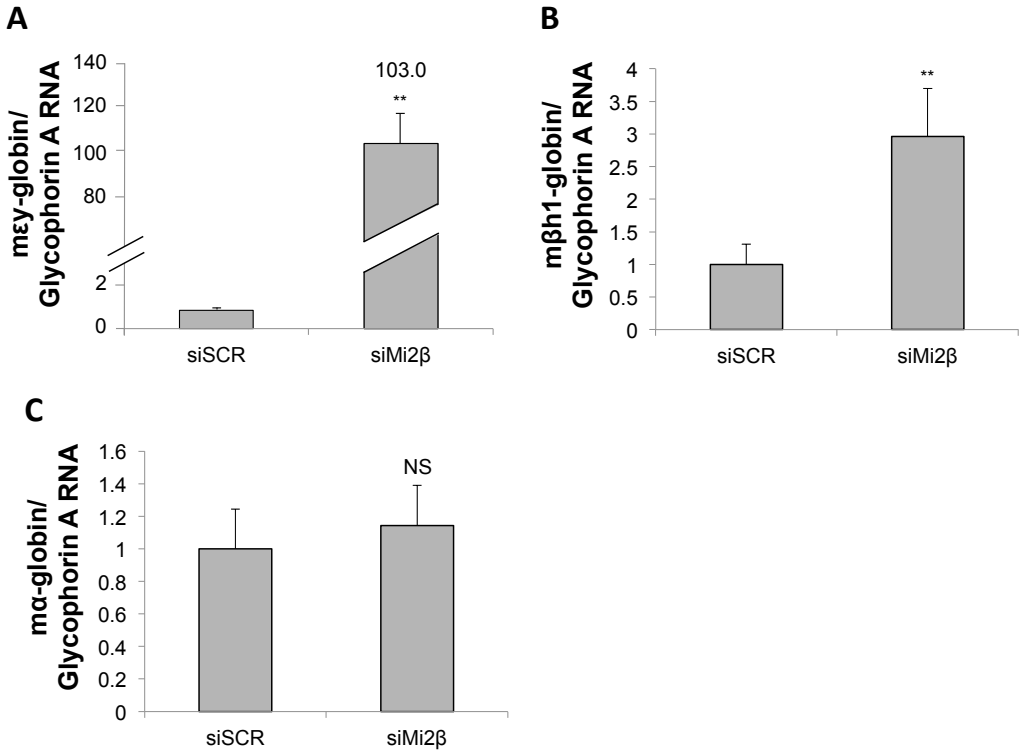

Figure 24. Mi2β regulates the expression of endogenous mouse β-type globin genes. CID cells that were transiently transfected as described in Figure 23, were then assayed for endogenous εy-, βh1-, and α-globin RNA levels by qPCR. (A) Transient knockdown of Mi2β in CID cells leads to increased expression of the murine εy (mεy) gene by 103-fold normalized to Glycophorin A. (B) Mi2β knockdown leads to a 3-fold increase in murine βh1 (mβh1) gene expression. (C) Murine α-globin (mα) RNA level is unchanged upon Mi2β knockdown. The data in Figure 24 are expressed as εy-, βh1-, and α-globin RNA normalized to Glycophorin A, a murine erythroid-specific housekeeping gene. Error bars represent the standard deviation of three independent experiments. *signifies $p < 0.05$ and **signifies $p < 0.02$ according to the students t-test. NS= Not statistically significant.

Because both Mi2β and its isoform, Mi2α (CHD3), have been shown to be incorporated into NuRD complexes (Tong et al. 1998; Wade et al. 1998; Zhang et al. 1998), we examined the role of Mi2α in γ-globin gene silencing. The level of Mi2α/CHD3 RNA was found to be ~10-15-fold lower than Mi2β RNA by qPCR, and knockdown of Mi2α had only a minor (~1.5-fold) effect on γ-globin gene expression in CID cells (Figures 25A-B), suggesting that the great majority of γ-globin gene silencing requires only the Mi2β (CHD4) isoform.

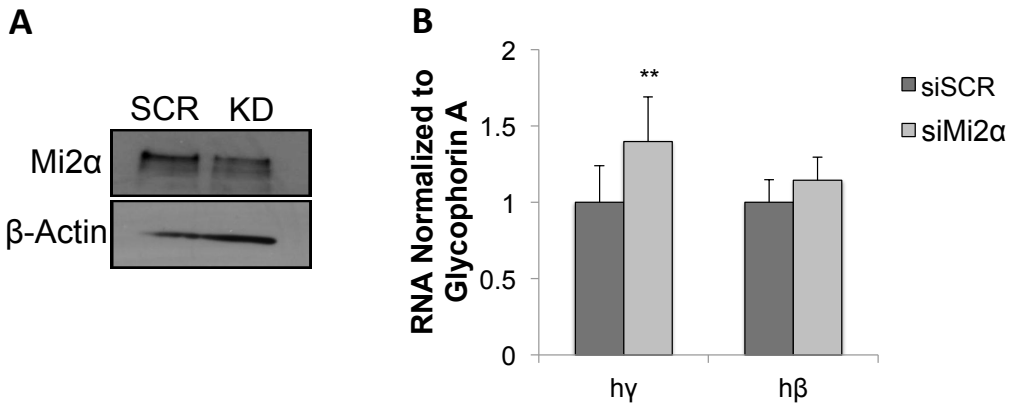

Figure 25. Mi2α knockdown exerts only a small effect on γ-globin gene expression in CID cells. (A) Western blot showing Mi2α protein level knockdown in CID cells. (B) qPCR data showing Mi2α knockdown leads to a ~1.5-fold induction of γ-globin (hγ) gene expression and no significant increase in β-globin (hβ) gene expression. **signifies $p < 0.02$ according to the student's t-test.

Based on the observation that Mi2β knockdown had a larger effect on γ-globin expression than did MBD2 knockdown, we explored the possibility that loss of Mi2β, the largest component of the MBD2/NuRD complex, could lead to destabilization of the complex and reduce the levels of other components, thereby creating a larger effect than loss of an individual component. Western blot (Figure 26A) and MBD2 co-precipitation assays (Figure 26B-C) in cells in which Mi2β was knocked down by ~80-90%, showed that other components of the MBD2/NuRD complex are present in normal abundance and able to interact despite depletion of Mi2β.

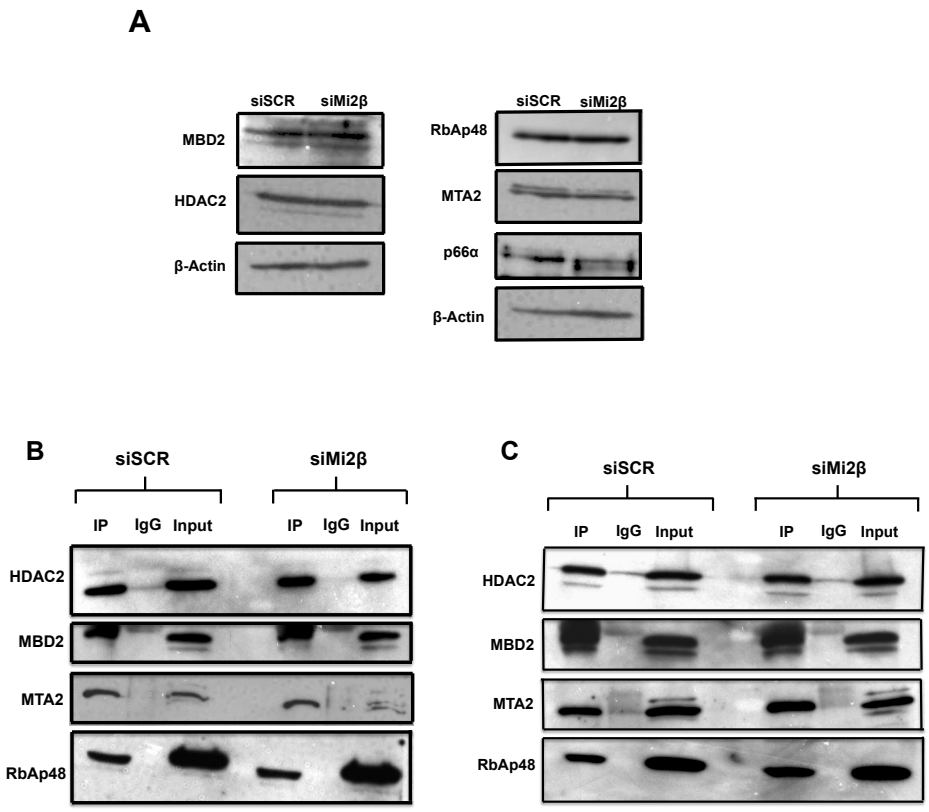

Figure 26. Mi2β knockdown does not lead to destabilization of the MBD2/NuRD complex. (A) Western blot of CID cell extracts showing MBD2/NuRD complex components 72 hours after Mi2β knockdown. (B-C) Western blots showing MBD2/NuRD complex components following co-immunoprecipitation reactions with either MBD2 antibody or IgG control for samples transfected with either siMi2β or siSCR control. To account for the potential increased stability of preformed MBD2/NuRD complexes, experiments were carried out at both 24 hours (B) and 72 hours (C) post-transfection.

B. Mi2β silences the γ-globin gene in human hematopoietic progenitor-derived primary erythroid cells

To determine the role of Mi2β on γ-globin gene silencing in the context of primary adult human erythroid cells, we stably knocked down Mi2β in CD34+ human hematopoietic progenitor derived erythroid cells via lentivirus-mediated shRNA infection. Two different Mi2β shRNA constructs were tested. Both resulted in increased γ-globin gene expression at day 10 of erythroid differentiation, and the amount of increased γ-globin expression obtained was proportional to the degree of knockdown of Mi2β (Figure 27A). Mi2β knockdown with construct #2 resulted in a ~20-fold increase in γ/γ+β-globin mRNA expression compared to ~9-fold in the case of MBD2 knockdown (Figures 27B and 27C). Of note, at day 10 of differentiation, the level of γ/γ+β- globin gene expression is <1% in both untreated and scramble shRNA controls. High performance liquid chromatography peformed in a representative experiment showed a HbF level of 13.2% of total hemoglobin when Mi2β was knocked down by ~25-30% (Table 7).

