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The Role of Methyl CpG Binding Domain Protein 2 (MBD2) in the Regulation of Embryonic and Fetal β-type Globin Genes

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The Role of Methyl CpG Binding Domain Protein 2 (MBD2) in the Regulation of Embryonic and Fetal β-type Globin Genes

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 my thesis to my family; Mom, Dad, Prathi, Nishu, Chengalpattu avva and thatha, and Pallipattu avva and thatha. Each of you has inspired me at every stage of my life and this thesis would not have been possible without you. I love you all.

I specially want to dedicate this thesis to my husband, Mahesh. I love you and I thank God for you every day.

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List of Abbreviations

bp ..... Base pair

C ..... Celsius

cDNA ..... Complementary DNA

CFU-E ..... Colony-forming unit, erythroid

ChIP ..... Chromatin Immuno-Precipitation assay

CpG ..... Cytosine-Guanine dinucleotide

DMSO ..... Di-Methyl Sulfoxide

DNA ..... Deoxyribonucleic acid

FBS ..... Fetal Bovine Serum

G418 ..... aminoglycoside antibiotic

GAPDH ..... Glyceraldehyde 3-phosphate

gDNA ..... Genomic DNA

GFP ..... Green Fluorescent Protein

H_20 ..... Water

HDAC ..... Histone Deacetylase

HPFH ..... Hereditary persistence of fetal hemoglobin

HS ..... DNAse I hypersensitive sites

IgG ..... Immunoglobulin G

K562 ..... Human chronic myelogenous leukemia

kb ..... Kilobase

KLF ..... Kruppel-like Factor

LCR ..... Locus Control Region

M ..... Molar

MBD1 ..... Methyl Binding Domain Protein 1

MBD2 ..... Methyl Binding Domain Protein 2

MBD3 ..... Methyl Binding Domain Protein 3

MBD4 ..... Methyl Binding Domain Protein 4

MCBP ..... Methyl-CpG Binding Protein ml milli-liter (10-3 Liter)

mL ..... Milliliter

mM ..... Millimolar

ncRNA ..... Non-Coding Ribonucleic Acid ng Nano gram

ng ..... Nanogram

nM ..... nano-Molar (10-9 Molar)

PBS ..... Phosphate buffered saline

PCR ..... Polymerase Chain Reaction

Q-RT PCR ..... Quantitative (real-time) Reverse Transcriptase Polymerase Chain Reaction

RNA ..... Ribonucleic acid

rpm ..... Revolutions per minute

RT-PCR ..... Reverse Transcriptase Polymerase Chain Reaction

shRNA ..... Short hairpin (double-stranded) Ribonucleic Acid

siRNA ..... Short interfering (double-stranded) Ribonucleic Acid

TAE ..... Tris-acetate-EDTA electrophoresis buffer

U ..... Unit

WT ..... Wildtype

α ..... Alpha

β ..... Beta

δ ..... Delta

ε ..... Epsilon

µg ..... micro-grams (10-6 grams)

µL ..... Microliter

µM ..... micro Molar(10-6 molar)

ρ ..... Rho

Abstract

The Role of Methyl CpG Binding Domain Protein 2 (MBD2) in the Regulation of Embryonic and Fetal β-type Globin Genes

By Merlin Nithya Gnanapragasam, M.Sc.,

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

Virginia Commonwealth University, 2010

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The reexpression of the fetal γ-globin gene in adult erythrocytes is of therapeutic interest due to its ameliorating effects in β-hemoglobinopathies. We recently showed that Methyl CpG Binding Domain Protein2 (MBD2) contributes to the silencing of the chicken embryonic ρ-globin and human fetal γ-globin genes. We further biochemically characterized an erythroid MeCP1 complex that is recruited by MBD2 to mediate the silencing of these genes. These observations suggest that the disruption of the MeCP1 complex could augment the expression of the fetal/embryonic globin genes.

In the studies presented in chapter 2, we have pursued a structural and biophysical analysis of the interaction between two of the six components of the MeCP1 complex: MBD2 and p66α. These studies show that the coiled coil regions from MBD2 and p66α form a highly stable heterodimeric complex. Further, overexpressing the p66α coiled coil domain in adult erythroid cells can augment the expression of the chicken ρ-globin and human γ-globin genes, by disrupting the assembly of a functional MeCP1 complex. This indicates that the exogenously expressed p66α coiled coil peptide competes with the endogenous p66α for the interaction with the coiled coil domain of MBD2. These studies show that the coiled coil interaction between MBD2 and p66α could serve as a potential targets for the therapeutic induction of fetal hemoglobin.

The laboratory showed that knockout of MBD2 in transgenic mice carrying the human β-globin gene cluster, results in an elevated expression of γ-globin in adult erythrocytes. However, MBD2 does not directly bind to the γ-globin gene to mediate its silencing. In the work presented in chapter 3, we have tested the hypothesis that MBD2 may suppress γ-globin gene transcription in adult erythrocytes indirectly, by binding to and repressing transcription of intermediary gene/s which may be involved in γ-globin gene regulation. Employing microarray technology, we have identified Gab1 and ZBTB32 as candidate genes that may be involved in the MBD2 mediated silencing of γ-globin.

CHAPTER 1: Introduction and Literature Review

Hemoglobin is the most abundant protein constituent of vertebrate red blood cells and is essential for the life-sustaining oxygen transport capacity of blood. Hemoglobin molecule is a tetramer consisting of four polypeptide chains: two α- like and two β-like globin chains. Each chain has an embedded heme prosthetic group. It carries oxygen from the lungs to the capillaries and carbon dioxide from the capillaries to the lungs. In humans, α-globin genes are transcribed from the α-globin locus on the telomeric end of the short arm of chromosome 16, and the β-globin genes are expressed from the β-globin locus on the short arm of chromosome 11 (Stamatoyannopoulos G., Grosveld F. 2001, Nathan et al. 2003)

I. The human β- globin locus and hemoglobin switching:

The human β-globin locus on chromosome 11 contains the genes that encode the β-chain of hemoglobin. They are located in the order of their expression during development: 5' ε, γ, δ, and β 3' (**Figure 1**). These genes are relatively small and comprise three exons and two introns, coding for globin chains which are 146 amino acids long (Stamatoyannopoulos G., Grosveld F. 2001, Tuan et al. 1985).

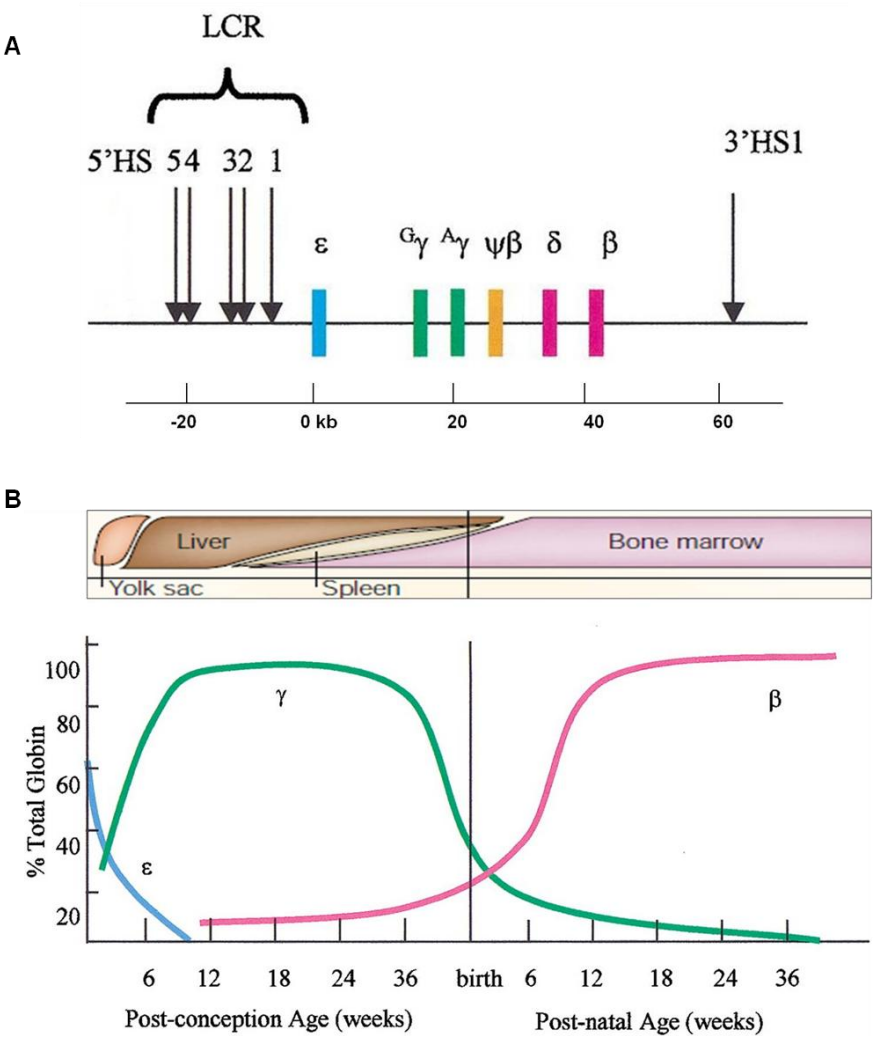

Figure 1 Human β-globin locus: A) Vertical arrows represent the DNase I hypersensitive sites HS1-5 defining the locus control region (LCR) which acts as an enhancer. Filled boxes are the five functional β-like globin genes arranged 5' to 3' in their order of expression during development. B) The lower figure depicts the spatial and temporal regulation of the genes. ε-globin is expressed in primitive erythrocytes of yolk sac and γ-globin in definitive erythrocytes of fetal liver. Gγ- and Aγ-globin genes are expressed at 7:3 ratio and differ at a single amino acid residue. After birth, β- (98%) and δ- (2%) globins are expressed in the bone marrow. Adapted from (Harju, McQueen & Peterson 2002, Weatherall 2001a).

The embryonic β-type globin gene, ε-globin is expressed exclusively in the earliest wave of erythroid cells from the yolk sac until 5 weeks post conception. This wave of primitive erythropoiesis arises from hemangioblast precursors in the blood islands of the yolk sac which give rise to primitive erythroid progenitors (EryP-CFC) which then enter the circulation and mature in the blood stream (**Figure 2**) (Peschle et al. 1985, McGrath, Palis 2008, Palis 2008). This transient lineage of cells maintains oxygen exchange during early embryonic development (McGrath, Palis 2008, Kingsley et al. 2006, Kingsley et al. 2004, Fraser, Isern & Baron 2007).

At approximately five weeks post conception the primitive erythroid lineage is replaced by definitive erythroid lineage. Two waves of erythroid progenitors emerge in the developing embryo to constitute the definitive lineage (**Figure 2**). The first wave is the transient wave of definitive erythroid precursors (BFU-E) that emerge from hemangioblast precursors in the yolk sac. These cells then seed the fetal liver where they mature and enucleate in the erythroblast islands and give rise to the first circulating definitive erythrocytes (Palis 2008, Wong et al. 1986, Keller et al. 1993, Palis et al. 1999). The second wave consists of a continuous stream of definitive erythroid precursors that originates from hematopoietic stem cells. These stem cells arise from the aorta-gonad-mesonephros (AGM) region while some of these progenitors have been speculated to also arise from the placenta. These cells then seed the fetal liver where they mature and enucleate while attached to the macrophage cells in the erythroblastic islands and are subsequently released into circulation (Palis 2008, Kurata et al. 1998, Orkin, Zon 2008). These cells express predominantly the fetal β-type globin genes Gγ and Aγ, and a very low level of the adult β globin gene. Gγ and Aγ -

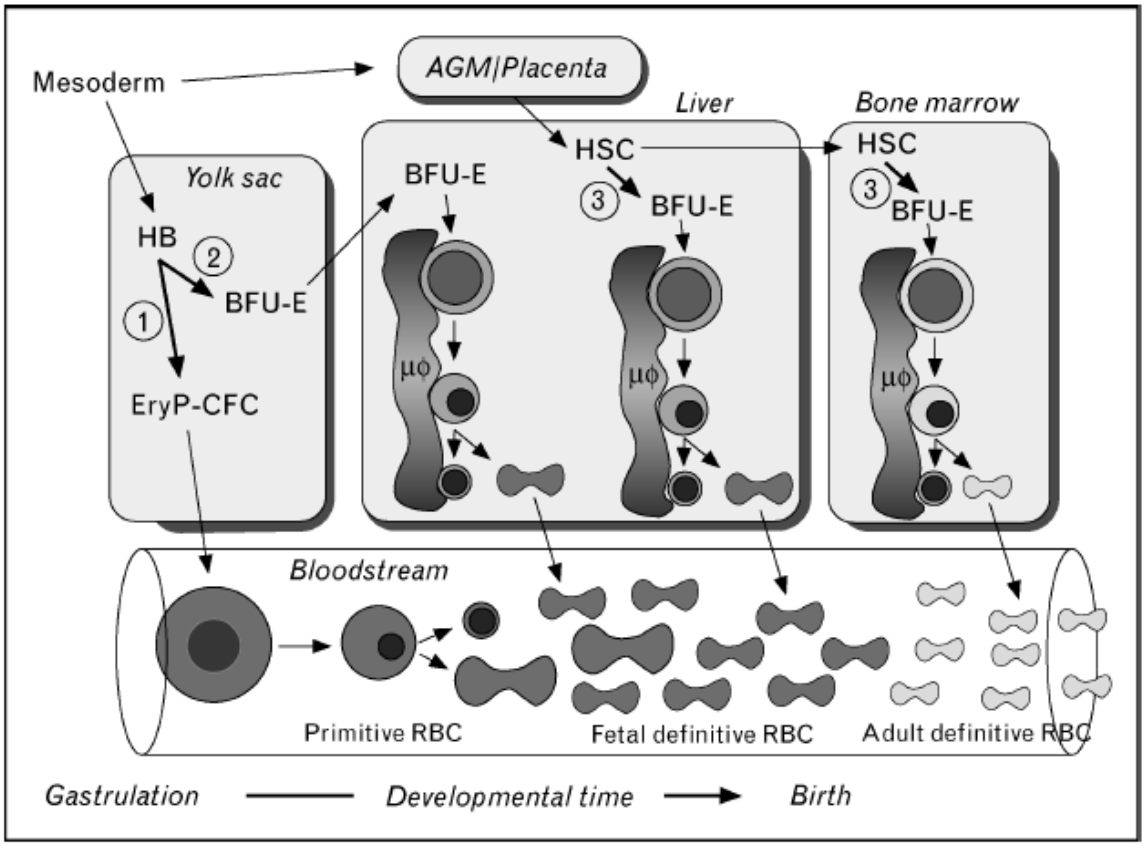

Figure 2 The three waves of erythroid progenitors in the mammalian embryo: The first wave consists of primitive erythroid progenitors (EryP-CFC) that originate from the yolk sac. They go on to mature in the blood stream. The second wave consists of a transient wave of definitive erythoid progenitors (BFU-E) that emerge from the yolk sac and seed the fetal liver. The third wave consists of a continuous stream of definitive erythroid progenitors produced in the fetal liver in late gestation and in the bone marrow after birth. Unlike the primitive erythropoiesis, the maturation in the definitive erythropoiesis happens in erythroblast islands which consist of macrophages. AGM: aorta gonad mesonephros region; μθ: macrophage cell. Adapted from (Palis 2008).

globins arose due to gene duplication in primate evolution and differ only in a single amino acid at position 136. Gγ is produced at a three times higher amount than Aγ in these cells. These γ-globins assemble with α-globin subunits to form the $\alpha_2\gamma_2$ hemoglobin tetramer which is termed fetal hemoglobin (HbF) (Stamatoyannopoulos G., Grosveld F. 2001). These definitive erythroid cells continue to be produced until birth.