Because complete absence of Mi2β in the bone marrow of mice results in a block in erythroid differentiation at the pro-erythroblast stage (Yoshida et al. 2008), we sought to determine whether or not a partial knockdown of this protein would interfere with erythroid differentiation. Fluorescence-activated cell sorting using the transferrin receptor (CD71) and Glycophorin A (CD235a) surface markers showed that after Mi2β knockdown, erythroid differentiation proceeds similarly to scramble shRNA controls after 10 days of differentiation, as shown in Figure 27D.

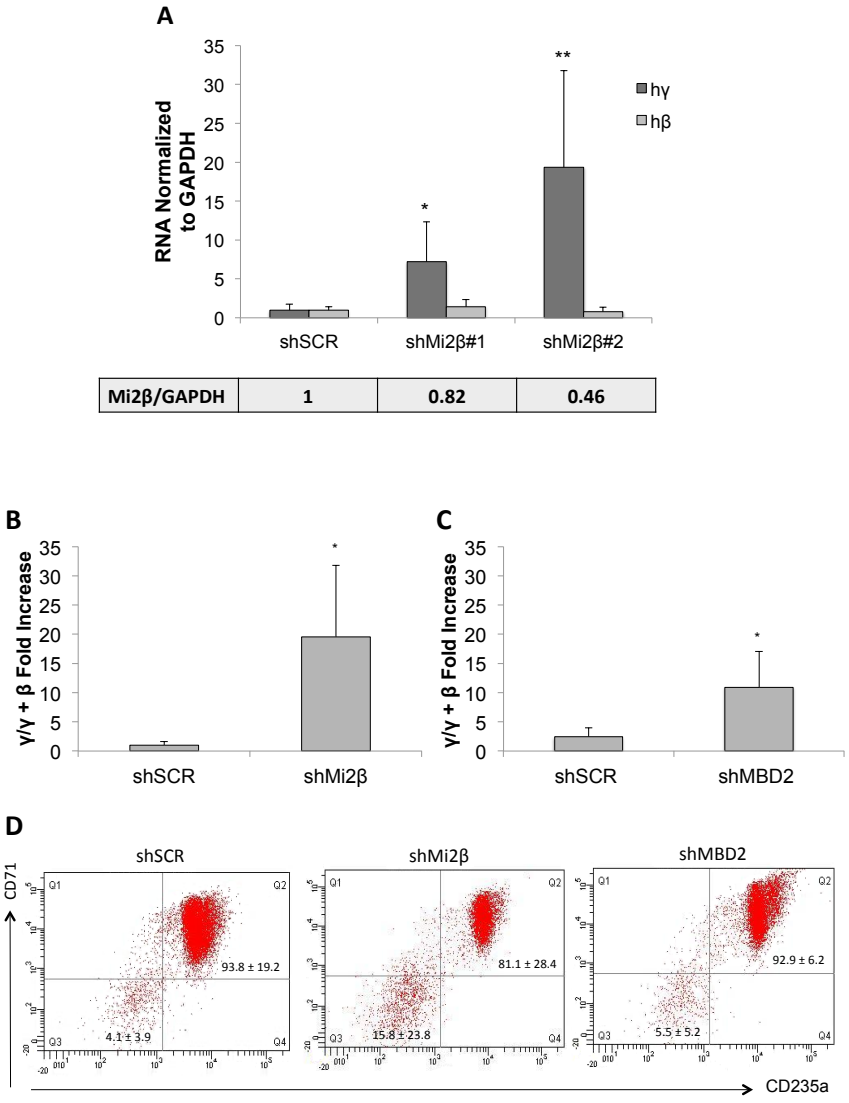

Figure 27. Mi2β regulates the expression of the γ-globin gene in human primary erythroid cells. CD34+ human hematopoietic progenitor cells were infected with lentivirus vectors harboring shRNA either for scramble control, two different Mi2β constructs, or MBD2. (A) Knockdown with shMi2β #1 leads to a 7.2-fold induction of γ-globin gene expression determined by qPCR. Knockdown with shMi2β #2 leads to a ~20-fold increase in γ-globin expression and a slight decrease in β-globin gene expression. Shown below are the Mi2β expression levels of each construct that were determined before cells were differentiated. (B) Partial knockdown of Mi2β (construct #2) leads to a 20-fold increase in γ/γ+β-globin gene expression. (C) Knockdown of MBD2 leads to a 9-fold increase in expression of γ/γ+β-globin gene expression. (D) FACS analysis showing erythroid differentiation of 81.1% of CD34+ progenitor cells in which Mi2β is knocked down compared to 93.8% of scramble shRNA control cells and 92.9% of cells in which MBD2 is knocked down. ± signifies standard deviations for at least three independent experiments. Error bars represent the standard deviation of at least three independent experiments. *signifies $p<0.05$ and **signifies $p<0.02$ according to the students t-test.

shSCR

Procedure:	Ref Range:	Units:	
Hemoglobin A	[95.0-99.0]	%	95.6
Hemoglobin A2	[1.2-3.0]	%	3.1
Hemoglobin F	**[0.0-2.0]**	**%**	**1.3**
Mi2β mRNA		**%**	**100**

shMi2β

Procedure:	Ref Range:	Units:	
Hemoglobin A	[95.0-99.0]	%	84.8
Hemoglobin A2	[1.2-3.0]	%	2.0
Hemoglobin F	**[0.0-2.0]**	**%**	**13.2**
Mi2β mRNA		**%**	**74.8**

Table 7. Mi2β knockdown increases HbF levels in human primary erythroid cells. Results obtained by high performance liquid chromatography assay carried out in the VCUHS CLIA approved clinical hematology laboratory showing a >10-fold increase in HbF after Mi2β knockdown (shMi2β) compared to scramble control (shSCR). qPCR assay showed the level of knockdown in the shMi2β treated primary erythroid cells was ~25%.

Interestingly, as erythroid differentiation proceeds, two distinct populations of cells were observed by flow cytometry of both control and Mi2β-knockdown cell populations. The major population was differentiated down the erythroid pathway and expressed both transferrin receptor and Glycophorin A (Figure 28A, quadrant 2), while the minor population did not stain for either (Figure 28A, quadrant 3). When analyzed by morphology, the minor population was found to consist of normally differentiated myeloid cells (Figures 28D-F, Quadrant 3). The size of this myeloid cell population varied among different patient-specific CD34+ progenitor batches, suggesting that a variable fraction of CD34+ cells in each batch assayed was committed to myeloid differentiation before exposure to erythroid differentiation medium. The percentage of myeloid cells was generally higher in Mi2β knockdown samples but this was highly variable across experiments and there was overlap with controls (Figure 28A).

The differentiated Mi2β knockdown erythroid cells in quadrant 2 were found to have ~40% Mi2β knockdown (Figure 28B) and a very high γ/γ+β-globin gene expression level (15% - 45% γ-globin RNA) (Figure 27B). In contrast, the myeloid cells in quadrant 3 showed a much higher Mi2β knockdown (~90%) (Figure 28C). Thus the difference may reflect more Lentiviral Mi2β shRNA expression in the myeloid compartment, which could impart a slight growth advantage. Overall, these results show that partial Mi2β knockdown does not inhibit terminal erythroid differentiation. These results differ sharply from the effect of conditional knockout of Mi2β in murine hematopoietic cells in which there is a complete block in erythroid differentiation at the proerythroblast stage (Yoshida et al. 2008).

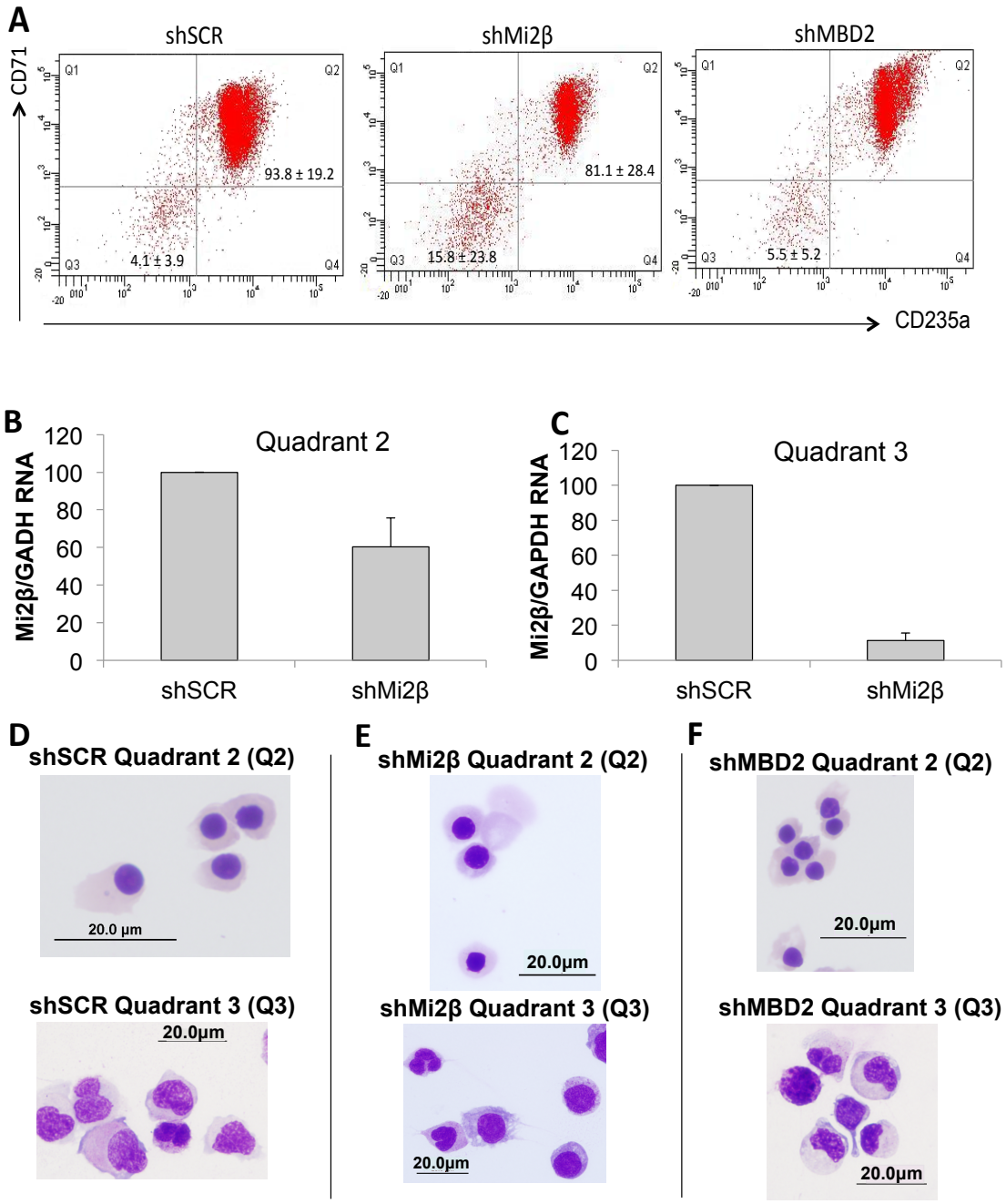

Figure 28. Higher Mi2β knockdown imparts a slight growth advantage to myeloid cells. (A) FACS analysis showing erythroid differentiation of shSCR-, shMi2β-, and shMBD2-treated CD34+ cells (B) qPCR analysis showing a ~40% knockdown level of Mi2β RNA in double-positive cells taken at the end of differentiation (Quadrant 2). (C) qPCR analysis showing a 90% knockdown of Mi2β RNA in double-negative cells taken at the end of differentiation (Quadrant 3). (D-F) Wright- Giemsa stain of scramble control, Mi2β knockdown and, MBD2 knockdown cell populations. Photo micrographs were generated using an Olympus (Center Valley, PA) BX41 compound microscope and Olympus DP71 digital camera at 100x magnification. Images were acquired with Olympus DP Controller software.

The level of Mi2β knockdown in cells found in quadrant 2 and quadrant 3 of Figure 28A is consistent with the level of GFP expression from the lentiviral vector used to transfect Mi2β shRNA (Figure 29). Lower levels of Mi2β knockdown (Figure 28B) correlate with lower GFP expression levels (Figure 29B, magenta color). These double positive (CD71+CD235a+) differentiated erythroid cells are also smaller in size compared to double negative (CD71-CD235a-) cells (Figure 29A, magenta color). In contrast, the cells that were negative for CD71 and Glycophorin A were larger myeloid cells (Figure 29A, blue color) showing a much higher Mi2β knockdown (~90%) (Figure 28C), and a higher GFP level (Figure 29B, blue color).

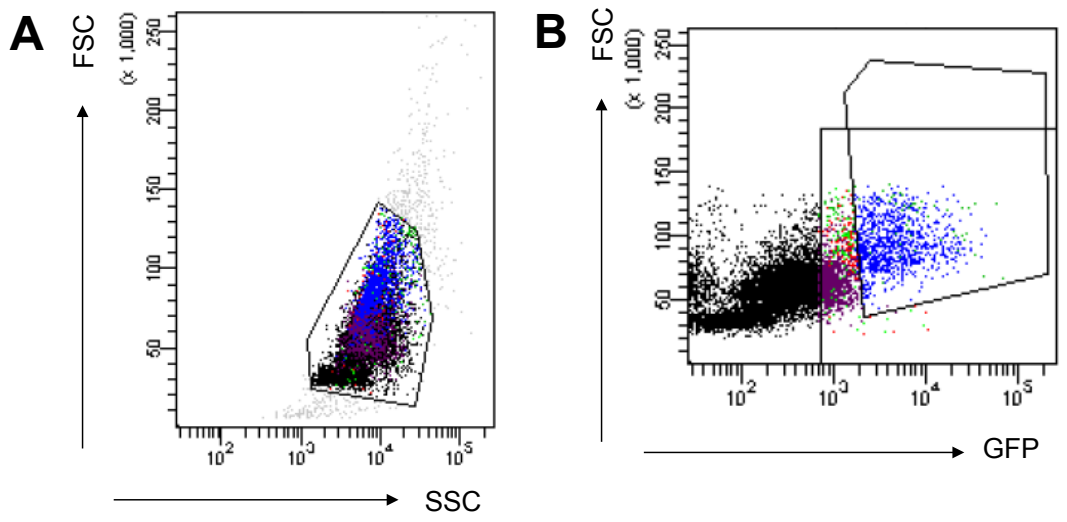

Figure 29. GFP expression in cells undergoing erythroid cell differentiation versus myeloid cell differentiation. (A) Forward scatter vs. side scatter plots showing that differentiated erythroid cells, depicted in magenta (Figure 28E, Quadrant 2), are smaller in size than cells lacking erythroid markers depicted in blue (Figure 28E, Quadrant 3). (B) GFP expression levels showing that the larger cells (blue) have higher GFP than differentiated erythroid cells (magenta).

Interestingly, a small number of experiments showed a loss of Mi2β knockdown as the cells approached terminal erythroid differentiation (Figure 30). Fluorescence-activated cell sorting using CD71 and CD235a markers was conducted every 3-4 days of differentiation to isolate erythroid cells, and Mi2β knockdown was determined by qPCR at each stage of differentiation. A representative experiment shows the level of Mi2β knockdown decreases as the cells progress through differentiation (Figure 30A). At day 12 of erythroid differentiation, the same set of shMi2β-treated cells were tested for γ-globin gene expression and showed ~17% γ/(γ+β)-globin RNA ratio when compared to shSCR (~0.01% γ/(γ+β)-globin) (Figure 30B). This suggests Mi2β's silencing effect on γ-globin gene expression occurs at early stages of differentiation and Mi2β knockdown does not appear to be essential during the later stages of differentiation to exert a significant effect. This event, however, did not occur in all experiments conducted, and perhaps the level of knockdown at the start of differentiation determines whether the cells are able to maintain a high level of Mi2β knockdown, or whether they are able to re-express Mi2β.

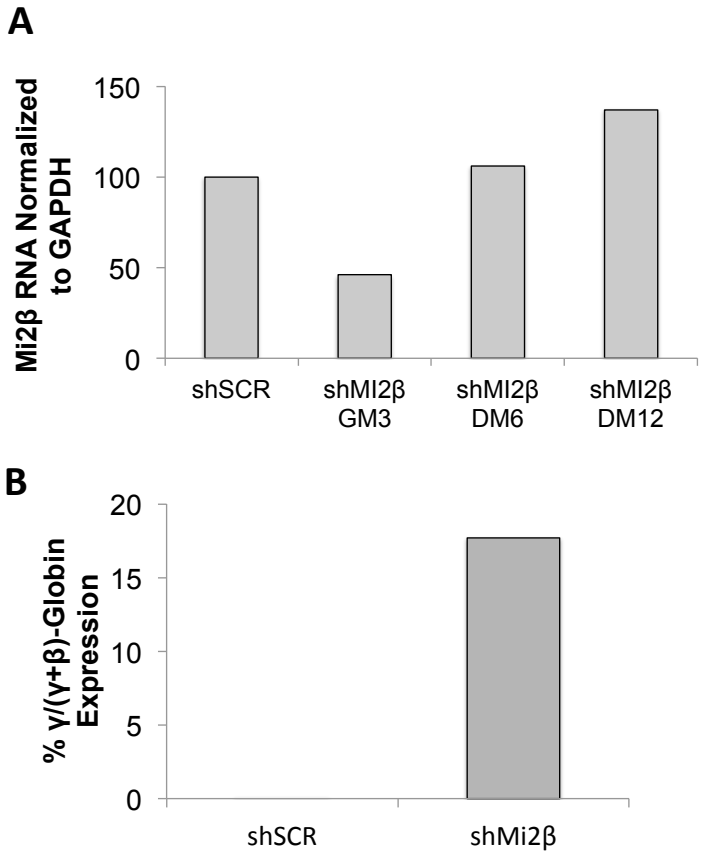

Figure 30. The silencing effect of Mi2β on γ-globin gene regulation occurs during earlier stages of erythroid differentiation. (A). qPCR showing ~50% knockdown of Mi2β in CD34+ cells at day 3 of growth medium (GM3), but higher level of Mi2β expression at days 6 and 12 of differentiation (DM6 and DM12 respectively). Each value was normalized to shSCR controls on each of the days the cells were tested. (B) qPCR showing ~17% γ/(γ+β)-globin mRNA expression in shMi2β knockdown samples at day 12 of differentiation, compared to 0.01% γ/(γ+β)-globin expression in shSCR sample control.

C. Mi2β affects the levels of two important transcription factors involved in silencing embryonic and fetal β-type globin gene expression, BCL11A and KLF1/EKLF

To further investigate the mechanism(s) through which Mi2β/CHD4 exerts such a large effect on γ-globin gene silencing, we studied its effect on KLF1/EKLF and BCL11A, two important regulators of γ-globin gene silencing. Knockdown of Mi2β in CID cells decreases both KLF1/EKLF and BCL11A protein levels as shown by western blot (Figure 31A). Consistent with the hypothesis that the silencing effect of Mi2β is at least partially independent from the MBD2/NuRD complex, loss of MBD2 does not result in any decrease of KLF1 or BCL11A, in either CID cells (Figure 31B), or in primary erythroid cells of βYAC containing MBD2 knockout mice (Figure 31C). In fact, cells with MBD2 knockdown or knockout appear to express slightly increased levels of BCL11A and KLF1 (Figures 31B and 31C).