Around the time of birth, the site of definitive hematopoiesis shifts from fetal liver to bone marrow (Stamatoyannopoulos G., Grosveld F. 2001, Palis 2008, Orkin, Zon 2008). In these cells, the fetal γ-globin expression gradually is silenced and adult β-globin becomes the predominant globin. By six months of age, γ-globin expression is reduced to less than 1% of the total β-type globin expression. Among the fetal β-type globin produced in these cells, Aγ is produced in a higher amount than Gγ at a ratio of 3:2 (Stamatoyannopoulos G., Grosveld F. 2001). The predominant β-type globin produced is the β-globin, which along with α-globin subunits forms the $\alpha_2\beta_2$ tetramer termed as HbA1. This hemoglobin constitutes 95-98% of the total hemoglobin in these adult erythroid cells. A low level of another adult β-type globin gene, δ-globin is also produced and along with α-globin subunits forms the $\alpha_2\delta_2$ tetramer termed as the HbA2 hemoglobin which constitutes 2-3% of the total hemoglobin in these cells (Stamatoyannopoulos G., Grosveld F. 2001).

This process, by which red cells in the blood stream containing predominantly one hemoglobin are gradually replaced by cells containing predominantly another type, is termed “hemoglobin switching”(Nienhuis, Stamatoyannopoulos 1978). The first switch from hemoglobin produced in the transient embryonic primitive erythroid cells to that

produced in the definitive erythroid cells is found to be common among vertebrates (Sankaran, Xu & Orkin 2010). However, the second switch from hemoglobin produced in the fetal to adult definitive erythroid cells is found to occur only in primates and humans due to the evolution of unique fetal β-type globin genes (Johnson 2002a). The rationale for the evolution of these fetal β-globin genes is still not entirely clear. The changes in the type of the globin subunits synthesized lead to the assembly of hemoglobin with different physiological properties. The fetal hemoglobin (HbF) has a higher oxygen affinity compared to hemoglobin (HbA). It is speculated that this increased oxygen affinity favors the oxygen delivery to the fetus in the placental circulation (Sankaran, Xu & Orkin 2010, Bank 2006). However, this reasoning is questionable since the placental mammals which have gestational periods even longer than primates do not have the fetal type hemoglobin (Sankaran, Xu & Orkin 2010).

A set of five ~250bp DNAseI hypersensitive sites (HS) are located ~6-20 kb upstream of the ε-globin gene (Stamatoyannopoulos G., Grosveld F. 2001). This region is termed as the locus control region (LCR). The LCR is required for the robust expression of the beta globin genes, thus acting as an enhancer for the locus (Forrester et al. 1986, Grosveld et al. 1987). The property that distinguishes the LCR from a classical enhancer is its ability to offer the position independent and copy number dependent expression of a linked transgene (Stamatoyannopoulos G., Grosveld F. 2001, Grosveld et al. 1987, Fraser, Grosveld 1998). The hypersensitive sites in the LCR can directly loop and interact with the β-globin gene that is actively transcribed (Palstra et al. 2003, Vakoc et al. 2005, Tolhuis et

al. 2002). Moreover the LCR has also been shown to facilitate the interaction of the globin genes with transcription factories (Ragoczy et al. 2006).

The human β-globin locus is one of the most extensively studied loci in the mammalian genome (Stamatoyannopoulos G., Grosveld F. 2001, Bank 2006). The primitive to definitive and the fetal to adult hemoglobin switch in the human β-globin locus have served as useful models to understand gene regulation during development and differentiation (Stamatoyannopoulos G., Grosveld F. 2001, Sankaran, Xu & Orkin 2010). Despite the extensive insight derived from numerous studies, the regulation of the genes in this locus is still not completely understood. The continued pursuit in understanding the regulation of these genes will shed more light on hemoglobin switching in particular and mammalian gene regulation in general. In addition, the knowledge obtained can be used in devising a more efficient treatment strategy for individuals with β-hemoglobinopathies. Specifically, understanding the regulation of the fetal γ-globin gene is of interest, since the elevated expression of γ-globin is shown to have ameliorating effect in β-hemoglobinopathies.

II. β-hemoglobinopathies:

β-hemoglobinopathies are common genetic disorders which contribute to significant mortality and morbidity throughout world. Approximately 7% of the world population are carriers for these disorders and annually there may be about 300,000 to 400,000 births of infants with sickle cell anemia or serious forms of thalassemia (Weatherall et al. 2006, Weatherall, Clegg 2001). The high frequencies of these genetic

disorders are due to the heterozygote protection against malaria. Hence the alleles causing these disorders are concentrated in countries in which positive selection pressure existed due to high prevalence of malaria. The resurgence of malaria in various parts of the world will serve to maintain the frequency of the genes which cause these hemoglobinopathies. In fact, even if this selective force is removed it will take many generations for the gene frequencies of these disorders to fall dramatically (Weatherall, Clegg 2001).

The β-hemoglobinopathies can be categorized into one of the two groups. The first group, is characterized by structurally abnormal hemoglobin due to aberrant amino acid substitutions. Although there are over 700 structural variants of hemoglobin subunits, most are rare and benign (Weatherall, Clegg 2001). The most common and severe disease in this group is sickle cell disease (SCD). The second group comprising β-thalassemias, is characterized by impaired synthesis of the β-globin chain.

A. Sickle cell disease (SCD):

SCD encompasses a group of conditions resulting from the inheritance of a mutated β-globin, known as the sickle β-globin gene (β^S-globin). This mutation leads to substitution of valine for glutamic acid in position 6 in the β-globin chain(Weatherall, Clegg 2001, Madigan, Malik 2006). In homozygous sickle cell anaemia (SCA), both alleles of the β^S-globin are inherited, resulting in production of mutant hemoglobin termed HbS. Inheritance of a single β^S allele results in the heterozygous, benign, sickle cell trait. Co-inheritance of other β-globin gene mutations (e.g. HbC and HbE) causes a relatively benign SCD, with mild hemolytic anemia and splenomegaly (Madigan, Malik 2006).

i. Incidence:

The sickle cell gene is distributed widely throughout the Sub-Saharan Africa, the Middle East and parts of the Indian subcontinent. (**Figure 3**). The carrier frequencies are found to range from 5% to 40% or more of these populations (Weatherall et al. 2006). As discussed earlier, the high frequency of the sickle cell genes has been evolutionarily favored especially in the populations exposed to malaria due to the heterozygote protection against malaria. It is one of the most common genetic blood disorders in the United States affecting 1 in 1,800 African Americans. The incidence of SCA in the USA is 1/625 African-American births (Madigan, Malik 2006). Patients with SCD have a markedly decreased life expectancy and their quality of life is greatly compromised by their disease (Fathallah, Atweh 2006). In the USA, the peak incidence of death among those affected with these disorders appears to be at 1–3 years of age, usually due to infection. Among affected adults in the USA, the median age of death is 42 years for males and 48 years for females (Weatherall, Clegg 2001)

ii. Pathophysiology:

In its deoxygenated state, HbS is extremely insoluble. This leads to polymer formation within the RBC which causes the shape of the RBC to change to the sickled form that gives the disease its name. Sickling is accompanied by increased rigidity, loss of deformability, increased adhesiveness to endothelial cells and red cell membrane damage. All of this adversely affects the flow properties of the red cells through micro-vasculature

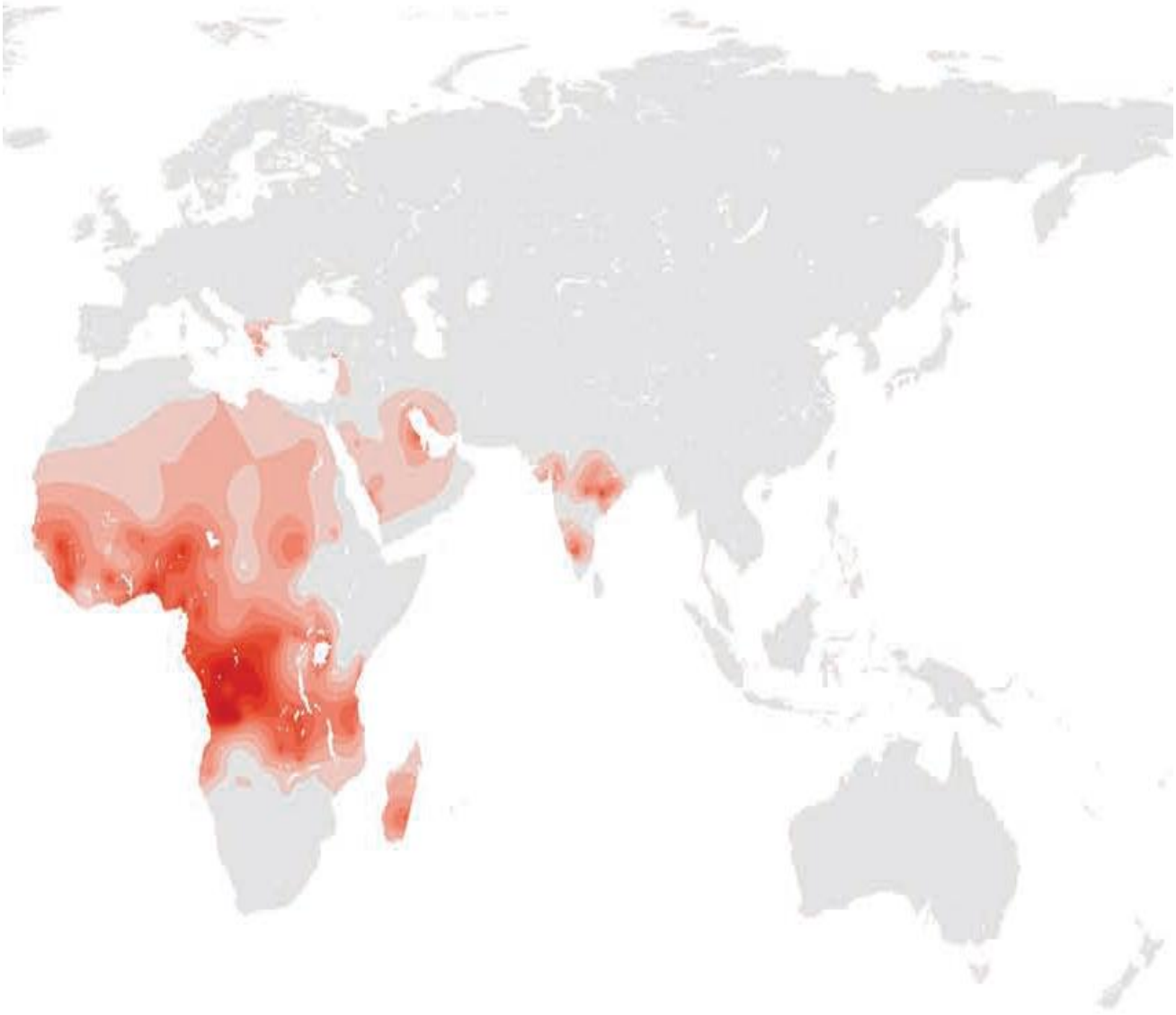

Figure 3 Map of HbS allele frequency. HbS allele in the homozygous state causes sickle cell anemia. Adapted from (Piel et al. 2010).

and causes vaso-occlusion. Membrane damage causes the red cell life span to be short , 15 days instead of 120 days, resulting in hemolytic anemia (Weatherall, Clegg 2001, Madigan, Malik 2006).

B. Beta thalessemias:

β-thalassemias are among the most intensively studied monogenic disorders (Weatherall 2001b). β-thalassemias, are characterized by an impaired rate of synthesis of the β-globin chain which results in dyserythropoiesis. Most of the β-thalassemias are inherited in an autosomal recessive fashion. Homozygous patients often die in their teens or early twenties (Atweh et al. 2003).

The deficiency or absence of the β-globin chain arises due to mutations that reflect every level of β-globin gene function such as transcription, processing of the mRNA, translation, and post translational stability of the β-globin polypeptide. The mutations include gene deletions, mutations in promoter regions, cap site, 5' untranslated region, splice site intron exon boundaries, splice site consensus sequences, cryptic splice sites in exons and introns, polyadenylation signal and mutations that result in abnormal translation of β-globin mRNA and unstable β-globin chains (Stamatoyannopoulos G., Grosveld F. 2001, Urbinati, Madigan & Malik 2006, Sackey 1999).