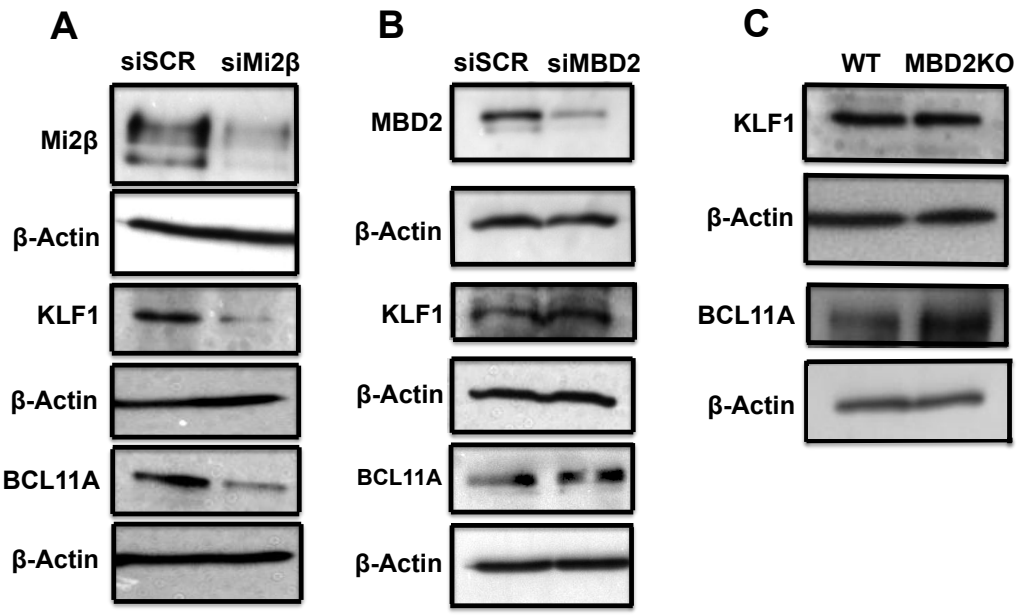

Figure 31. Mi2β positively regulates the expression of KLF1 and BCL11A in CID cells. (A) Western blot showing a decrease in murine KLF1 and murine BCL11A protein levels after Mi2β knockdown in CID cells. (B) Western blot showing no change in murine KLF1 and murine BCL11A protein levels after MBD2 knockdown in CID cells. (C) Western blot showing no change in murine KLF1 or murine BCL11A in primary adult mouse erythroblasts from MBD2 knockout mice.

The effect of Mi2β knockdown on BCL11A and KLF1 expression was also observed in CD34+ human hematopoietic progenitor derived erythroid cells, where Mi2β knockdown downregulates both BCL11A and KLF1 mRNA and protein levels (Figure 32A & 32B respectively). Likewise, MBD2 knockdown does not decrease expression of either KLF1 or BCL11A in human primary erythroid cells (Figure 32C). Interestingly, these results suggest that Mi2β acts as a positive regulator of the BCL11A and KLF1 genes in contrast to its negative regulatory role as part of the MBD2/NuRD complex (Gananapragasam et al. 2011).

In order to investigate whether or not Mi2β directly activates the BCL11A and/or KLF1 genes through interactions at their promoter regions, we carried out chromatin immunoprecipitation (ChIP) assays to examine occupancy of Mi2β in their proximal promoter regions. As shown in Figure 33, Mi2β is significantly enriched at the proximal promoter region of both the BCL11A and KLF1 genes in human primary erythroid cells. Thus Mi2β appears to interact directly with and induce expression of both the BCL11A and KLF1 genes.

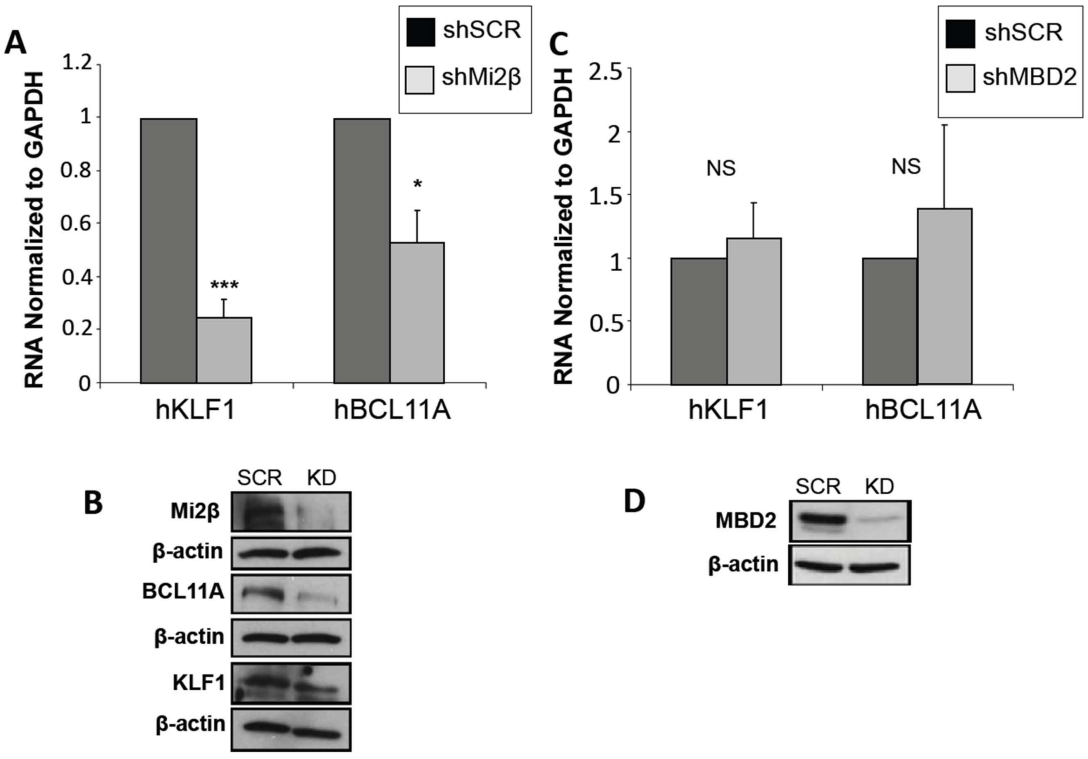

Figure 32. Mi2β positively regulates the expression of KLF1 and BCL11A in human CD34+ hematopoietic progenitor derived primary erythroid cells.
(A) qPCR analysis showing mRNA levels of KLF1 and BCL11A following Mi2β knockdown are decreased by 70% and 40% respectively. (B) Western blot showing a decrease in the levels of BCL11A and KLF1 protein upon Mi2β knockdown in human primary erythroid cells. (C) The mRNA levels of KLF1 and BCL11A following MBD2 knockdown are not affected. (D) Western blot showing the level of MBD2 protein knockdown in human primary erythroid cells. Error bars represent the standard deviation of three or more independent experiments. *signifies $p < 0.05$ and **signifies $p < 0.02$ and *** signifies $p < 0.001$ according to the students t-test.

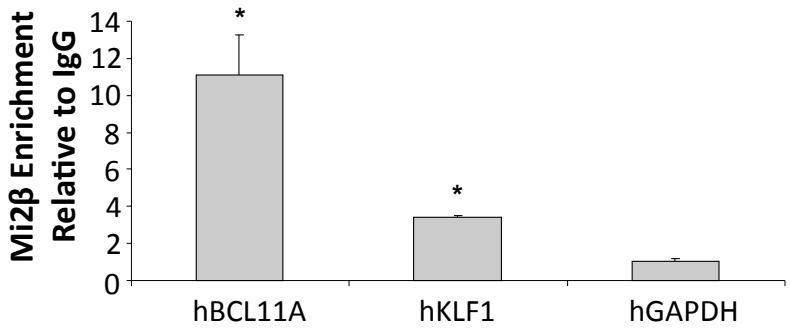

Figure 33. Mi2β binds to BCL11A and KLF1 promoter regions in human primary erythroid cells. Chromatin immunoprecipitation assay and subsequent qPCR showing significant enrichment in the BCL11A and KLF1 promoter/exon 1 regions after 2 days of differentiation of primary human erythroid cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a negative control and enrichment values are normalized to IgG. Error bars represent the standard deviation of at least three independent experiments. *signifies $p<0.05$.

In order to confirm Mi2β's effect on γ-globin expression is partially through activation of KLF1 expression, we co-transfected CID cells with siMi2β and a KLF1 expression vector. A 3-fold over-expression of KLF1 24 hours post-transfection (Figure 34A) is able to diminish the increase in γ-globin gene expression in siMi2β treated samples at 72hours post-transfection compared to samples treated with siMi2β alone (Figure 34B). This confirms Mi2β acts, at least partially, by activating KLF1.

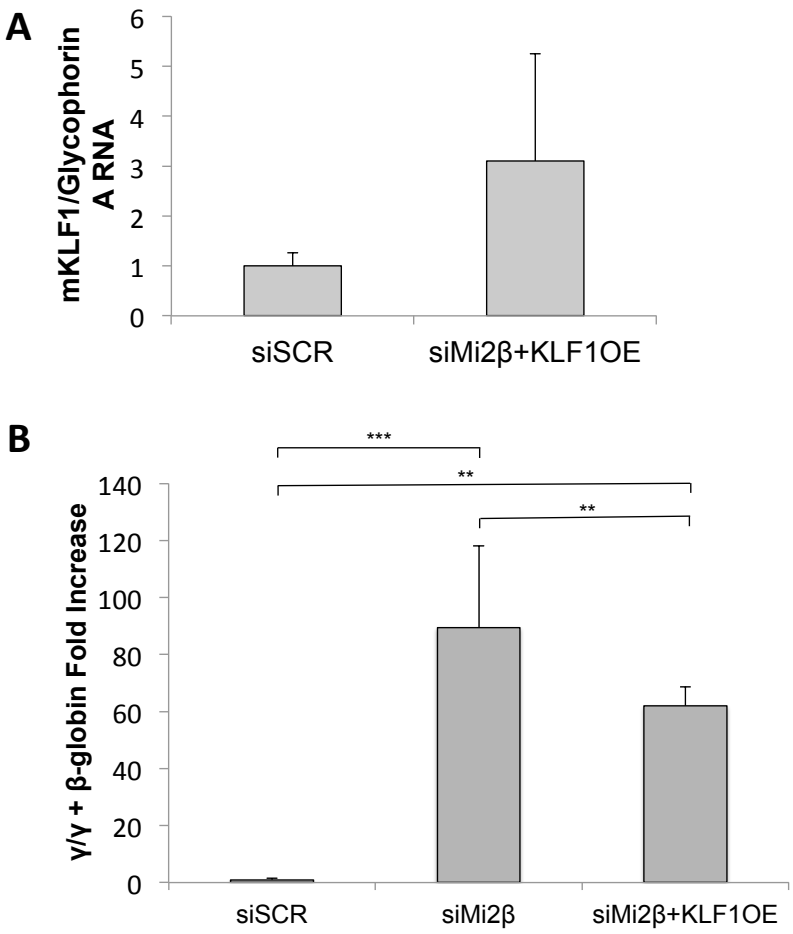

Figure 34. KLF1 overexpression significantly decreases γ/γ+β-globin gene expression after Mi2β knockdown in CID cells. (A) qPCR results showing that KLF1 RNA is expressed ~3-fold higher in samples transfected with Mi2β siRNA and KLF1 expression vector (siMi2β + KLF1 OE) than scramble control cells (siSCR) at 24 hours post-transfection. (B) qPCR data showing that forced KLF1 expression in Mi2β knockdown cells leads to a significant decrease in γ/(γ+β)-globin expression compared to Mi2β knockdown alone at 72 hours (62-fold induction vs. 89-fold induction). Error bars represent the standard deviation of three independent experiments. **signifies $p < 0.02$, and ***signifies $p < 0.001$ according to the student's t-test.

D. Mi2β affects γ-globin gene expression in a manner largely independent of the FOG-1/GATA-1/NuRD complex

Since GATA-1 and FOG-1 occupy the distal promoter region of the γ-globin gene and play a repressive role on its expression through the NuRD complex in βYAC mice (Harju-Baker et al. 2008), we explored the extent to which Mi2β acts through the FOG-1/GATA-1/NuRD complex to exert its silencing effect on the γ-globin gene. Chromatin immunoprecipitation assays in CID cells showed that Mi2β occupies the γ-globin gene promoter region in CID cells as shown in Figure 35A, consistent with reported results in βYAC transgenic mice (Bottardi et al. 2009). To determine the relative extent to which the silencing effect of Mi2β is mediated through the FOG-1/GATA-1/NuRD complex, FOG-1 was knocked down in CID cells. In contrast to the effect of Mi2β knockdown, this resulted in only a very small (~3-fold) increase in γ-globin gene expression (Compare Figure 35B to Figure 23A). This suggests that the strong silencing effect of Mi2β is mediated only in small part by the FOG-1/GATA-1/NuRD complex. In order to determine whether knockdown of Mi2β results in an additive or synergistic disruption of some type of cooperative effect mediated by interaction between the MBD2/NuRD and GATA-1/FOG-1/NuRD complexes, we simultaneously knocked down MBD2 and FOG-1 in CID cells. Combined knockdown of these two proteins resulted in a ~7-fold induction of γ-globin mRNA, which is much lower than with Mi2β knockdown and only slightly different than the effect of MBD2 knockdown alone (Compare Figure 35C to Figure 23B).

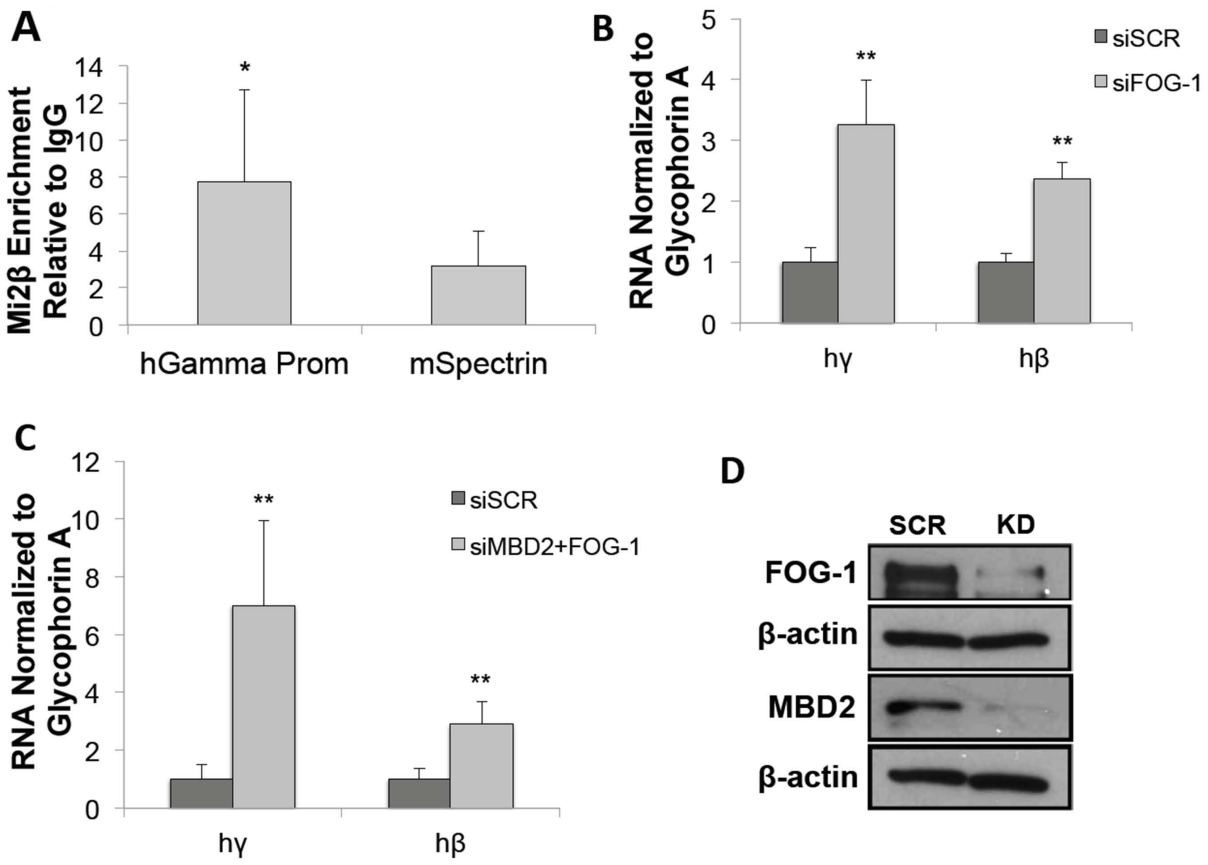

Figure 35. Mi2β occupies the γ-globin gene promoter and acts in a partially independent manner from GATA-1/FOG-1/NuRD.
(A) ChIP assays showing significant Mi2β enrichment at the γ-globin promoter region in CID cells. The mouse α-spectrin gene was used as a negative control. (B) qPCR results showing knockdown of FOG-1 leads to a ~3-fold induction of the human γ- globin (hγ) and a ~2.5-fold induction of the human β-globin (hβ) gene. (C) Simultaneous knockdown of MBD2 and FOG-1 leads to a ~7-fold induction of γ-globin and a ~3-fold induction of β-globin. (D) Western blot showing both FOG-1 and MBD2 knockdown in CID cells. Error bars represent the standard deviation of three or more experiments. *signifies $p < 0.05$ and **signifies $p < 0.02$ according to the students t-test.

E. Mi2β affects γ-globin gene expression independently of other MBD3/NuRD complexes

Since Mi2β is part of both MBD2- and MBD3/NuRD complexes and it acts in part through the GATA-1/FOG-1/NuRD complex to repress γ-globin expression, we tested the hypothesis that Mi2β could be acting partially through an MBD3/NuRD complex. However, significant knockdown of MBD3 in CID cells (Figure 36A, shown by MBD3 mRNA levels due to a lack of an MBD3-specific antibody) shows no change in γ-globin expression and a slight but significant increase in β-globin expression (Figure 36B). MBD3 knockdown also leads to no change on KLF1 and BCL11A protein levels in contrast to Mi2β knockdown (Figure 36C). This suggests the effect of Mi2β on these transcription factors is independent of both the MBD2- and MBD3/NuRD complexes (Figure 36C). Simultaneous knockdown of both MBD2 and MBD3 show a significant increase in both γ-globin as well as β-globin gene expression in a similar fashion than MBD2 knockdown alone (compare Figure 36D to Figure 23B). These data, along with previous studies (Gnanapragasam et al. 2011), suggests MBD3 does not have an effect on γ-globin expression, and Mi2β's silencing of γ-globin is largely independent of the MBD3/NuRD complex.

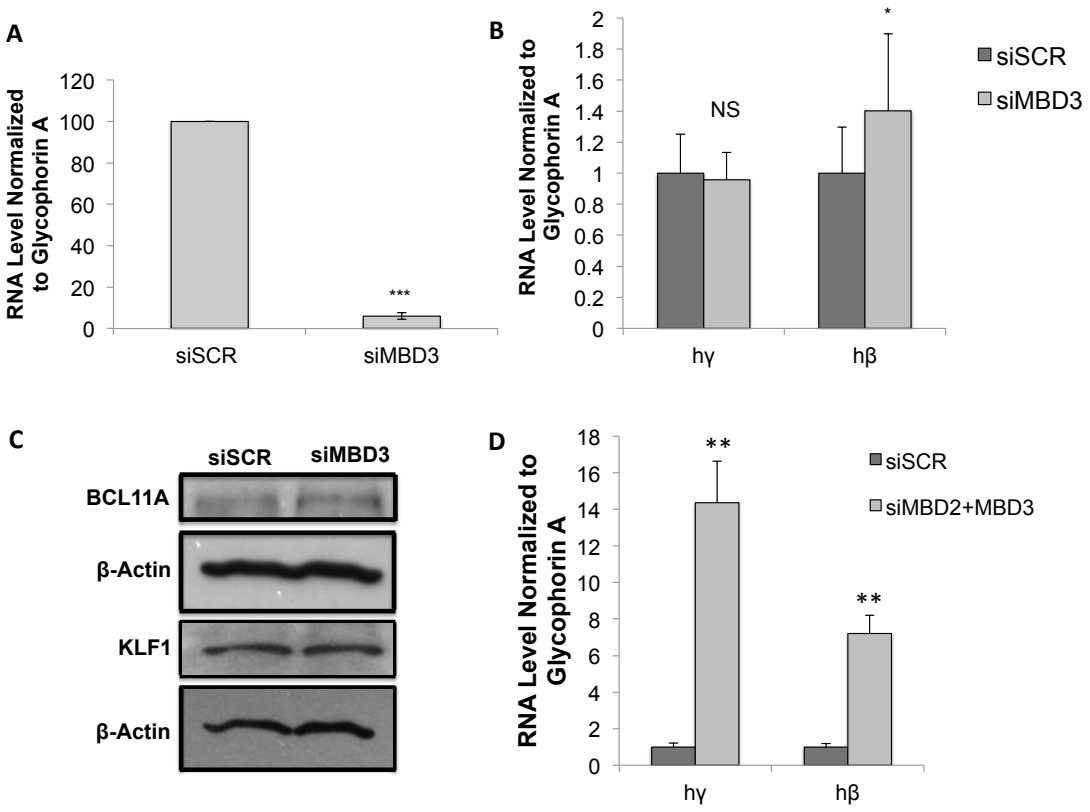

Figure 36. Mi2β acts independently of the MBD3/NuRD complex to exert its effect in γ-globin gene expression.
(A) qPCR showing ~90% MBD3 knockdown in CID cells 24 hours after siRNA transfection. (B) qPCR showing knockdown of MBD3 does not affect the expression of γ-globin but leads to a slight increase in the expression of β-globin gene. (C) Western blots showing no change in BCL11A and KLF1 protein levels following MBD3 knockdown in CID cells. (D) qPCR showing simultaneous knockdown of MBD2 and MBD3 leads to ~14-fold induction of γ-globin gene expression and ~7-fold induction of β-globin expression.