Over 180 different mutations have been identified that can give rise to the clinical phenotype of β-thalassemia. 20 different alleles are found to account for 80% of β-thalassemias world wide (Stamatoyannopoulos G., Grosveld F. 2001). The mutations which cause no β-chain to be produced are termed β^0 and those which lead to the

production of small amounts of the β-chain are termed β^{+}. β-thalassemia is classified based on severity into β-thalassemia major (cooley's anemia), minor (β-thalassemia trait) and intermedia. β-thalassemia major is severe and occurs when both the β-globin alleles are mutated. Since many β-thalassemia mutations are prevalent in populations which have high incidence of β-thalassemias, many patients with β-thalassemia major are compound heterozygotes for two different mutations. β-thalassemia minor is usually symptomless and these individuals have only one of their β-globin alleles mutated. β-thalassemia intermedia has moderate severity and may occur due to homozygous or heterozygous mutations (Stamatoyannopoulos G., Grosveld F. 2001, Urbinati, Madigan & Malik 2006, Sackey 1999).

i. Incidence:

As mentioned earlier, it is highly likely that the β-thalassemia causing alleles have been maintained in populations due to the heterozygote protection offered against malaria (Weatherall 2001b). β-thalassemia has a high incidence in the Mediterranean basin, parts of Africa, throughout the middle east, the Indian sub-continent, south-east Asia, Melanesia and into the Pacific islands (**Figure 4**). The carrier frequency in these areas ranges from 1% to 20% of the population (Weatherall, Clegg 2001).

ii. Pathophysiology:

The decreased β-chain synthesis leads to excess alpha chains and the excess chains form tetramers which are very insoluble. The tetramers accumulate and precipitate within the RBC leading to the increased fragility and cell death. Thus the life span of the RBCs

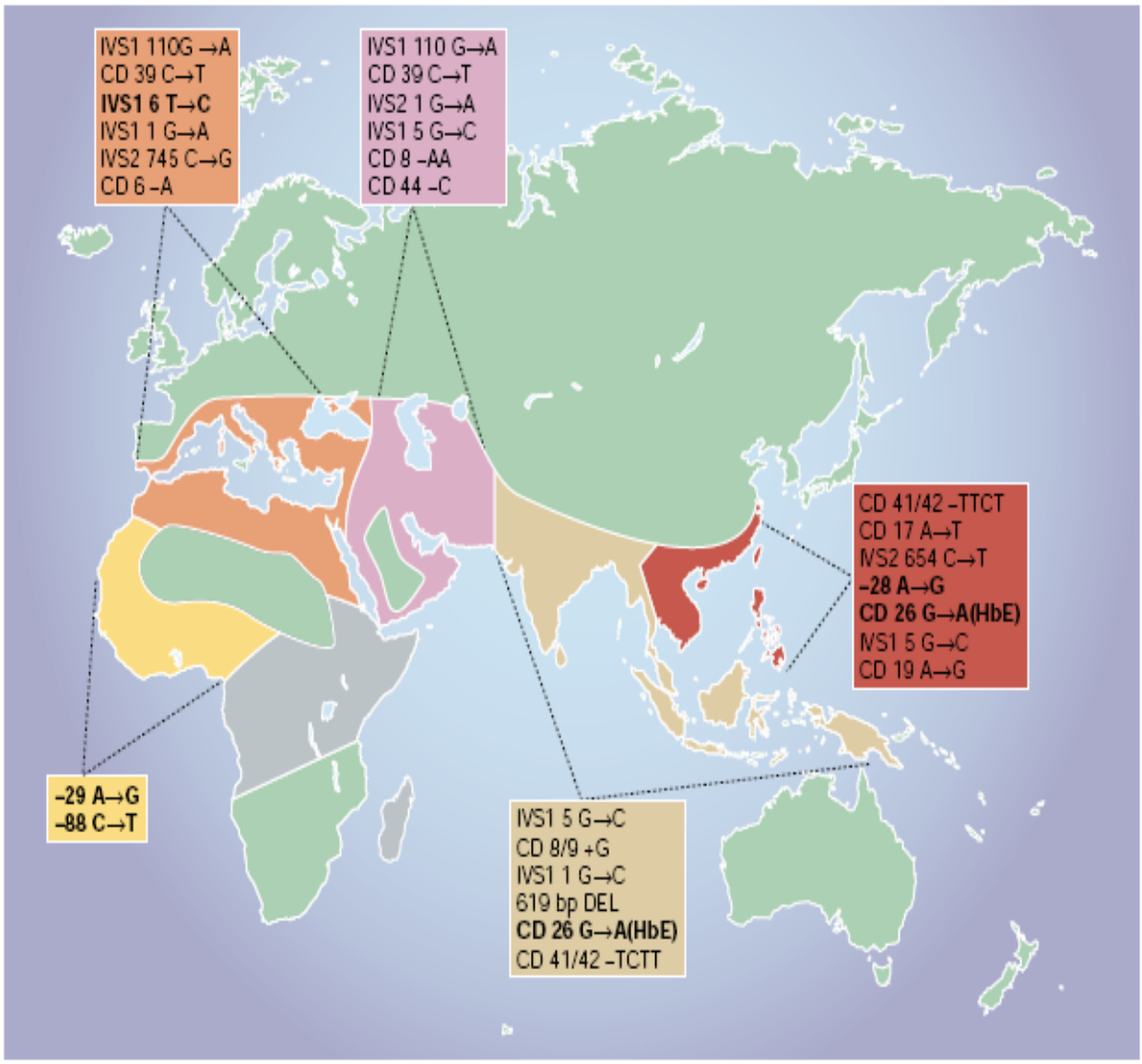

Figure 4 The map of β-thalassemia mutations. The mutations depicted in bold are the common mild mutations in the region. HbE is a harmless hemoglobin variant unless interacting with α- or β-thalassemia. β-thalassemia is also present in the regions shaded in grey. However, the molecular pathology is unknown in these regions. Adapted from (Weatherall 2001a).

is very short and they may be destroyed within the marrow leading to ineffective erythropoiesis (Weatherall 2001b, Urbinati, Madigan & Malik 2006, Sackey 1999).

The lack of beta chains lead to decreased hemoglobin content per cell, hypochromia and microcytosis. Attempts to increase the red cell mass result in expanded marrow cavities and extramedullary erythropoiesis in the liver and spleen (Urbinati, Madigan & Malik 2006, Sackey 1999). The individuals with these thalassemias become iron-loaded even with sparing use of transfusions, due to the increased iron absorption from diet. Iron toxicity affects the liver (cirrhosis), pituitary (hypogonadism and growth failure), heart (arrhythmia and cardiomyopathy) and bone (pathologic fractures) (Urbinati, Madigan & Malik 2006, Sackey 1999, de Silva et al. 2000, Premawardhena et al. 2005).

C. Management strategies for β-hemoglobinopathies:

Therapy for sickle cell disease involves the management of the the acute clinical events such as vaso-occlusive crisis, acute chest syndromes and aplastic crisis that often follows the infection with parvovirus B19. Additionally, management of the many forms of organ damage that results from occlusive ischemia is necessary. Chronic transfusion and intensive iron chelation is administered to reduce the amount of sickle sickle hemoglobin and thereby ameliorating the symptoms (Madigan, Malik 2006).

Treatment for β-thalassemia syndromes involves regular blood transfusions, splenectomy, iron chelation therapy and folic acid supplementation (Urbinati, Madigan & Malik 2006).

The only curative therapy currently available for individuals with severe β-hemoglobinopathies involves bone marrow or cord blood stem cell transplantation from an HLA identical sibling. However this is a high risk procedure that may be unacceptable except in the most severe cases (Stuart Fox MD et al. , Stuart Fox MD et al. , Bunn 1997, Platt, Guinan 1996).

D. Fetal hemoglobin induction, an effective treatment strategy:

Alternative treatment options for β-hemoglobinopathies are gene therapy and fetal hemoglobin induction (Atweh et al. 2003, Mabaera et al. 2008). Gene transfer for the correction of the mutated genes requires additional research to determine its safety and viability (Bank, Dorazio & Leboulch 2005). Initial studies resulted in detrimental side effects and more research have since been dedicated to devising safer gene transfer strategies (Bank, Dorazio & Leboulch 2005). Induction of fetal hemoglobin holds great promise for treating these diseases. Since the disorder arises due to an insufficient production of adult β-globin chain or production of an aberrant adult β-globin chain, upregulation of the repressed fetal β-type globin chain can compensate for the defects (Stamatoyannopoulos G., Grosveld F. 2001).

The concept that the increased levels of fetal hemoglobin (HbF) can be useful in ameliorating the symptoms of the disorders arose from pioneering observations in patients with β-hemoglobinopathies. Initial observations indicated that the symptoms of β-hemoglobinopathies in children manifested only after a few months of birth coinciding with the decline of the level of HbF (Stamatoyannopoulos G., Grosveld F. 2001, Stuart

Fox MD et al.). Moreover, higher steady state levels of fetal hemoglobin were observed to correlate with less severe phenotype in populations with the β-hemoglobinopathies (Premawardhena et al. 2005, Miller et al. 1987, Pembrey et al. 1978, Pembrey et al. 1978, Kar et al. 1986, Castro et al. 1994, Platt et al. 1994). In addition, rare mutations in the β-globin locus were identified which lead to a highly elevated HbF production, termed as hereditary persistence of fetal hemoglobin (HPFH). When individuals with β-hemoglobinopathies coinherit the HPFH mutations, it results in a relatively benign form of disease (Stamatoyannopoulos G., Grosveld F. 2001, Nathan et al. 2003). Taken together, these studies demonstrate that HbF has an ameliorating effect in sickle cell disease and β-thalassemias.

In SCD, fetal hemoglobin is a potent inhibitor of the polymerization of deoxyhemoglobin S. As a result, it causes a reduction in sickling, vascular occlusion and prevention of pulmonary hypertension (Mabaera et al. 2008, Poillon et al. 1993). In β-thalassemia, elevated levels of γ-globin chains combine with the redundant alpha chains leading to reduced dyserythropoiesis and lower blood transfusion requirement, which prevents iron overload induced cardiac failure (Mabaera et al. 2008). While 10-15% HbF has dramatic ameliorating effects in individuals suffering from β-hemoglobinopathies, even 5% HbF can increase the life expectancy of these individuals (Platt et al. 1994, Ley et al. 1982b, Gaston, Rosse 1982). Due to the ameliorating effects of HbF in individuals with β-hemoglobinopathies, treatment strategies aimed at reactivating fetal hemoglobin production in adult erythrocytes have been rigorously studied.

The first approach to reactivate fetal hemoglobin in adult erythroid cells occurred soon after the initial cloning of the human globin genes. Site-specific DNA methylation within or adjacent to these genes was found to correlate with transcriptional repression (McGhee, Ginder 1979a, Ginder, McGhee 1981, Razin, Riggs 1980, Shen, Maniatis 1980b, Vanderploeg, Flavell 1980). Thus, it was hypothesized that inhibiting DNA methylation using cytidine analogues such as 5-azacytidine (Taylor, Jones 1982), may allow the reactivation of the γ-globin gene. The β-type globin genes were the first group of genes for which the treatment with 5- azacytidine, an irreversible inhibitor of DNA methyltransferase, was shown to alter developmentally established patterns of expression by increasing levels of the fetal γ-globin gene. This was first demonstrated in baboons and subsequently in patients with β-thalassemia and sickle cell anemia (Charache et al. 1983, Desimone et al. 1982, Ley et al. 1982a). However, concerns were raised about the mutagenic potential of 5-azacytidine (Stamatoyannopoulos G., Grosveld F. 2001). Thus current focus is on the less toxic deoxycytidine analogue, 5-aza-2-deoxy-cytidine (decitabine), and is currently being tested in clinic.

Nathan and Stamatoyannopoulos demonstrated that stress erythropoiesis created by S-phase inhibitors such as Hydroxyurea induce HbF (Stamatoyannopoulos G., Grosveld F. 2001, Letvin et al. 1985, Letvin et al. 1984, Papayannopoulou et al. 1984). Hydroxyurea, although cytotoxic showed tremendous success in inducing HbF and was eventually approved by FDA for use in the treatment of sickle cell disease (Nathan 2005, Stamatoyannopoulos 2005).

More agents have been tested for their ability to induce fetal hemoglobin. Short chain fatty acids such as butyrate have shown potential success (Perrine et al. 1989, Perrine, Greene & Faller 1985, Atweh et al. 1999). The mechanism of action of these agents in the induction of HbF remains unclear and one of the prevailing hypothesis is that they induce HbF by inhibiting histone deacetylases (Stamatoyannopoulos G., Grosveld F. 2001).

While 5-azacytidine has carcinogenicity, the other agents which have been shown to induce HbF such hydroxyurea and butyrate also have undesirable properties (Stamatoyannopoulos G., Grosveld F. 2001). Hydroxyurea is cytotoxic and is ineffective in several sickle cell disease patients and has only a minor benefit for individuals β-thalassemia. Butyrate is also ineffective in several patients and has toxicity (Stamatoyannopoulos G., Grosveld F. 2001).

Continued research aimed at discovering agents which can induce HbF with minimal or no toxicity will have a huge impact on the treatment and cure of β-hemoglobinopathies. A limitation to this goal is the lack of understanding of the fetal to adult β-globin switch in its entirety. Research focused on furthering our understanding on the mechanism of fetal to adult β-globin switch is vital to the development of better agents that induce HbF in adult erythroid cells.

III. Human γ-globin gene regulation:

The tissue and developmental stage specific expression of the individual genes in the β-globin gene cluster has been shown to be developmentally regulated by a complex

interplay between *cis* elements: the β-globin LCR and the downstream sequences adjoining the individual genes, and transacting factors (Stamatoyannopoulos G., Grosveld F. 2001). However, the exact mechanism of globin gene switching during development is still not completely understood.

A. β-YAC transgenic mouse model:

In order to study the human β-globin switching during development transgenic β-YAC mice have been used extensively. The murine β-globin locus consists of four genes: the embryonic β-type globin genes, εy (orthologous to the human ε) and βh1 (orthologous to the human γ) and the adult β-type globin genes, β-major and β-minor. In mice, unlike primates and humans, only a single hemoglobin switch from embryonic to adult β-type globins occurs. Murine development lacks the fetal β-type globins present in primates and humans. With respect to the site of hematopoiesis two switches occur in mice similar to humans. The embryonic to adult hemoglobin switch in mice takes place at day 10.5 post-conception in mice. During this period the site of erythropoiesis shifts from the yolk sac to the fetal liver (Chada, Magram & Costantini 1986, Farace et al. 1984). During later stages of fetal life, the site of definitive erythropoiesis shifts from fetal liver to bone marrow (**Figure 5**).