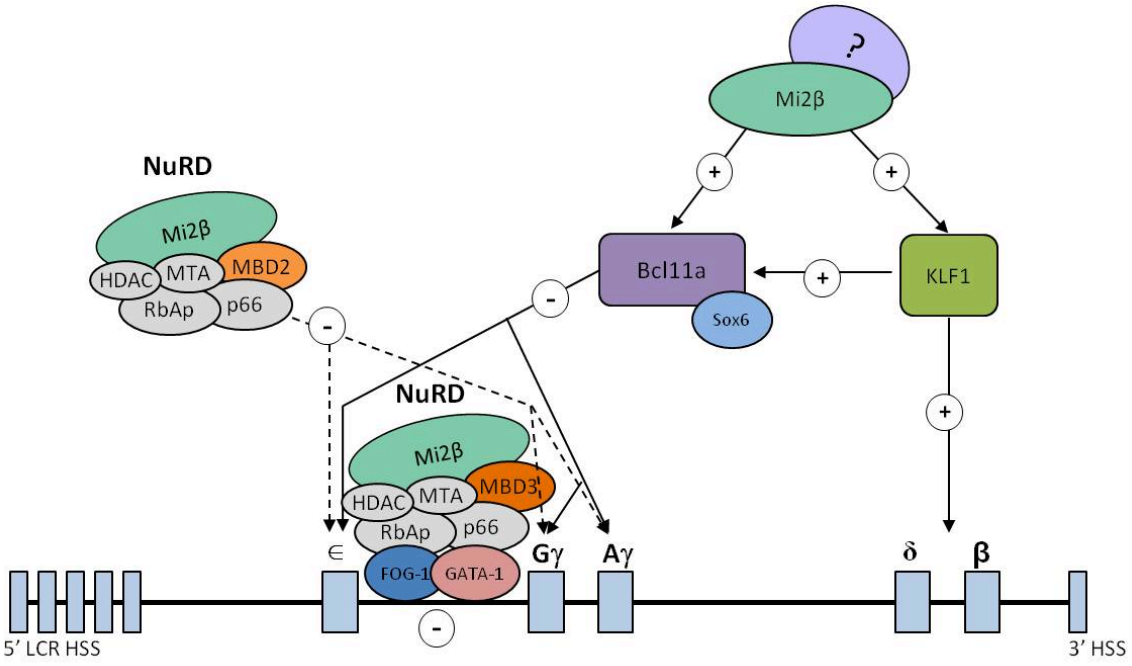

Figure 37. Working model of Mi2β-mediated developmental globin gene silencing through multiple mechanisms. Mi2β is a critical component of the MBD2/NuRD complex which regulates developmental globin gene silencing independently of BCL11A and KLF1-EKLF in an indirect manner. Mi2β binds to the distal promoter region of the γ-globin gene as part of the MBD3/NuRD/GATA-1/FOG-1 silencing complex. Mi2β binds to and activates expression of BCL11A and KLF1/EKLF, which in turn silence γ-globin gene expression. Solid arrows represent direct interactions and dotted arrows represent indirect interactions

IV. Discussion

The developmental switch from the fetal γ-globin expression, to the adult β-globin gene expression, is regulated by multiple factors including DNA methylation, transcription factors such as KLF1, BCL11A, GATA-1, TR2/TR4 (Sankaran, Xu & Orkin 2010; Ginder, Gnanapragasam & Mian 2008), and epigenetic modifiers such as the MBD2/NuRD complex (Kransdorf et al. 2006; Rupon et al. 2006).

As one of the major components of the MBD2/NuRD complex, Mi2β plays a critical functional role in this repression (Gnanapragasam et al. 2011). Disruption of the interaction between the MBD2 and p66α coiled coil domains results in a displacement of both p66α and Mi2β from the NuRD complex and leads to a significant de-repression of the γ-globin gene in CID cells (Gnanapragasam et al. 2011). In this study, we have found a novel role for Mi2β, which appears to be independent of its function in MBD2/NuRD and MBD3/NuRD complexes. We observed that knockdown of Mi2β has a greater effect in de-repressing γ-globin gene expression than does knockdown of MBD2 and MBD3. Although the GATA-1/FOG-1/NuRD complex is not essential for silencing human γ-globin expression in transgenic βYAC bearing adult erythroid cells (Miccio & Blobel 2010), it contributes to the silencing of the γ-globin gene through a direct interaction of GATA-1 in the distal promoter region (Harju-Baker et al. 2008). Here we show that knockdown of FOG-1 in CID cells induces a small increase in γ-globin gene expression, in contrast to Mi2β knockdown. Further, combined MBD2 and FOG-1 knockdown results in much less γ-globin gene induction than Mi2β knockdown alone. Given that knockdown of MBD3 in CID cells does not lead to an increase in γ-globin gene expression (Figure 36B), a major part of the silencing effect of Mi2β appears to be independent of both the MBD2/NuRD,

and MBD3/NuRD complexes.

In this report we show that knockdown of Mi2β results in down regulation of both BCL11A and KLF1/EKLF, which exert strong γ-globin gene silencing effects in transgenic βYAC mice and primary human erythroid cells (Lee et al. 2000; Sankaran et al. 2008; Sankaran et al. 2009; Xu et al. 2010; Zhou et al. 2010; Borg et al. 2010; Xu et al. 2011). The similar effect of Mi2β knockdown in both murine CID cells and human primary erythroid cells shows that Mi2β acts as an activator of the BCL11A and KLF1 genes in both murine and human hematopoietic cells. As MBD2 knockdown results in slightly increased levels of BCL11A expression, and MBD3 knockdown results in no change in either of these transcription factors, these results confirm that Mi2β regulates the BCL11A and KLF1/EKLF genes independently of the MBD2/NuRD and MBD3/NuRD complexes. Mi2β occupies the proximal promoter regions of both BCL11A and KLF1, a result consistent with a direct positive regulatory effect.

Mi2β's silencing effect in γ-globin regulation appears to be critical during the early stages of CD34+ cell differentiation, since loss of Mi2β knockdown during the first 6 days of culture allows a significant increase of γ-globin expression at day 12 of differentiation. These data are consistent with the pattern of expression seen with BCL11A and KLF1, where the level of expression of these two genes begins to increase during the early stages of differentiation (Xu et al. 2010; Siatecka & Bieker 2011). Mi2β occupies the promoter regions of BCL11A and KLF1 at day 2 of erythroid differentiation, and therefore low expression or absence of Mi2β during the beginning stages of erythroid differentiation most likely diminishes the activating role of Mi2β on BCL11A and KLF1, resulting in an increase in γ-globin expression. Other mechanisms of action cannot be ruled out.

The concept of Mi2β as a gene activator is supported by previous reports for the CD4 gene in T-cells (Williams et al. 2004), T helper 2 cell identity (Hosokawa et al. 2013) and the adult β-globin gene in mice (Miccio & Blobel 2010). We have shown previously (Gnanapragasam et al. 2011) as well as in this report (Figure 36B) that a partial knockdown of MBD3 in CID cells has little effect on the expression of the γ-globin gene (Gnanapragasam et al. 2011). Therefore, it seems unlikely that the role of Mi2β as an activator of the BCL11A and KLF1/EKLF genes is mediated through the GATA-1/FOG-1/NuRD or the MBD3/NuRD complex.

How Mi2β switches its function from a repressor to an activator remains to be determined. One possibility lies on post-translational modifications of Mi2β or its binding partners. In drosophila, the Mi2β's homologue (dMi2) is known to be a phosphoprotein in which the ATPase activity is regulated via phosphorylation (Bouazoune & Bhrem 2005). In mice, other transcription factors involved in globin gene regulation undergo post-translational modifications allowing them to switch from transcriptional activators to repressors (Bierker 2001).

Interestingly, in addition to a marked increase in γ-globin and ε-globin expression after Mi2β knockdown in CID cells, a small increase in β-globin RNA was observed, but expression of no other tested erythroid-specific genes was increased (Figures 23E and 24C). Together these results suggest that the silencing effect of Mi2β on erythroid-specific genes in CID cells may be restricted to the β-globin locus. In contrast to the results in CID cells, we observed a decrease in β-globin gene expression in primary human erythroid cells with ~50% Mi2β knockdown, consistent with the predicted effect of decreased KLF1 expression.

Based on the results presented in this report in conjunction with the literature, we propose a testable working model in which Mi2β acts through multiple pathways to silence γ-globin gene

expression, as shown in Figure 37. Mi2β is an important component of the MBD2/NuRD complex which acts as a repressor of γ-globin expression in adult erythroid cells. It also is a component of the GATA-1/FOG-1/MBD3/NuRD complex which binds upstream of the γ-globin promoter region and exerts a negative regulatory effect. A third and previously unreported function of Mi2β described here is through its positive regulation of the KLF1 and BCL11A genes. In murine erythroid cells, the latter appears to be responsible for the great majority of the silencing effect of Mi2β while in the primary human erythroid model this effect is less pronounced. An intriguing observation is that knockdown of Mi2β by less than 50% in primary erythroid cells exerts a large effect on γ-globin gene expression. This suggests that full γ-globin gene silencing is dependent on maintaining a tightly controlled level of Mi2β.

There are a large number of potential molecular targets for therapeutically increasing fetal hemoglobin levels in patients with β-globin gene disorders. In this report we focus on the chromatin remodeling complex component, Mi2β, as a potential target. Chromatin remodeling complexes could prove to be good targets for therapeutic induction of fetal hemoglobin expression because even partial disruption of a complex component could affect expression of multiple genes that are involved in γ-globin gene silencing. Indeed, Mi2β appears to silence γ-globin gene expression through multiple pathways and, as shown in this report, its partial depletion relieves silencing in human erythroid cells. Complete depletion of Mi2β might have catastrophic consequences, through blocking erythroid differentiation, as shown in conditional knockout mice (Yoshida et al. 2008). Knockdown of Mi2β in human primary erythroid cells at times showed a loss of knockdown as differentiation proceeded. A possible reason behind this finding is that cells with higher levels of Mi2β knockdown are detrimental for the cells, and therefore cells develop ways to silence the lentiviral vector expression and regulate Mi2β

expression. Consistent with a partial Mi2β knockdown sparing a block in erythroid differentiation, a very recent report showed a partial Mi2β knockout in βYAC transgenic mice resulted in increased γ-globin gene expression without adversely affecting erythropoiesis (Costa et al. 2012). Since Mi2β exerts its chromatin remodeling and helicase functions through its ATPase enzymatic activity, targeting its ATPase function is certainly appealing. Small molecules capable of targeting the ATPase domain of Mi2β are a possible approach. Other possible targets are Mi2β's tPHD domains, which have been shown to be vital to the function of the ATPase activity of this protein (Morra et al. 2012; Watson et al. 2012). As shown in Figure 22, disrupting the interaction of the PHD domains with histone 3 tails could prove to be a successful target.

In summary, identifying specific epigenetic mechanisms of γ-globin gene silencing as potential therapeutic targets seems promising. Indeed DNA methylation inhibitors and histone deacetylase inhibitors induce embryonic/fetal globin gene expression and have shown clinical efficacy (DeSimone et al. 1982; Ley et al. 1982; Ginder, Whitters & Pohlman 1984; Perrine et al. 1993). We have extended previous work that identified the chromatin remodeling protein, Mi2β, as an important factor in developmental β-type globin gene silencing through its role in NuRD complexes. Importantly, we show here that a major mechanism for its silencing effect is through a direct positive regulation of KLF1/EKLF and BCL11A. The striking finding that as little as 50% reduction in Mi2β expression results in a large increase in γ-globin gene expression in primary human erythroid cells suggests that it may serve as a useful molecular target for therapeutic induction of HbF in patients with β-globin gene disorders.

Chapter 4: Summary, Future Directions and Perspectives

Sickle cell anemia and β-thalassemia are among the most common single gene disorders worldwide. Sickle cell anemia is characterized by abnormally "sickle" shaped red blood cells, which cause vaso-occlusion and ischemia in small vessels leading to painful crises. Auto infarction and necrosis of the spleen, kidney failure, strokes and acute chest syndrome are among possible complications (Nathan et al. 2003). Hydroxyurea is the standard treatment for sickle cell anemia but it has highly variable effects on patients (Charache, et al 1992; Steinberg et al. 1997; Ware et al. 2002). The most severe form of β-thalassemia (β-thalassemia major) is caused by a complete absence of expression of the β-globin gene and it is characterized by severe anemia. The standard treatment for patients with β-thalassemia major is life-long transfusions. Iron accumulation due to transfusions results in iron deposit in several organs, leading to damage (Rund & Rachmilewitz 2005). Sickle cell anemia and β-thalassemia benefit from an increase in fetal hemoglobin, which is composed of two fetal γ-globin and two α-globin chains. Understanding the molecular mechanisms behind the silencing of γ-globin gene expression will therefore prove to be important when attempting to develop new therapies for these conditions.

Advances in the field have led to a better understanding of the regulatory functions leading to γ-globin gene silencing. Among the many regulatory mechanisms leading to this process, DNA methylation is known to play a role in the regulation of embryonic and fetal

globin gene expression (Mavilio et al. 1983; McGhee & Ginder 1979; Shen & Maniatis 1980; van der Ploeg & Flavell 1980; DeSimone et al. 1982; Charache et al. 1983; Ley et al. 1982). The methyl-CpG-binding domain protein 2 (MBD2), along with its remodeling complex (NuRD) binds to and negatively regulates the ρ-globin gene in avian systems, and it indirectly regulates the expression of γ-globin gene in humans (Singal et al. 2002; Kransdorf et al. 2006; Rupon et al. 2006; Gnanapragasam et al. 2011). Adult MBD2 knockout mice crossed bearing the human β-locus (βYAC) exhibit a significant increase in γ-globin gene expression (Rupon et al. 2006). Among other important factors that regulate the expression of globin genes, KLF1 is a critical regulator of the β-locus. KLF1 binds to, and positively regulates the expression of β-globin gene as well as BCL11A (Zhou et al. 2010; Borg et al. 2010). BCL11A, a transcription factor known to bind to the β-globin locus, plays a significant role in the regulation of γ-globin gene expression (Sankaran et al. 2008; Sankaran et al. 2009; Xu et al. 2010). BCL11A knockout mice show a delay in the γ- to β-globin switch (Sankaran et al. 2009), and crossing BCL11A KO mice with sickle cell mice results in a drastic amelioration of the sickle cell disease phenotype (Xu et al. 2011). The delay in γ-globin gene silencing seen in the BCL11A knockout mice early in life, however, is not fully maintained through adulthood (Esteghama et al. 2013). This suggests BCL11A plays a more significant role during the embryonic switch in mice, but further layers of repression may play a role in silencing γ-globin expression throughout adulthood.

In the studies presented in Chapter 2, we investigated whether targeting MBD2 *in vivo* could lead to amelioration of the sickle cell anemia phenotype in mice. Knocking out MBD2 in a sickle cell anemia mouse model showed promising results by significantly increasing survival compared to sickle cell disease (SCD) control mice. Blood smears of SCD mice crossed with MBD2ko mice also showed a lower number of sickle cells present in the peripheral blood.

Despite an increase in survival, some of the phenotype typical of sickle cell anemia including splenomegaly, liver and kidney damage were still present in SCDMBD2-/- mice. Perhaps the greatest disadvantage in using the BERK sickle cell mouse model is their low fetal hemoglobin base line (less than 1%), which is significantly less than in adult humans with sickle cell anemia (2-8%). A greater level of induction in γ-globin gene expression is therefore needed in order to fully correct the symptoms of these mice. SCD mice also show a more severe phenotype compared to sickle cell disease patients including greater liver necrosis and higher levels of hemolytic anemia. In order to overcome this challenge, we will first determine whether genetic modifiers play a role in the expression of γ-globin gene. The MBD2 KO mouse model used in our laboratory is on a FVB/C57BL/6 BALB/C mixed genetic background. Because other studies have reported higher baseline levels of γ-globin gene expression on BERK mice under a C57BL/6 background (Xu et al, 2011), our approach will be to back-cross our mice at least three generations into a ~90% C57BL/6 genetic background. Following the backcrosses, we will measure HbF and harvest organs in order to determine whether there is a more drastic improvement on the pathology of these mice. On the event that the genetic background does not affect γ-globin gene expression, a different mouse model will be considered (Ryan et al. 1997).

Once in a pure genetic background, the role of MBD2 on endothelial nitric oxide synthase (eNOS) will be investigated in BERK mice. MBD2 binds to, and negatively regulates the expression of the eNOS gene. Absence of MBD2 in mice has been shown to confer protection from hind-limb ischemic injury (Rao et al. 2011). Endothelial cells from large vessels of SCDMBD2+/+ and SCDMBD2-/- mice will be tested for eNOS levels by immunohistochemistry. Microcirculation studies will also be carried out to compare sickling and blood flow in the two groups of mice. The effects of Mi2β on SCD mice will also be explored. In

chapter 3, we have shown that ~50% Mi2β knockdown causes a significant increase in HbF production without disrupting erythroid differentiation of human primary erythroid cells. In order to determine whether Mi2β haploinsufficiency is able to ameliorate the symptoms of sickle cell anemia, mice heterozygous for Mi2β will be crossed with BERK SCD mice. Similar experiments will be conducted with this mouse model as shown in Chapter 2.

Given the limitations of the transgenic mouse models discussed in the introduction of this thesis, there is a need for whole animal models in order to study human erythropoiesis. An interesting study will therefore be to investigate the effects of knocking down MBD2 and/or Mi2β in a "humanized" mouse model of hematopoiesis. Previous studies have shown that stromal cells appear to regulate survival, migration and differentiation of hematopoietic stems cells via transcription factors and cell cycle regulators (Dazzi et al. 2006). Osteoblasts in the bone marrow also appear to play a role in hematopoiesis (Wilson & Trumpp 2006). This is supported by the observation that CD34+ cells are not viable after differentiation in culture. A more humanized *in vivo* model will allow us to determine whether the bone marrow environment affects gene expression pattern and viability of these cells. This will be accomplished by the engraftment of human hematopoietic progenitor cells treated with shMBD2 or shMi2β in the bone marrow of immunocompromised mice. A tetracycline-inducible shRNA system will also be used to control knockdown levels. The mouse strain NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, available in the Jackson Laboratories, has a severe combined immune deficiency mutation (*scid*) and show greater engraftment of human hematopoietic stem cells compared to other strains previously studied. Aside from gene expression analysis to determine the effect of knocking down NuRD complex components on γ-globin expression, we will also be able to determine any detrimental effects of targeting NuRD complex components on the bone marrow *in vivo*. Prior studies with

MBD2-/- mice have shown changes in Th1 and Th2 gene regulation (Hutchins et al. 2002), and ablation of Mi2β hinders proper myeloid differentiation of mouse bone marrow cells (Yoshida et al. 2008). In order to determine whether targeting MBD2 or Mi2β has detrimental effects in bone marrow cells, myeloid markers will be used to monitor proper myeloid differentiation.

Further studies with the NOD/SCID mouse model will be conducted. CD34+ cells from sickle cell anemia patients will be used instead of healthy donors in order to knockdown MBD2 or Mi2β and engraft them in NOD/SCID mice as described above. These experiments will allow us to understand better whether targeting these proteins will alleviate the pathophysiologic effects of sickle cell anemia in human cells *in vivo*.