70 to 248 kb constructs of the entire human β-globin locus as ligated cosmids, yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), and phage artificial chromosomes (PACs), have been used to study the regulation of the human β-globin genes

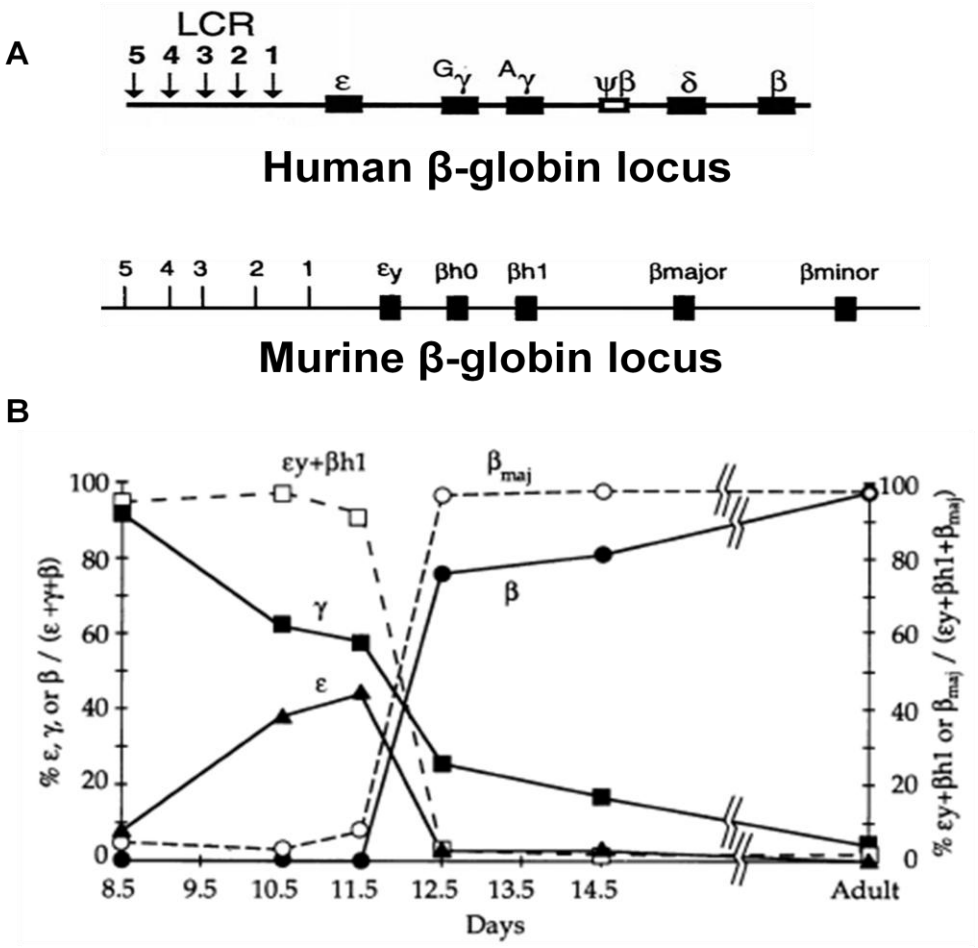

Figure 5 Endogenous mouse β-type globin and human β type globin switching in whole locus (β-YAC) transgenic mice during development. A) The murine β-globin locus. The numbers represent the DNAse1 hypersensitive sites which constitute the Locus control region (LCR). The black boxes represent the β-type globin genes in the locus. B) The graph shows the changes in the expression of human and endogenous murine β-globin genes during development in the β-YAC transgenic mice. The y axis on the left depicts the percentage of the human β-globin genes. The expression of the human β-globin genes are represented in the graph by solid lines. The y axis on the right depicts the level of the endogenous mouse β-globin genes. The expression of the murine β-globin genes is represented by dotted lines. Adapted from (Harju, McQueen & Peterson 2002).

(Gaensler et al. 1991, Peterson et al. 1993a, Peterson et al. 1993a, Gaensler, Kitamura & Kan 1993, Peterson et al. 1998, Peterson et al. 1997, Peterson et al. 1993b, Peterson et al. 1993). Despite the lack of fetal β-globins in mice compared to humans, the evolutionary conservation enables transgenic mice containing the human β-globin locus to show correct temporal expression patterns of the human β-globin genes during development (Stamatoyannopoulos G., Grosveld F. 2001, Peterson et al. 1993a). The ε-globin gene is expressed solely in the yolk sac during primitive erythropoiesis. Expression of the γ-globin gene begins in the yolk sac during primitive erythropoiesis and continues in the definitive period in the fetal liver. The γ-globin expression finally switches off around day 17 post-conception. The expression of the adult β-globin gene begins at day 12 post-conception in the fetal liver and is the only predominant β-type globin gene expressed after birth in the adult erythrocytes of the bone marrow (**Figure 5**).

The ability of the transgenic mice to correctly regulate human β-globin transgenes has had great impact on the study of globin gene regulation. The studies using these transgenic mice have contributed greatly to our understanding of the cis- and trans-regulatory elements involved in the regulation of the human β-globin genes.

B. Cis acting sequences:

The cis acting sequences include the regulatory sequences that are immediately flanking the individual genes globin gene sequences and the more distant β-globin LCR which is important for the robust expression of all the genes in the locus (Stamatoyannopoulos G., Grosveld F. 2001, Bank 2006). The regulatory elements in each

of the genes are thought to interact with the LCR to aid the developmental expression of the genes. Each of these regulatory elements is composed of binding sites for multiple erythroid specific and ubiquitous transcriptional activators and repressors (Stamatoyannopoulos G., Grosveld F. 2001, Sankaran, Xu & Orkin 2010, Bank 2006). These transcription factors further interact with each other, with other cofactors (co-activators or co-repressors) and chromatin remodeling enzymes to form multimeric complexes which can affect the chromatin structure of the locus and the assembly of the transcription initiation complex. The transcription initiation complex is assembled at the TATA box which is located approximately 30 bp upstream of the transcription initiation site of each of these β-globin genes. The frequency of the initiation of transcription of each of these genes is dependent upon the interaction of the initiation complex with transcription factors and cofactors bound to the promoters and the distant regulatory sequences ie., the LCR (Stamatoyannopoulos G., Grosveld F. 2001). Thus, the level of transcription of the globin genes is the product of the frequency of interaction of the LCR with a given gene and the stability of the interaction (Stamatoyannopoulos G., Grosveld F. 2001). A looping model has been proposed to explain this interaction. According to the looping model the initiation of transcription is achieved by the interaction between the LCR and the genes and the looping out the intervening DNA (**Figure 6**) (Stamatoyannopoulos G., Grosveld F. 2001, Bank 2006). Recent studies using chromatin conformation capture (3C) and tagging and recovery of associated proteins (RNA TRAP) have provided confirmation that the LCR and a particular beta globin gene depending on the developmental stage, form an active chromatin hub (ACH) and interact with each other

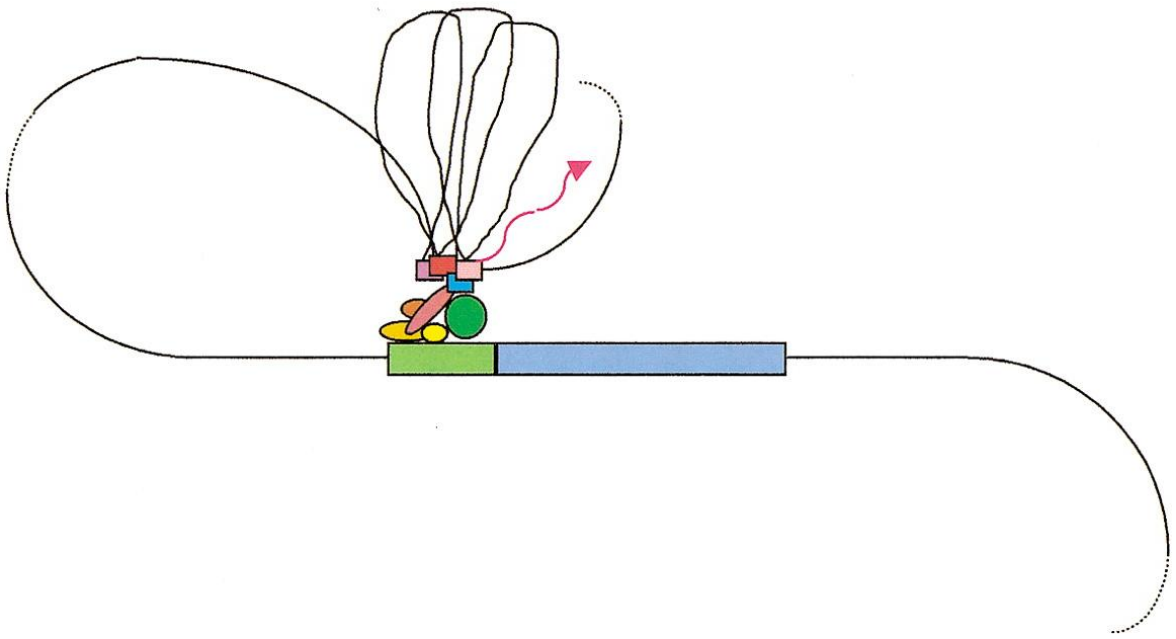

Figure 6 Looping model of LCR function: In the looping model the transcription factors (denoted by colored ovals and circles) bind to the DNAse1 hypersensitive sties (represented by the small colored boxes) of the LCR and to the globin gene promoter (green rectangle). These transcription factors and their co-activators contribute to the direct interaction of the LCR and the globin gene promoter by looping out the intervening DNA between the LCR and the gene. The mRNA transcript is denoted by the wavy arrow. Adapted from (Harju, McQueen & Peterson 2002).

through a looping mechanism (Engel, Tanimoto 2000, Noordermeer, de Laat 2008).

C. Trans-acting factors:

A variety of molecular factors that regulate transcription have been found to be involved in the β-globin genes regulation and the switching process. This includes both tissue-specifically expressed proteins and ubiquitously expressed proteins.

GATA1 is one of the first factors which were shown to be important for β-globin gene switching (Evans, Felsenfeld 1989, Tsai et al. 1989, Martin, Tsai & Orkin 1989). In fact, one of the studies that led to the discovery of GATA1 was due to an analysis of factors binding to the -175HPFH site in the promoter of γ-globin gene (Martin, Tsai & Orkin 1989). A recent study reported a GATA1 zinc finger mutation in a patient with congenital erythropoietic porphyria and elevated HbF which also indicates a role of GATA1 in switching (Phillips et al. 2007). One of the mechanisms by which GATA1 plays a role in hemoglobin switching appears to be by facilitating the chromosomal looping in the β-globin locus. GATA1 is a principal transcription factor which binds to promoters of many erythroid specific genes (Stamatoyannopoulos G., Grosveld F. 2001, Sankaran, Xu & Orkin 2010, Martin, Tsai & Orkin 1989, Martin, Orkin 1990) and has been shown to have a role in both activation and repression of these genes (Welch et al. 2004). It is also required for normal erythropoiesis in both mouse and humans (Weiss, Keller & Orkin 1994, Fujiwara et al. 1996, Nichols et al. 2000, Pevny et al. 1991, Simon et al. 1992, Yu et al. 2002).

FOG1 (Friend of GATA1) protein was identified as a critical binding partner of GATA1 (Tsang et al. 1997). FOG1 has been shown to mediate both the activation and repression of transcription by GATA1 by recruiting chromatin remodeling proteins (Miccio et al. 2010, Miccio, Blobel 2010). GATA1 has been shown to bind to upstream regions of γ-globin genes in a FOG1 dependent manner. FOG1 has been shown to recruit the NuRD co-repressor complex, thus mediating the transcriptional silencing by GATA1 of these genes (Miccio et al. 2010, Miccio, Blobel 2010, Hong et al. 2005, Hong et al. 2005, Rodriguez et al. 2005). These GATA1 binding regions are shown to be necessary for HbF silencing in transgenic mice (Harju-Baker et al. 2008).

Human genetic studies were performed in individuals with hereditary persistence of hemoglobin (HPFH) who do not have mutations within the β-globin locus, to identify modifier loci which may have a role in globin gene switching. These studies have identified SNPs at two loci for their association with high γ-globin levels (Menzel et al. 2007, Thein et al. 2007, Thein et al. 2009, Lettre et al. 2008, Uda et al. 2008, So et al. 2008). One is the HBS1L-MYB region on chromosome 6 and the other is BCL11A on chromosome 2 (Menzel et al. 2007, Thein et al. 2007, Thein, Menzel 2009). The biological effect of HBS1L-MYB region on globin switching is not clear although some studies suggest that the MYB protein level may be responsible for the elevated γ-globin level (Jiang et al. 2006, Wahlberg et al. 2009).

BCL11A (B-cell lymphoma/leukemia 11A) is a zinc finger transcriptional factor. It is indispensible for lymphoid development. Its role in β-globin gene switching was revealed by genome wide genetic association studies which revealed strong association of

SNPs in this gene with high HbF levels (Menzel et al. 2007, Lettre et al. 2008, Uda et al. 2008). Subsequent studies have shown that it functions as a regulator of β-globin switching (Sankaran et al. 2008a, Sankaran et al. 2009, Xu et al. 2010). Knockdown of BCL11A expression in human adult erythroid cells causes an induction of γ-globin (Sankaran et al. 2008b). The expression of the full-length isoform of this gene is developmentally restricted to erythroid cells expressing adult β-globin genes (Sankaran et al. 2009). Further, knockout of this gene in β-YAC transgenic mice disrupts proper silencing of the mouse endogenous embryonic β-type globin genes and human fetal γ-globin genes in the adult erythroid cells of the fetal liver (Sankaran et al. 2009). BCL11A has been shown to physically interact with NuRD co-repressor complex, erythroid transcription factors GATA1 and FOG1 and HMG-box protein SOX6 to bring about silencing of the human fetal and mouse embryonic globin genes (**Figure 7B**) (Xu et al. 2010). However, BCL11A does not bind to the promoters of the β-globin genes. It has been shown to occupy the upstream LCR and γ-δ intergenic region of the β-globin locus indicating long-range interactions as an important mechanism of silencing by this protein (Xu et al. 2010).

SOX6 is a member of the Sry-related high mobility group (HMG) box transcription factors. This group of factors serves as determinants of cell fate and differentiation in various lineages (Schepers, Teasdale & Koopman 2002, Wegner 1999). The role of SOX6 in globin gene regulation was first observed in Sox6 knockout mice. The expression of mouse embryonic globin genes were elevated in adult erythroid cells in fetal liver, affecting εy more than βh1 (Yi et al. 2006). It was also shown to directly bind to εy promoter to silence the gene (Yi et al. 2006). However, a role for SOX6 in the regulation

of human β-globin genes has not yet been thoroughly studied. A role for SOX6 in human γ-globin regulation was revealed in a recent study which suggests that varying levels of SOX6 correlate with human γ-globin expression in human erythroid progenitors (Sripichai et al. 2009). Moreover, recent studies have revealed that SOX6 co-occupies the the β-globin cluster with BCL11A and GATA1 and co-operates with BCL11A in silencing the γ-globin gene in human adult erythroid progenitors (**Figure 7B**) (Xu et al. 2010). This study also revealed that the knockdown of SOX6 alone leads to a moderate elevation of γ-globin expression in human adult erythroid progenitors (Xu et al. 2010). The precise mechanism of the regulation of γ-globin gene expression by SOX6 is not clear. HMG domain factors are capable of binding to the minor groove of DNA and can cause a drastic bend of DNA leading to conformational changes (Ferrari et al. 1992, Connor et al. 1994, Thomas 2001, Anonymous, Bagga 2000, Drew 2000). Thus SOX6 may function as an architectural protein which could mediate long range interactions in the β-globin locus by bridging distant regions of DNA with the associated proteins and thus assembling higher order complexes (Xu et al. 2010).