In the studies presented in Chapter 3 we discovered a novel role for Mi2β, the chromatin remodeling protein of the NuRD complex. In addition to its role as a negative regulator of γ-globin gene expression through the MBD2/NuRD complex, Mi2β also acts as an activator of BCL11A and KLF1, further contributing to the silencing effect on the γ-globin gene. It will be important to confirm that Mi2β exerts its effect on γ-globin largely through its regulation of KLF1 and BCL11A. In order to accomplish this, experiments with forced expression of BCL11A and KLF1 in siMi2β-treated cells will be carried out to determine if the substantial increase in γ-globin gene expression is diminished. Transient forced expression of KLF1 in siMi2β-treated CID cells results in a partial decrease in γ-globin gene expression when compared to siMi2β treated CID cells (Figure 34). Further experiments must be carried out with forced expression of both KLF1 and BCL11A in CID cells as well as in human primary erythroid cells. In particular, stable expression systems must be used in human primary erythroid cells in order to determine the effect of over expressing KLF1 and BCL11A in shMi2β-treated cells. It is expected that KLF1 and BCL11A mediate a large portion of the effect carried out by the knockdown of Mi2β,

although the involvement of other factors in this process cannot be ruled out. In the event that forced expression of KLF1 and BCL11A in a Mi2β knockdown setting does not ablate Mi2β's effect on γ-globin expression, the role of other factors acting through Mi2β will be investigated. In order to do this, a microarray comparing WT primary human erythroid cells and shMi2β treated cells will give rise to potential candidates. Erythroid-specific candidates will then be investigated and validated.

The mechanism by which Mi2β acts as an activator rather than a repressor remains to be determined. A plausible hypothesis is that the majority of Mi2β remains tightly bound to NuRD complexes. A minor pool of free-floating Mi2β is able to interact with other proteins or co-factors that allow Mi2β to act as an activator. This idea is supported by experiments conducted in CID cells showing that Mi2β knockdown does not affect the composition of the NuRD complex (Figure 26). To further support this hypothesis, we will perform ChIP assays of other NuRD complex components on the γ-globin or Ugt8 (a known target of the MBD2/NuRD complex) promoter regions after Mi2β knockdown. This will allow us to determine if partial absence of Mi2β affects other NuRD complex components' ability to bind to these genes.

Post-translational modifications could also play a role in allowing Mi2β to change from a repressor to an activator. In humans, Mi2β is known to be phosphorylated at Ser-1349 by the kinase Ataxia telangiectasia mutated (ATM) following double strand breaks (Urquhart et al. 2011). In drosophila, dMi-2 has been shown to be a phosphoprotein, and its ATPase and nucleosome remodeling activity are enhanced when phosphorylated. In order to test whether post-translational modifications play a role in Mi2β's activating/repressing role, we will first look for potential post-translational modification consensus sites. In the case of phosphorylation, kinase inhibitors could be used to block phosphorylation. Binding of Mi2β to the γ-globin

promoter region (repressive role) as well as binding of Mi2β to BCL11A/KLF1 (activating role) could then be measured to determine if binding affinity is affected. γ-globin and BCL11A/KLF1 gene expression can also be measured following the kinase inhibitor treatment to determine potential changes. Ultimately, mapping other post-translational modifications could be achieved by mass spectrometry.

Further studies will be carried out to investigate whether Mi2β interacts with other partners in order to exert its effect as an activator. Recent studies have shown Mi2β interacts with p300 and GATA-3 in Th2 T helper cells when acting as a transcriptional activator, suggesting its ability to form complexes independently of the NuRD complex (Hokosawa et al. 2013). In order to determine whether or not Mi2β interacts with other NuRD complex components while acting as an activator, ChIP assays will be conducted to test if other NuRD complex components show enrichment in the BCL11A and/or KLF1 promoter regions. In the event that no other NuRD components are present supporting the view of Mi2β acting independently of the NuRD complex in its role as an activator, we will search for other potential Mi2β binding factors. We will first conduct ChIP assays to determine enrichment of GATA factors and p300 in the BCLL1A and KLF1 promoter regions since they are known to interact with Mi2β in other systems. In order to investigate whether other factors also interact with Mi2β, Mi2β will be tagged in order to conduct a tandem affinity purification (TAP) assay. Since Mi2β interacts with the MBD2- and MBD3-NuRD complex, the proteins belonging to these complexes will need to be pre-cleared by conducting consecutive immunoprecipitation reactions. The purification product will then be subjected to mass spectrometry in order to identify potential partners of Mi2β.

In order to determine whether targeting Mi2β induces γ-globin gene expression *in vivo*, we will screen for small molecules that will inhibit the ATPase catalytic domain of Mi2β. In order to do this, the ATPase domain must be cloned in an expression vector and the expressed domain must be purified. High throughput screening will then be carried out in which molecules with favorable chemical properties such as solubility, decreased toxicity and stability are tested. Potential small molecule candidates will be tested *in vitro* in human CD34+ hematopoietic progenitor cells for effective inhibition of Mi2β. In the event we find a candidate(s) that effectively inhibits Mi2β, leading to an increase in γ-globin expression without disrupting erythroid differentiation in human primary erythoid cells, these molecules will be further tested in sickle cell disease mice.

Perspectives:

Due to the need for more effective treatments for hemoglobinopathies such as sickle cell anemia and β-thalassemia, there has been a long-standing search for understanding the molecular mechanisms behind the fetal γ-globin gene silencing. The main challenges in finding a target to successfully de-repress γ-globin expression in humans are 1) the complexity of how this locus is regulated, in which many factors play a combinatorial effect on gene expression 2) finding potential targets that will not have negative systemic effects 3) finding "targetable" proteins that will provide easier ways for drug development and delivery.

The first challenge when attempting to find ways to relieve γ-globin silencing is the complexity of its regulation. As reviewed in the introduction of this thesis, many layers of repression lead to successful silencing of γ-globin gene expression. As it appears, many

regulatory factors contribute to an overall global effect on the regulation of γ-globin gene expression. In fact, individually knocking down several of these factors in human primary erythroid cells appears to de-repress γ-globin gene expression in a similar manner (Xu et al. 2013). Finding a link between different branches of regulation is therefore imperative in order to find a target that will have a therapeutic effect of a larger magnitude. Epigenetic mechanisms offer the advantage of having broader effects, since epigenetic factors and their chromatin remodeling functions regulate many genes. Our studies emphasize the role of the MBD2/NuRD complex, which indirectly regulates γ-globin gene expression. Mi2β, a major component of the NuRD complex contributes to the β-locus gene regulation through multiple pathways, including its repressive functions via the GATA-1/FOG-1/MBD3/NuRD complex, the MBD2/NuRD complex, its association with BCL11A, as well as its activating role in BCL11A and KLF1 gene expression (shown in Figure 12). The role of Mi2β on γ-globin regulation through different mechanisms makes this molecule a very promising therapeutic target.

The second challenge in finding potential therapeutic targets for β-globin disorders is avoiding systemic detrimental effects. Two important transcription factors, BCL11A and KLF1, are thought to play a particularly important role in the regulation of γ-globin expression. BCL11A was shown to have a rather significant role in γ-globin gene silencing during mouse embryogenesis (Sankaran et al. 2009). However, BCL11A is also an important factor in other tissues and in regulating B-cell function (Liu et al. 2003). KLF1 is also known to play a significant role in both β- and γ-globin expression but complete absence of this transcription factor leads to β-thalassemia. Complete absence of KLF1 may also result in detrimental effects in erythropoiesis, since KLF1 is important for the regulation of many erythroid-specific genes. In fact, a dominant negative mutation in its zinc finger domain ("neonatal anemia" or nan) is known

to cause hereditary spherocytosis characterized by hemolytic anemia (Heruth et al. 2010). Targeting MBD2 offers the advantage of achieving an increase in γ-globin expression without resulting in detrimental systemic effects. Apart from globin genes, MBD2 only regulates a small group of genes such as murine IL-4 and gut genes (Hutchins et al. 2002; Berger et al. 2007). Aside from a mild nurturing phenotype, MBD2 knockout mice appear to behave normally. We have shown that Mi2β plays a significant role in γ-globin gene silencing, although a potential disadvantage of targeting this protein is that complete absence of Mi2β can affect erythroid differentiation, or other processes in tissues not included in this study. However, we have shown that a partial Mi2β knockdown is able to increase γ-globin gene expression to a large extent without affecting erythroid differentiation. The fact that complete absence of Mi2β is not necessary to achieve an increase in HbF may prove to be beneficial, because it is difficult to completely eliminate the expression of genes in an *in vivo* setting.

Another challenge in finding a potential new treatment for β-globin disorders is drug development and delivery. As reviewed in the introduction of this thesis, a handful of transcription factors play an important role in the repression of γ-globin but their lack of enzymatic activity poses a challenge to find ways to target them. Using RNAi to target these transcription factors through a lentiviral system is plausible. However, difficulties similar to the ones encountered with gene therapy treatments attempted at introducing the β-globin gene in β-thalassemia patients make this a difficult option. The vectors must be erythoid specific, and prolonged expression is needed. Silencing of the integrated gene/RNAi sequence after an extended period of time has been a long-standing challenge for gene therapy treatments. New vectors have proved to be more efficient at stable expression and have shown success in patients (Payen & Leboulch 2012), but long-term studies will be needed to assess this problem. The use

of insulators and the integration of these genes within cellular promoters rather than using viral promoters has decreased the risk of activating proto-oncogenes, yet insertion of these vectors in intronic regions can lead to disruption of genes (Payen & Leboulch 2012). Although gene therapy or the use of RNAi to target transcription factors may prove to be efficacious in the treatment of β-thalassemia or sickle cell anemia, it may be difficult to bring such technology to the developing world, in areas where this condition is most prevalent. The use of small molecules has become an area of interest in the past few years. They have proved to be successful in the treatment of cancer, and many small molecule drugs are already FDA approved and a few are in clinical trials. Imatinib (Gleevec), a small molecule currently used for chronic myelogenous leukemia is an example of such success (Fausel 2007). Because using an effective small molecule could provide an easier delivery method (could be given orally), small molecules may be an effective way to treat sickle cell anemia or β-thalassemia in the developing countries. Considering Mi2β has an ATPase domain that could be targeted by a small molecule, it is a great candidate as a possible therapeutic target for β-type globin gene disorders. MBD2 has a methyl-binding domain (MBD), which could also be targeted by a small molecule. Although small molecule inhibitors for DNA-protein interactions have been developed in the past (Ng et al. 2007), they are conventionally thought to be more difficult to target. A small peptide disrupting the MBD2 interaction with other NuRD complex components may be a more effective way to target this protein.

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