KLF1**,** also known as EKLF is a member of the Krüppel-like zinc-finger transcription factor family of proteins. It has been shown to play an important role in erythropoiesis and β-globin gene transcription (Miller, Bieker 1993, Nuez et al. 1995, Perkins 1996, Perkins, Sharpe & Orkin 1995, Wijgerde et al. 1996). KLF1 knockout mice die from severe β-thalassemia due to a severe reduction of β-globin gene expression (Nuez et al. 1995). This is because KLF1 acts as an activator of the adult β-globin gene in adult erythrocytes by binding to a critical promoter element CACCC of the adult β-globin gene

and HS1-HS3 of the LCR (Im et al. 2005). It was in fact originally discovered as a result of studies in individuals with β-thalassemias who had the CACCC motif mutated (Miller, Bieker 1993). Recent studies by Borg *et al.*and Zhou *et al.* have found a link between KLF1, BCL11A and HPFH (Borg et al. 2010, Zhou et al. 2010). Borg et al performed linkage analysis for HPFH in a large Maltese family and defined the cause as a heterozygous truncating mutation in KLF1 which only partially reduced the KLF1 level (Borg et al. 2010)s. Zhou et al., devised a KLF1 knockdown mouse model harboring the human β-globin locus and showed that a partial reduction in the KLF1 levels as opposed to complete knockout had a minimal effect on β-globin expression and erythropoiesis while elevating fetal γ-globin levels (Zhou et al. 2010). While both these studies revealed that a partial reduction in KLF1 levels can lead to an increase in γ-globin expression, they also showed that this effect was mediated indirectly through BCL11A. They showed that KLF1 binds to BCL11A to elevate the transcription of the gene. Taken together, these studies have revealed that subtle reductions in KLF1 can have an effect on γ-globin expression without affecting β-globin expression and erythropoiesis. Thus KLF1 seems to have an important role in hemoglobin switching through two pathways: by directly activating the adult β-globin gene and by indirectly repressing γ-globin gene through BCL11A in adult erythroid cells (**Figure 7A**).

NF-E4 was initially identified as a critical transcription factor in chicken β-globin switching (Choi 1988, Gallarda et al. 1989). A human homologue of this protein, p22NF-E4 has been suggested to be involved in human β-globin switching (Zhao et al. 2004, Zhou et al. 2004). This protein along with a ubiquitous transcription factor CP2 constitutes a

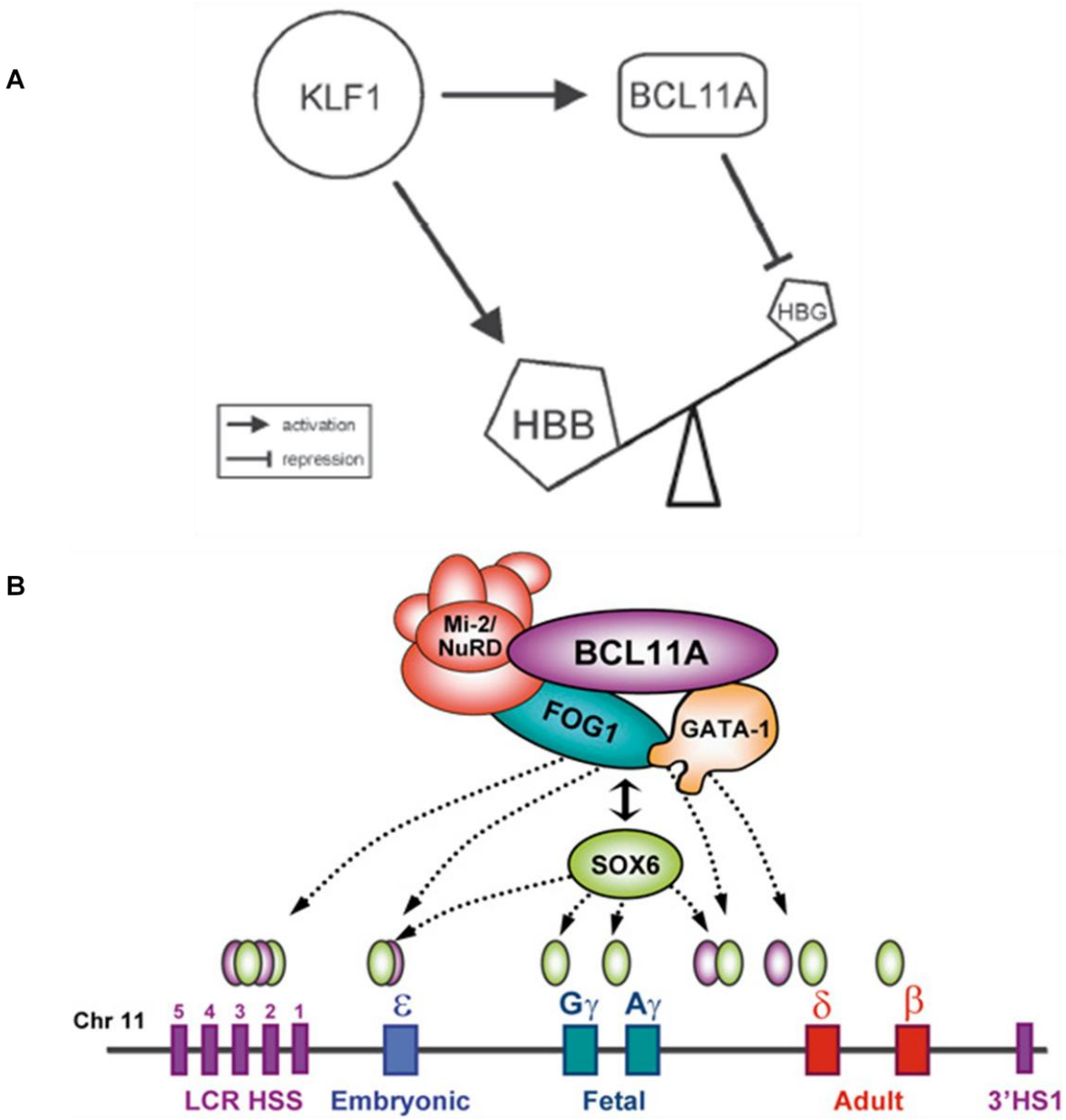

Figure 7A) Model for the regulation of β-globin genes by KLF1 and BCL11A in human adult erythroid cells: KLF1 activates adult β-globin gene and BCL11A in adult erythroid cells. BCL11A represses human fetal γ-globin gene. Thus, while KLF1 directly activates adult β-globin gene expression by binding to the gene, it is also involved in the silencing of the fetal globin gene by activating BCL11A. B) Model for BCL11A mediated silencing of the γ-globin gene: BCL11A interacts with Mi-2/NuRD and erythroid transcription factors such as FOG1, GATA1, and SOX6. However, BCL11A does not bind to the fetal globin gene. It has been shown to bind to the upstream LCR and the γ- δ-globin intergenic regions, suggesting the involvement of long range interactions in its silencing. Adapted from (Xu et al. 2010, Borg et al. 2010).

fetal transcription factor complex termed as stage selector protein (SSP) (Zhou et al. 2000). Enforced expression of this protein in transgenic mice delays the fetal to adult switch. However, this switch is eventually completed in the adult bone marrow (Zhou et al. 2004). The mechanism of the regulation of the hemoglobin switch by this protein is not clear.

A mass spectrometry screen was performed to identify the binding partners of NF-E4 protein. PRMT5 arginine methyltransferase was identified on the screen which indicated a potential role for this protein in globin gene regulation (Zhao et al. 2009). This study further showed that PRMT5 induces the repressive histone mark, H4R3me2s, which serves as a template for the binding of DNMT3A methyltransferase, leading to subsequent DNA methylation. Reduction of PRMT5 levels, or its enzymatic activity has been shown to cause elevation of γ-globin levels (Zhao et al. 2009, Rank et al. 2010).

A recent study has shown that Friend of PRMT1 (FOP) is one of the targets for methylation by PRMT arginine methyltransferases (van Dijk et al. 2010). FOP has been shown in this study to have a role in the silencing of γ-globin gene in adult erythroid cells. FOP is a chromatin-associated protein with a critical role in transcriptional regulation, including estrogen-dependent gene induction in breast cancer cells. The exact mechanism by which this protein may regulation γ-globin expression is unclear. It is speculated that this protein could be a crucial target for PRMT mediated γ-globin regulation (van Dijk et al. 2010).

The proximal promoters of ε- and γ-globin genes and not the adult β-globin genes contain direct repeat sequences similar to the DR1 binding sites for non-steroid nuclear hormone receptors. Mutations in these sites have been associated with HPFH syndrome

(Filipe 1999). Moreover, mutations of these sites in β-YAC transgenic mice lead to the elevated expression of ε-globin gene in adult erythroid cells (Filipe 1999, Tanimoto 2000). This indicates that the proteins that bind to these sites may have a role in the regulation of embryonic and fetal β-globin genes. COUP-TF orphan nuclear receptors have been shown to bind to these sites and SCF- mediated repression of COUP-TFII has been shown to be involved in inducing γ-globin genes in human adult progenitors (Filipe 1999, Aerbajinai et al. 2009; 2009).

TR2 and TR4 are another class of nuclear orphan receptors which have been shown to hetrodimerize and bind to the DR1 sites in the ε- and γ-globin promoters. One of the HPFH mutations was identified in the DR1 site in γ-globin promoter and was shown to disrupt TR2 and TR4 binding (Tanabe et al. 2002). TR2 and TR4 proteins are present in a complex termed as DRED (direct repeat erythroid definitive) complex, which was initially identified as a repressor complex bound at ε-globin promoter (Tanimoto 2000).

Ikaros-PYR complex transcription factor is involved in hemoglobin switching through a chromatin remodeling complex called PYR complex (Bank 2006, O'Neill 1999, O'Neill et al. 2000). The PYR complex consists of the SWI-SNF and NuRD chromatin remodeling complexes. This complex has been shown to bind to a DNA sequence upstream of the human δ-globin gene (O'Neill 1991). Ikaros knockout mice have a modest delay in β-globin switching and in β-YAC transgenic mice there is to a delay in the fetal to adult β-globin switch (Lopez 2002, Keys et al. 2008). However, these mice exhibit multiple hematopoietic defects.

Many of these trans-factors regulate the β-globin genes, by recruiting chromatin remodeling complexes such as SWI-SNF and NuRD. Our lab has identified MBD2 as another such factor which regulates human fetal γ-globin expression and chicken embryonic ρ-globin expression by recruitment of the NuRD complex. MBD2 binds to methylated DNA and recruits the NuRD corepressor complex to bring about chromatin remodeling to silence genes. DNA methylation and MBD2 and their role in the regulation of embryonic and fetal globin genes will be discussed in detail below.

IV. DNA methylation:

An epigenetic trait can be defined as a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence (Berger et al. 2009). DNA methylation is the first epigenetic mechanism to be discovered (Allis, Jenuwein & Reinberg 2007). It was in the late 1940s that the modification of DNA by covalently bound methyl groups was described (HOTCHKISS 1948, WYATT 1951). In 1962, it was identified that the CpG dinucleotide is the principal target for the methylation of DNA in vertebrates (**Figure 8A**) (DOSKOCIL, SORM 1962). Spurred by the discovery of X-inactivation, DNA methylation was first proposed to act as an epigenetic mark in 1975 by Riggs, Holliday and Pugh (Riggs 1975, Holliday, Pugh 1975). A direct inverse correlation between DNA methylation and gene expression was first described in the case of globin genes (Ginder, McGhee 1981, Razin, Riggs 1980, Vanderploeg, Flavell 1980, McGhee, Ginder 1979b, Shen, Maniatis 1980a).

DNA methylation has since been implicated in the transcriptional control of developmentally regulated genes, imprinted genes, X-inactivation and in the maintenance of genomic integrity by preventing the spread of the parasitic DNA elements like transposons (Ginder, Gnanapragasam & Mian 2008, Li, Bestor & Jaenisch 1992, Okano et al. 1999, Warnecke, Bestor 2000, Walsh, Bestor 1999, Venolia et al. 1982, Mohandas, Sparkes & Shapiro 1981). DNA methylation also has an important role in tumorigenesis by aberrantly silencing tumor suppressor genes (Baylin 2005, Egger et al. 2004).

DNA methylation is carried out by two major classes of DNA methyltransferase (DNMT) enzymes, which catalyze the transfer of a methyl group from S-adenosyl-methionine (SAM) to cytosine. The first group, which includes DNMT3a and DNMT3b, carry out de novo methylation on completely unmethylated substrate DNA (Okano et al. 1999, Hansen et al. 1999, Xu et al. 1999). The second group, DNMT1, copies methylation onto the newly synthesized DNA strand at replication, thus passing on the methylation pattern to daughter cells (Li, Bestor & Jaenisch 1992, Bestor et al. 1988). Evidence suggests some overlap in *de novo*/maintenance activity exists between the methyltransferases (Lian et al. 2002; Jair et al. 2006).

DNA methylation is essential for mammalian embryonic development. DNMT1 null mice die at mid-gestation (Li, Bestor & Jaenisch 1992, Bestor et al. 1988). These embryos show reduced DNA methylation levels. While DNMT3b knockout is embryonic lethal, DNMT3a knockout mice die shortly after birth (Xu et al. 2010, Okano et al. 1999, Hansen et al. 1999). The DNMT null mice have reduced DNA methylation levels leading

to defects in X-chromosome inactivation, aberrant expression of imprinted genes and elevation in the expression of transposons(Suzuki, Bird 2008).

DNA methylation mediates transcriptional repression through two main mechanisms (**Figure 8A**). The first is through direct interference with binding of transcription factors to the promoter region of a gene (Tate, Bird 1993, Brero, Leonhardt & Cardoso 2006). The second mode of repression is more common and involves methyl cytosine- binding proteins (MCBPs) that are attracted to the methyl cytosine signal (Ginder, Gnanapragasam & Mian 2008, Klose, Bird 2006).

A. Methyl cytosine binding proteins:

Methyl Cytosine Binding Proteinss (MCBPs), along with their associated corepressor factors and complexes mediate the repressive effect of DNA methylation through enzymatic modification of adjacent histones, recruitment of chromatin remodeling proteins, or direct inhibition of transcription initiation (Ginder, Gnanapragasam & Mian 2008). Two major classes of these proteins have been characterized: the methyl CpG binding domain (MBD) proteins, MBD1, MBD2, MBD4, and MeCP2, which share a related 80 amino-acid methyl-CpG binding domain (MBD) (Ginder, Gnanapragasam & Mian 2008, Klose, Bird 2006), and the Kaiso-like proteins, Kaiso, ZBTB4, and ZBTB38, which bind methylated CpGs via a conserved zinc finger motif which confers sequence specificity (Prokhortchouk et al. 2001, Filion et al. 2006).

The first methyl CpG binding activity was discovered in a complex termed MeCP1 (Meehan et al. 1989). It was later identified that MBD2 was the methylated DNA-binding

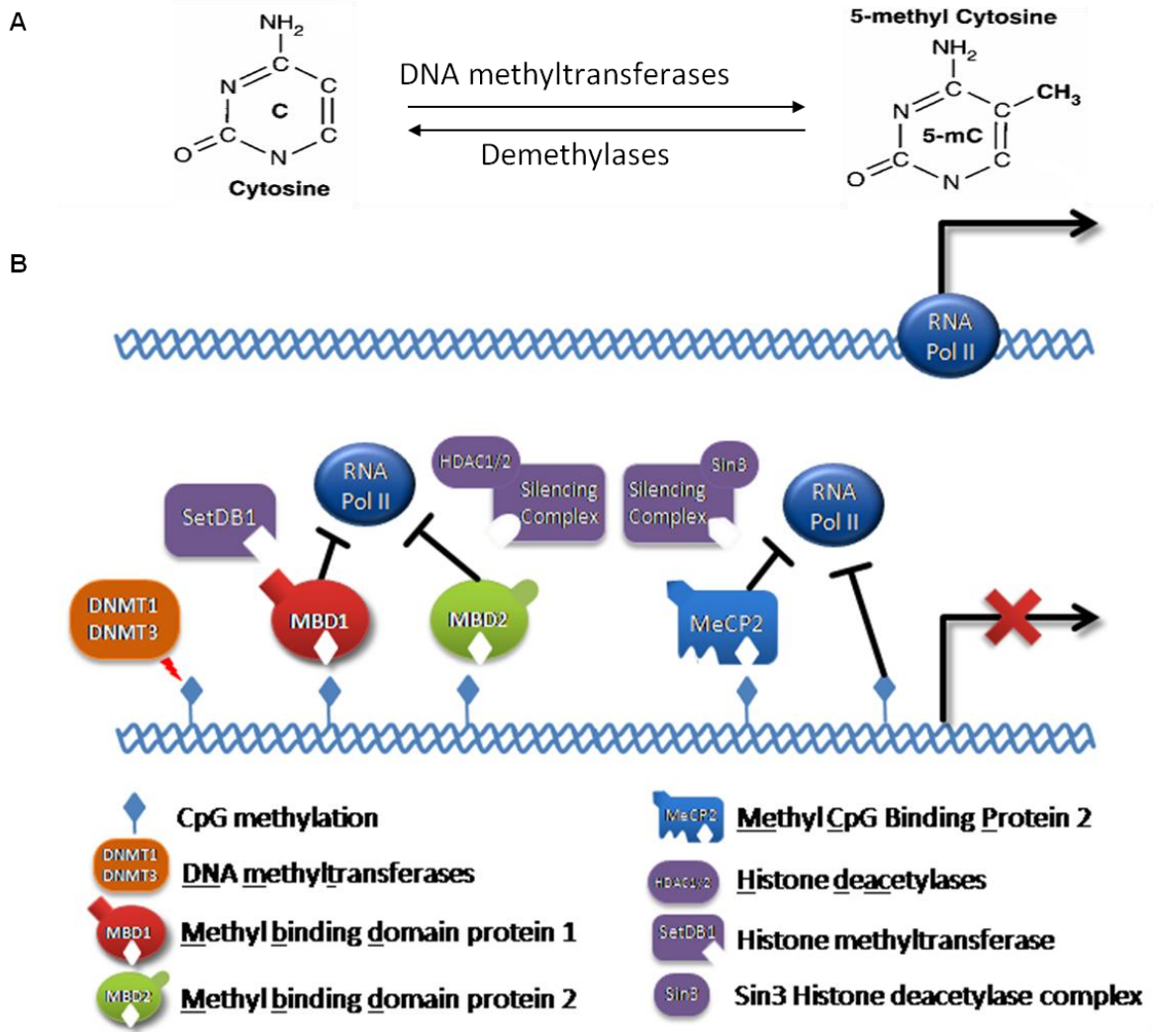

Figure 8 DNA methylation: A) Methyl Cytosine is synthesized by DNA methyl transferases (DNMT1, 3a and 3b) using SAM acts as the methylation donor. Demthylases are still under investigation. B) The mechanism of DNA methylation mediated transcriptional silencing: The top strand represents a transcriptionaly active gene. The bottom strand is methylated at the CpG residues by the action of DNA methyltransferases (DNMT1, 3a and 3b). DNA methylation or the recruitment of methyl CpG binding proteins can directly inhibit recrutiment of RNA polymerase and transcription factors. The main mechanism of repression is by the recruitment of co-repressor complexes by the methyl CpG binding proteins. These complexes contain histone deacetylases and chromatin remodeling proteins. Adapted from (Ginder, Gnanapragasam & Mian 2008).

component of this complex. MeCP2 was the first methyl-CpG binding domain protein to be cloned and is considered to be the founding member of the methyl-CpG binding domain family of proteins (Boyes, Bird 1991). In this family, only MBD3 has a non-functional MBD domain due to two critical amino acid substitutions with the domain (Saito, Ishikawa 2002). Thus, it is unable to preferentially bind to methylated CpGs.

Interestingly MBD3 knockout mice die embryonically (Hendrich et al. 2001). MBD4 on the other hand, is the only member of this family which is not involved in transcriptional regulation. Although it does preferentially bind to methylated DNA, it is more commonly associated with DNA repair/glycosylase activity in mammals (Hendrich et al. 1999). Knockout of MBD4 in mice causes an approximate three-fold increase in 5mC to T transitions (Millar et al. 2002, Wong et al. 2002).

MeCP2 has been shown to exhibit preference for methyl-CpGs flanked by short A-T rich stretches, while the other MBD proteins do not seem to exhibit any sequence preference beyond their affinity for methylated DNA (Klose, Bird 2006, Li, Bird 2007). Kaiso family of proteins do not show any sequence conservation with the methyl CpG binding domain protein family. In contrast to MBD family proteins, they bind symmetrically methylated CpGs in a sequence specific manner using a zinc finger motif (Prokhortchouk et al. 2001, Filion et al. 2006, Prokhortchouk et al. 2006).

While loss of DNA methylation in indispensible for embryonic development in mice, the Methyl CpG binding proteins with the exception of MBD3, have been shown to be dispensable for embryonic development (**Table 2**). Functional redundancy between the

Protein	Function	Associated Proteins
MeCP2	Transcriptional repression	Sin3A, Brahma
MBD1	Transcriptional repression	SETDB1/CAF-1
MBD2	Transcriptional repression	NuRD Complex
MBD3	Transcriptional repression (Does not bind methylated DNA)	NuRD Complex
MBD4	DNA Repair	MLH1
Kaiso	Transcriptional repression	N-CoR Complex

Table 1 Co-repressor complexes recruited by Methyl CpG binding proteins

Protein	Model system	Experimental approach	Phenotype	Reference
MeCP2	*Mus musculus*	Knockout	Neural, RTT-like phenotype	Guy et al. (2001)
	Xenopus laevis	Antisense knockdown	Improper neural patterning, embryonically lethal	Stancheva et al. (2003)
MBD1	*Mus musculus*	Knockout	Minor neural defects, increased genomic instability	Zhao et al. (2003)
MBD2	*Mus musculus*	Knockout	Mild maternal phenotype, abnormal differentiation, reduced tumorigenesis	Hendrich et al. (2001), Sansom et al. (2003)
MBD3	*Mus musculus*	Knockout	Failure in differentiation of pluripotent cells embryonically lethal	Hendrich et al. (2001), Kaji et al. (2007)
	Xenopus laevis	Antisense knockdown	Defective eye formation, embryonically lethal	Iwano et al. (2004)
MBD4	*Mus musculus*	Knockout	No apparent phenotype, increased mutation rate	Millar et al. (2002), Wong et al. (2002)
Kaiso	*Mus musculus*	Knockout	No apparent phenotype, reduced tumorigenesis	Prokhortchouk et al. (2006)
	Xenopus laevis	Antisense knockdown	Premature activation of zygotic transcription	Ruzov et al. (2004)

Table 2 The phenotypes caused by the loss of function of Methyl-CpG binding proteins ((Bogdanovic, Veenstra 2009)**.**

proteins has been speculated to be one of the reasons for this observation in mammals. However, in a recent study it was shown that knockout of three of the methyl CpG binding proteins, MeCP2, MBD2 and Kaiso, is dispensible for embryogenesis (Cabalerro et al. 2009)

While MBD proteins are not important during embryonic development in mammals, they seem to be particularly important for nervous system functioning postnatal. MeCP2 null mice exhibit several neurological defects and die at around six weeks of age (Chen et al. 2001). These defects are specific to neuronal maturation and not differentiation. An MeCP2 mutation causes Rett syndrome in women (Amir et al. 1999). MBD1 null mice exhibit impaired adult hippocampal neurogenesis and increased genomic instability in neural stem cells (Zhao et al. 2003). MBD2 null mice display defects in maternal behavior (Hendrich et al. 2001). Mice deficient for Kaiso do not show any overt phenotypes (Prokhortchouk et al. 2006).

Biochemical analysis of MeCP2, MBD1, MBD2, and Kaiso has provided evidence that each can associate with a specific corepressor complex and bring about transcriptional silencing (**Table 1**) (Ginder, Gnanapragasam & Mian 2008, Klose, Bird 2006).

V. Methyl CpG Binding Domain Protein 2 (MBD2):

MBD2 recruits Nucleosomal Remodeling and Histone Deacetylase (NuRD) co-repressor complex to mediate transcriptional silencing. MBD2 along with NuRD is termed as Methyl-CpG binding protein complex1 (MeCP1). It is a large multiprotein complex

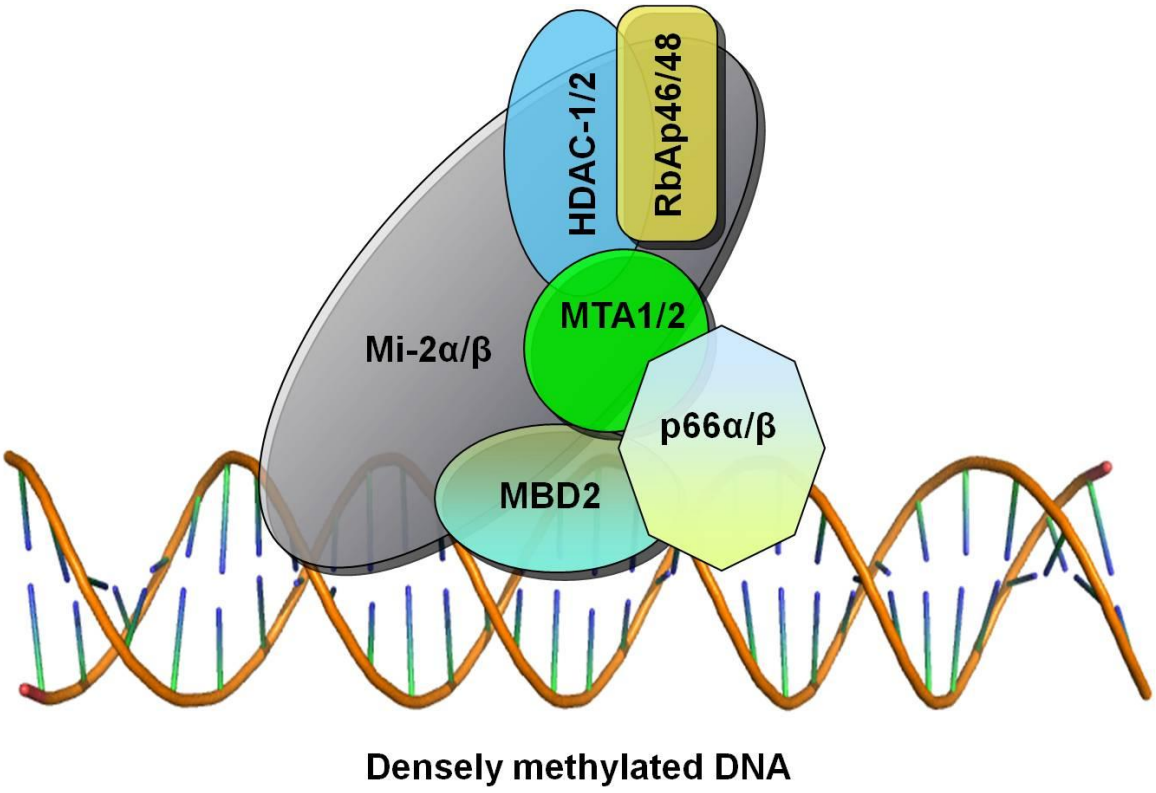

Figure 9 MeCP1 complex: Schematic representation of the six core components found in the MeCP1 complex.

(Wade et al. 1999). NURD complex, in the context of MeCP1 has been found to contain p66α/β transcriptional repressor, a large chromatin-remodeling protein (Mi-2α/β), histone deacetylases (HDAC1/2), histone binding proteins (RbAp46/RbAp48) and transcriptional repressor MTA1/2, whose activity in the NURD complex is not clear (**Figure 9**) (Ginder, Gnanapragasam & Mian 2008, Li, Bird 2007, Wade et al. 1999, Feng, Zhang 2001, Nan et al. 1998, Ng et al. 1999). Initial studies documented that MBD3 was also part of this complex. However, the emerging view is that MBD2-NuRD and MBD3-NuRD are distinct complexes (Le Guezennec et al. 2006).

Although MBD2 has been shown to bind to a sequence containing as few as three CpGs in vitro (Fraga et al. 2003), MeCP1 complex as a whole seems to require at least 15 CpGs per complex in vitro (Meehan et al. 1989, Wade 2001). Moreover, in vivo studies have documented MBD2 binding, only to methylated CpG island sequences (Ginder, Gnanapragasam & Mian 2008).

MBD2-/- mice are viable and fertile (Hendrich et al. 2001). They display abnormal maternal nurturing behavior (Hendrich et al. 2001). Molecular analyses of T cell differentiation in MBD2 knockout mice revealed the disruption of temporal and spatial expression patterns of interleukin 4 and interferon γ genes in T helper (TH) cells, which alters the immune response in these animals (Kersh 2006, Hutchins et al. 2002). Investigation of gene expression in the colon of MBD2-/- mice revealed aberrant overexpression of ExPa genes, a set of genes coding for digestive enzymes normally expressed only in duodenum and pancreas (Berger et al. 2007). A recent study has shown that the loss of MBD2 leads to dysregulation of adult olfactory epithelial progenitor-driven

neurogenesis (Macdonald et al. 2010). MBD2 null mice display enhanced proliferation of the progenitors and decreased life span of olfactory receptor neurons (Macdonald et al. 2010).

Loss of MBD2 causes a moderate but significant induction of Xist gene in SV-40 transformed fibroblast cell lines derived from the tails of mice deficient for MBD2. The product of Xist is a nontranslated RNA that triggers cis-inactivation of X chromosome. Thus, the Xist gene in the inactive X chromosome is expressed while the gene in the active X-chromosome is silenced. The silencing of Xist gene was restored by exogenous MBD2 by directly binding to the gene (Barr et al. 2007).

Loss of MBD2 has been shown to strongly suppresses the formation of intestinal adenoma in mice that are heterozygous for a mutation in Apc ($Apc^{min/+}$) mice while the $Apc^{min/+}$ mice which are wild type for MBD2 develop multiple intestinal neoplasia (Berger, Bird 2005b, Berger, Bird 2005a, Sansom et al. 2003). The mechanism for the suppression of the intestinal neoplasia by MBD2 has recently been documented. MBD2 has been shown to bind and silence the Lect2 gene whose product is an inhibitor of Wnt (Phesse et al. 2008). Increased Wnt signaling is a key event in intestinal cancer. In the absence of MBD2 the Wnt signaling pathway is attenuated leading to the suppression of intestinal neoplasia in the $Apc^{min/+}$ mice (Phesse et al. 2008). MBD2 is also involved in silencing tumor suppressor genes in many cancers. In fact, among all the methyl CpG binding proteins, MBD2 binds the largest known proportion of genes in human tumors (Esteller 2008, Ballestar, Esteller 2008, Ballestar, Esteller 2005, Lopez-Serra et al. 2008).

The studies performed in our lab have shown that MBD2 knockout causes the reexpression of human fetal γ-globin gene expression and chicken embryonic ρ-globin gene in adult erythrocytes (Rupon et al. 2006, Kransdorf et al. 2006). These studies will be discussed in detail in the following sections.

VI. The role of DNA methylation in β-globin switching:

The initial correlations between DNA methylation and gene expression were documented in the β-globin gene clusters. Site-specific cytosine methylation within or adjacent to these genes was found to correlate with the transcriptional repression (McGhee, Ginder 1979a, Ginder, McGhee 1981, Razin, Riggs 1980, Vanderploeg, Flavell 1980, Shen, Maniatis 1980a). The β-globin genes were also the first group of genes for which the treatment with 5-azacytidine, an inhibitor of DNA methylation, was shown to alter the developmentally established patterns of gene expression by increasing levels of the fetal γ-globin gene in adult erythrocytes. This was first demonstrated in baboons and subsequently in patients with β-thalassemia and sickle cell anemia (Charache et al. 1983, Desimone et al. 1982, Ley et al. 1982a).

Since then there has been a debate about whether DNA methylation is a primary mechanism involved in initiating globin gene switching or a secondary event (Enver et al. 1988). There is a general consensus that methylation can serve as a lock-off mechanism that may follow other events that initiate developmental globin gene repression (Singal, vanWert 2001, Rupon 2006). Once in place, DNA methylation can prevent transcription despite an optimum nuclear trans-factor environment.

A. DNA methylation and the regulation of Chicken β-globin locus:

Among vertebrates, the chicken β-globin gene cluster (5' ρ-βH-βA-ε-3') on chromosome 7, has been extensively studied with regard to the role of DNA methylation in the developmental regulation of β-globin switching. The adult globin genes, β^H and β^A, are flanked by the embryonic globin genes, ρ and ε (**Figure 10**). ρ-globin is expressed abundantly in primitive erythrocytes in the yolk sac from 36 h of embryogenesis to day 5 (Chan, Wiedmann & Ingram 1974). Definitive erythrocytes are produced from the fetal liver from day 5 and later from the bone marrow, and these cells express the adult β-globins (Chan, Wiedmann & Ingram 1974). Thus, the transcriptional silencing of the embryonic ρ-globin gene occurs concomitantly with activation of the adult β-globin genes on day 5 of embryonic development (Chan, Wiedmann & Ingram 1974, Groudine, Peretz & Weintraub 1981)

A strong inverse correlation exists between site-specific DNA methylation and expression of the chicken β-type globin genes (McGhee, Ginder 1979a, Ginder, McGhee 1981). Every CpG site in the 235 bp promoter and 248 bp proximally transcribed region (exon 1 and intron1) of the ρ-globin gene, is methylated in definitive erythrocytes but unmethylated in primitive erythrocytes (Ginder, McGhee 1981, Singal 1997, Singal, vanWert & Ferdinand 2002). The DNA methylation inhibitor, 5-azacytidine, induces the transcription of the ρ-globin gene and causes a concomitant loss of CpG methylation (Ginder 1984). The induction of ρ-globin gene transcription is increased 5-10-fold further when treatment with 5-azacytidine is followed by the treatment with histone deacetylase inhibitors such as butyrate or trichostatin A; but the histone deacetylase inhibitors alone do

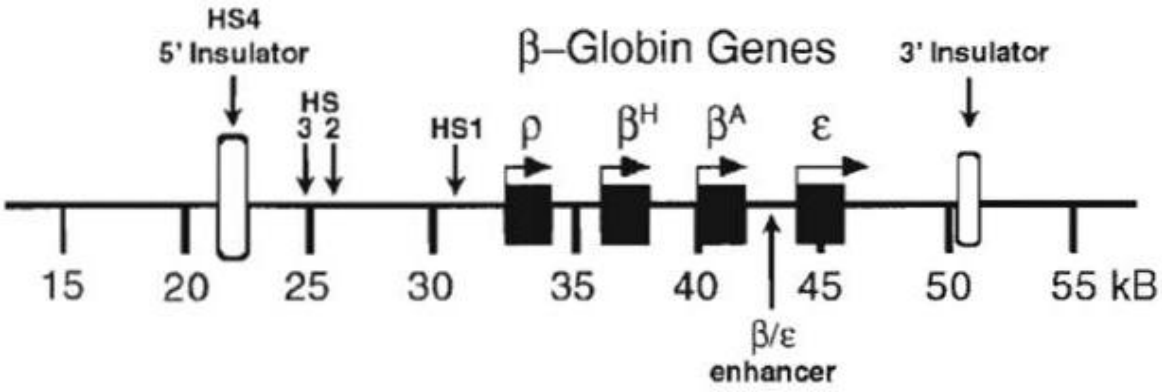

Figure 10 The map of the chicken β-globin locus. The arrows indicate the DNAse hypersensitive sites. The shaded boxes represent the β-type globin genes in the locus. Adapted from (Kransdorf 2006).

not have any effect on the ρ-globin gene transcription (Ginder 1984, Singal et al. 2002). These studies indicate that DNA methylation dominates over histone acetylation in the silencing the chicken embryonic ρ-globin gene.

B. DNA methylation and the mouse β-globin locus regulation:

A number of studies in other vertebrates such as mouse, baboons and humans support a role for DNA methylation in developmental β-type globin gene silencing. The chicken β-globin locus differs from the loci of mice, baboons or humans in the CpG density. Three (ρ-, β^A -, and ε-globin) of the four β-globin genes in the chicken locus contain CpG islands that overlap with the promoter. On the other hand, the β-globin loci of mice, baboons, and humans do not have CpG islands (Ginder, Gnanapragasam & Mian 2008). In mice, the small numbers of CpGs in the embryonic β-globin gene promoters as well as 5'HS2 of the LCR are hypomethylated in primitive erythroid cells which have high expression of the embryonic globins. On the other hand, the β-major promoter and the β-minor coding region are hypermethylated in these primitive cells correlating with the lack of expression of the genes in these cells (Hsu et al. 2007). In definitive mouse erythroid cells, 5'HS2 and the βmaj promoter are hypomethylated while the embryonic globins are hypermethylated in correlation with their expression patterns (Kiefer et al. 2008).

C. DNA methylation and the regulation of β-globin genes in baboons:

Among primates, the old world simian primates and not the prosimians or other species, have a highly conserved β-globin gene structure and pattern of expression

(Johnson 2002b). In fact, the γ-globin gene gained fetal stage-specific expression during the transition from prosimians to simians (Tagle et al. 1988). For this reason, baboons, which are simian primates, have served as excellent animal models for studies on β-globin gene regulation (Desimone, Mueller 1978, Barrie, Jeffreys & Scott 1981). In baboons, an inverse correlation is observed between DNA methylation of the ε- and γ-globin gene promoters and the expression of the genes during globin gene switching (Lavelle et al. 2006). Methylation of the ε- and γ-globin gene promoters, during ε-to-γ and γ-to-β globin switching respectively, is initiated in an incomplete manner with no observed preference for any individual CpG sites (Lavelle et al. 2006). These observations support a stochastic model in which initial low levels of methylation are predicted to increase over time as a consequence of both *de novo* and maintenance methylation (Lavelle et al. 2006). These data again suggest that DNA methylation may not be the initiating event in globin gene silencing, but is involved in a lock-off mechanism in β-globin gene regulation.

D. DNA methylation and the regulation of the human β-globin genes:

In the human β-globin locus, the same inverse correlation between gene expression and DNA methylation has been noted. The human fetal γ-globin genes are hypomethylated in tissues where they are highly expressed and hypermethylated in adult erythroid cells and non-erythroid tissues (Singal, Ginder , Mavilio et al. 1983, Mabaera et al. 2007). It was observed that in the primary cells from human yolk sac and fetal liver, the CpGs in the ε and γ-globin promoters respectively are hypo-methylated (Mavilio et al. 1983, Mabaera et al. 2007). On the other hand, the CpGs in the promoters of adult β-globin genes are

hypomethylated in the erythroid cells from adult bone marrow (Mavilio et al. 1983, Mabaera et al. 2007). This correlation was also observed during erythroid differentiation. In early differentiating adult bone marrow cells that express high levels of γ-globin, the γ-globin promoter is hypomethylated, and during later stages of differentiation when the gene is silenced the promoter is hypermethylated (Kiefer et al. 2008, Mabaera et al. 2007). In the case of infants of diabetic mothers, a delayed switch from fetal to adult globin is observed. These infants have hypomethylation of γ-globin gene compared to control infants in correlation with the expression status of γ-globin (Perrine et al. 1988).

The argument that DNA methylation may be a secondary event during globin gene switching in the human globin locus was strengthened by studies performed by Enver and colleagues (Enver et al. 1988). They used somatic cell hybrids obtained by fusing mouse erythroleukemia cells and human fetal erythroid cells, which express γ-globin initially but switch to β-globin with time in culture. Before this switch the γ-globin promoter remains unmethylated. However, during the switch when the hybrids no longer express γ-globin, the promoter still remains unmethylated although over time they become methylated; this suggests that methylation of DNA in this promoter may not be a primary event in the silencing. However, it is to be noted that in this study only two CpG sites (-53 and +1805) in the γ-globin promoter were studied. Recent data from studies of human β-globin YAC transgenic mice lacking the methyl cytosine binding protein 2 (MBD2), also support this argument. Knockout of MBD2 in mice caused delayed silencing of the fetal γ-globin gene during embryonic erythroid development. However, the DNA methylation in the promoter was only modestly decreased, suggesting that DNA methylation may be a secondary event

in the regulation of this gene (Rupon 2006). The role of MBD2 in globin gene regulation will be discussed in detail below.

VII. The role of MBD2 in β-globin gene regulation:

The studies performed previously in our laboratory demonstrated a role for MBD2 in the regulation of both the chicken embryonic ρ-globin gene and human fetal γ-globin gene. An erythroid methyl cytosine binding protein complex containing MBD2 assembles on the methylated chicken ρ-globin promoter and proximal transcribed region (exon1 and intron1) when incubated with chicken erythroid nuclear extracts *in vitro* (Singal 1997, Singal et al. 2002). This offered a mechanism for the DNA methylation mediated silencing of chicken embryonic ρ-globin gene.

In order to further understand the mechanism, the complex in primary chicken adult erythroid cells was biochemically characterized. The complex consisted of the canonical components of the MeCP1 complex, but did not contain MBD3 (**Figure 11B**) (Kransdorf et al. 2006).The view emerging based on this study and work from other laboratories is that MBD2-NuRD and MBD3-NuRD are two distinct complexes (Le Guezennec et al. 2006, Kransdorf et al. 2006). Chromatin immunoprecipitation assays confirmed that MBD2 is bound to the methylated ρ-globin gene in adult chicken erythrocytes *in vivo* similar to results *in vitro* (**Figure 11A**). As expected, the ChIP assays did not detect any MBD2 binding to the gene in 5 day chicken embryonic erythroid cells (Kransdorf et al. 2006).

The role of MBD2 in ρ-globin gene silencing was further probed using mouse erythroleukemia (MEL) cells. The cells were stably transfected with *in vitro* methylated ρ-

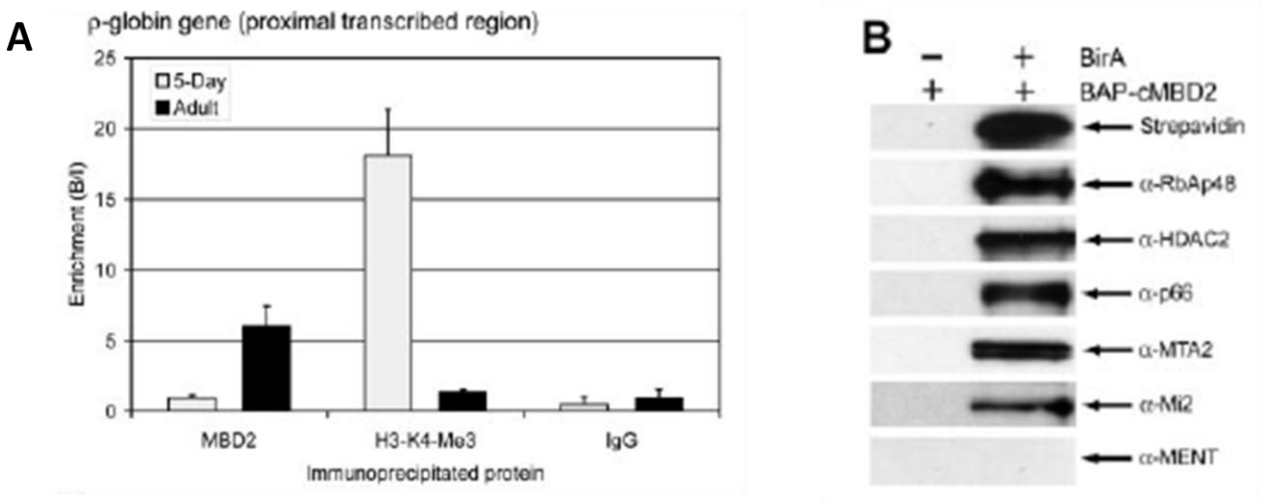

Figure 11: MBD2 and chicken ρ-globin gene regulation: A) Enrichment for MBD2, H3-K4-Me3, and IgG at the rho-globin gene in 5-day and adult erythrocytes as determined by ChIP assay B) Western blot analysis of the eluate from cMBD2 pull-down experiments in BAP-cMBD2 6C2 cells. The major components of the MeCP1 complex are seen in the eluate from pulldown. (Kransdorf et al. 2006)

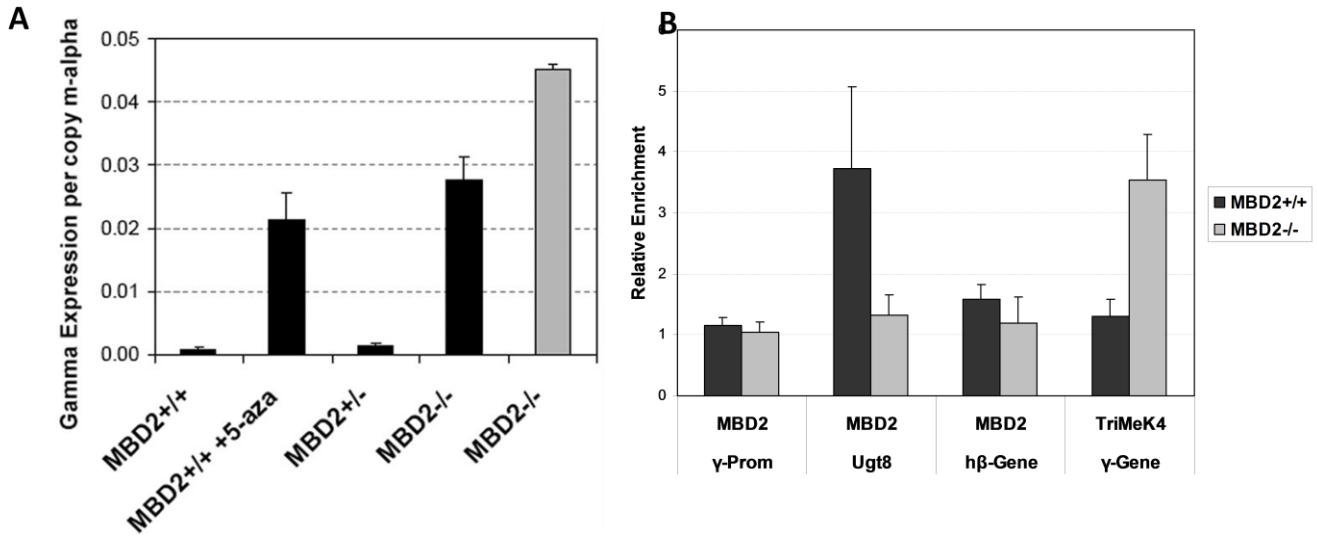

Figure 12 MBD2 and human γ-globin gene regulation: A)Quantitation of RNase protection assay (RPA) results. The level of γ-globin mRNA is expressed per copy of the endogenous mouse α-globin. B) Results from ChIP assay. Chromatin was immunoprecipitated with anti-MBD2, anti-TriMeK4, and control antibodies (IgG or normal serum) from MBD2 +/+ and MBD2 -/- splenic erythroblasts. The sequence-specific enrichment was determined by real-time PCR (Rupon et al. 2006).

globin gene causing transcriptional silencing of the gene. ChIP assays showed that MBD2 bound to the silent gene along with the other components of the NuRD complex (Kransdorf et al. 2006). Moreover, when MBD2 was knocked down using shRNA in these cells, transcriptional de-repression of the ρ-globin gene was observed indicating a critical role for MBD2 in its silencing (Kransdorf et al. 2006).

Next, the role of MBD2 in the silencing of human fetal γ-globin gene in adult erythrocytes was examined using β-YAC transgenic mice. MBD2 knockout β-YAC transgenic mice, showed an elevation in the levels of fetal γ-globin gene in adult erythrocytes, similar to the level obtained with treatment of wild type mice with DNA methylation inhibitor 5-azacytidine (**Figure 12**) (Rupon et al. 2006). Moreover, it was observed that in the absence of MBD2, the developmental silencing of γ-globin is delayed. Taken together, these observations showed that MBD2 plays a role in the DNA methylation mediated silencing of the fetal γ-globin gene.

In contrast to the observation in the chicken embryonic ρ-globin gene, MBD2 was not found to bind to the γ-globin promoter (Rupon et al. 2006, Kransdorf et al. 2006). MBD2 containing MeCP1 complex requires densely methylated DNA for its binding (Meehan et al. 1989). Thus, the absence of its binding to the gene is not surprising because there are no CpG islands in the γ-globin genes, unlike the chicken ρ-globin gene.

VIII. Scope of the thesis:

Previous studies from our laboratory have demonstrated that MBD2 plays a critical role in the silencing of the embryonic/fetal globin genes in adult erythrocytes (Rupon et al. 2006). Further, MBD2 containing MeCP1 complex was biochemically characterized in primary erythroid cells (Kransdorf et al. 2006). How the different components in the complex interact with each other and how these interactions contribute to the repressor activity of the MeCP1 complex is not very well understood. The work presented in Chapter 2 of this thesis has focused on understanding the interaction between two of the components of the MeCP1 complex: MBD2 and p66α. In addition, the importance of this interaction in the silencing of chicken embryonic ρ-globin and human fetal γ-globin has been examined.

We observed that knockout of MBD2 in β-YAC transgenic mice causes the re-expression of fetal γ-globin gene in adult erythrocytes (Rupon et al. 2006). However, MBD2 does not bind directly to the γ-globin gene. MBD2 requires multiple methylated cytosines for its binding and the gene lacks densely methylated CpGs (Meehan et al. 1989). This thesis focuses on understanding the mechanism by which MBD2 mediates the silencing of γ-globin gene without binding directly to the gene. The work presented in Chapter 3, examines the hypothesis that the loss of MBD2 results in transcriptional activation of a gene or genes that are normally silent in adult erythroid cells. The product of this gene(s) would, in turn, result in transcriptional activation of the γ-globin gene.

IX. Significance:

Many compounds currently available to treat patients with hemoglobinopathies carry short- or long-term potential risks of toxicity and, in addition, the responses to the agents are variable (Stamatoyannopoulos G., Grosveld F. 2001). Increasing the fetal hemoglobin (HbF) levels is a viable therapeutic strategy because it ameliorates the symptoms and increases the life span of individuals suffering from β-hemoglobinopathies (Stamatoyannopoulos G., Grosveld F. 2001). Although 5-azacytidine is successful in inducing HbF amounts in patients with sickle cell anemia and β-thalassemia, it has been used primarily in older patients with severe disease because its risk of carcinogenicity (Stamatoyannopoulos G., Grosveld F. 2001). MBD2 is found to increase HbF to levels similar to those attained with 5-azacytidine treatment in β-YAC transgenic mice (Rupon et al. 2006). Interestingly, it is not required for normal mammalian embryonic development and confers only a subtle phenotype in null mice (Hendrich et al. 2001). These observations together indicate that MBD2 may be an excellent target for therapeutic reactivation of the γ-globin gene in adult erythrocytes.

Thus, strengthening our understanding of how MBD2 and the MeCP1 complex regulates embryonic/fetal globin gene will contribute greatly to devising better therapeutic strategies to augment the fetal hemoglobin expression. Disrupting the function of MBD2 and the MeCP1 complex could also be therapeutically applied in the treatment of cancer since MBD2 plays a prominent role in silencing tumor suppressor genes in cancers (Esteller 2008, Ballestar, Esteller 2008, Ballestar, Esteller 2005, Lopez-Serra et al. 2008).

CHAPTER 2: Characterization of the role of the coiled coil interaction between MBD2 and p66α in the regulation of embryonic/fetal globin genes

I. Introduction:

MBD2 belongs to the family of methyl CpG binding domain proteins that recognize methylated CpGs through a ~60 amino acid methyl binding domain (MBD) (Wade et al. 1999). Although knocking out DNA methyltransferases (DNMTs) results in embryonic lethality, MBD2 is dispensable to embryonic development and contributes to only a subtle phenotype (Hendrich et al. 2001).

MBD2-/- females have abnormalities in maternal nurturing behavior (Hendrich et al. 2001). MBD2 knockout mice also exhibit disruption of temporal and spatial expression patterns of interleukin 4 and interferon γ during T cell differentiation (Kersh 2006), abnormal expression of digestive enzyme genes in the colon (Berger et al. 2007), dysregulation of adult olfactory epithelial progenitor-driven neurogenesis (Macdonald et al. 2010) and dysregulated expression of the Xist gene (Barr et al. 2007). Moreover, loss of MBD2 suppresses the formation of intestinal adenoma in $Apc^{min/+}$ mice by attenuating the Wnt signaling pathway (Berger, Bird 2005a). MBD2 is also involved in silencing tumor suppressor genes in many cancers (Esteller 2008, Ballestar, Esteller 2008, Ballestar, Esteller 2005, Lopez-Serra et al. 2008).

The role of MBD2 in globin gene regulation was first documented in the chicken embryonic ρ-globin gene (Kransdorf et al. 2006, Singal et al. 2002). The chicken β-globin gene cluster (5' ρ-β^H-β^A-ε-3') is located on chromosome 7. The adult globin genes, β^H and β^A, are flanked by the embryonic globin genes, ρ and ε. The ρ-globin is expressed in primitive erythrocytes in the yolk sac from 36 h of embryogenesis to day 5 (Chan, Wiedmann & Ingram 1974). Definitive erythrocytes are produced in the fetal liver on day 5 and later in the bone marrow, and these cells express the adult β-globins (Chan, Wiedmann & Ingram 1974).

Work in our laboratory has established that every CpG site in the 235 bp promoter and 248 bp proximally transcribed region (exon 1 and intron1) of the ρ-globin gene is methylated in adult erythrocytes but is unmethylated in primitive embryonic erythrocytes (Ginder, McGhee 1981, Singal 1997, Singal, vanWert & Ferdinand 2002). An erythroid Methyl Cytosine Binding Protein Complex containing MBD2 forms on the methylated promoter and proximal transcribed region of the ρ-globin gene in both *in vitro* and *in vivo* analyses (Kransdorf et al. 2006, Singal 1997, Singal et al. 2002). Moreover, when MBD2 was knocked down in mouse erythroleukemia cells harboring in vitro methylated ρ-globin gene (MEL ρ cells), derepression of the ρ-globin is observed (Kransdorf et al. 2006). These studies taken together indicate a critical role for MBD2 and the MeCP1 complex in the silencing of chicken embryonic ρ-globin gene.

The role of MBD2 in the regulation of human fetal γ-globin gene was examined using MBD2-/- β-YAC transgenic mice. The human β-globin locus on chromosome 11 contains the genes that encode the β-chain of hemoglobin. They are located 5' to 3' in the

order of their expression during development: 5' ε, γ, δ, and β 3'. Two switches in globin expression occur during development. The first occurs during the switch from primitive to definitive hematopoiesis at 5 weeks post- conception. At this time, γ-globin replaces ε globin as the major γ chain subunit. A change in the site of hematopoeisis form the yolk sac to fetal liver also occurs at this time. At the time of birth, the second switch takes place whereby β globin and a small amount of δ-globin replace γ-globin. By the time of birth, bone marrow is the primary site of hematopoiesis. The ability of transgenic mice, containing the entire human β-globin locus, to correctly regulate human β-globin transgenes during development, makes them a valuable model in the study of β-globin gene switching.

When MBD2-/- mice were crossed with β-YAC transgenic mice, human fetal γ-globin gene expression was elevated by at least 10 fold compared to wild type β-YAC transgenic mice. This level was similar to that obtained with the treatment of DNA methylation inhibitor 5-azacytidine (Rupon et al. 2006). However, in contrast to the chicken embryonic ρ-globin gene, MBD2 was not found to directly bind to the human fetal ρ-globin gene (Rupon et al. 2006). Nevertheless, these studies indicated that MBD2 plays a role in the DNA methylation mediated silencing of the fetal γ-globin gene.

The erythroid MeCP1 complex was biochemically characterized in chicken primary adult erythroid cells (Kransdorf et al. 2006). This was the first time the complex was studied in primary cells as previous characterizations were performed in cell lines. These studies revealed that this erythroid MeCP1 complex contains the canonical MeCP1 components such as MBD2, p66, Rbap, Mi2 and HDAC. However, this complex did not

contain MBD3. MBD3 has an MBD domain that differs from MBD2 in two critical amino acids in the methyl CpG binding (MBD) domain that render it unable to bind to methylated DNA. MBD2 and MBD3 were initially reported to be present in the same complex. However, recent studies including ours have revealed that MBD2 NURD and MBD3 NURD are mutually exclusive complexes (Le Guezennec et al. 2006).

It is not well understood how the individual components in the MeCP1 complex are assembled and how these components interact with each other to contribute to the transcriptional silencing. This knowledge will be crucial in devising strategies to disrupt the function of the complex.

We have sought to understand the interaction between two components in this complex: p66α transcriptional repressor and MBD2. Previous studies have shown that p66 knockdown can abrogate MBD2 mediated transcriptional repression (Brackertz et al. 2006). However, these studies were performed using MBD2 fused to the Gal4 DNA binding domain and by measuring activity of a luciferase reporter containing Gal4 DNA binding sites. The contribution of the interaction between p66 and MBD2 on MBD2 mediated silencing of endogenous genes has not yet been examined. The aim of this study is to understand the interaction between these two proteins in MBD2 mediated globin gene regulation.

The human MBD2a protein is 411 amino acids long. It contains an N-terminal glycine-arginine repeat region, a methyl-binding domain, a putative transcription repression domain, a region implicated in binding the RbAp46/48 homologue p55 in Drosophila (Marhold, Brehm & Kramer 2004),and a coiled-coil region (**Figure 13**). The

p66α/β proteins are 66 kDa transcriptional repressors and contain two highly conserved regions: an N-terminal coiled-coil domain (CR1) and a C-terminal GATA-like zinc finger domain (CR2) (**Figure 13**) (Brackertz et al. 2006, Feng et al. 2002, Brackertz et al. 2002). Previous studies have shown that the CR1 coiled coil domain of p66α, can directly bind the coiled coil domain of MBD2 (Brackertz et al. 2006, Feng et al. 2002, Brackertz et al. 2002). In this study we have examined the role of this interaction in the MBD2 mediated regulation of chicken embryonic ρ-globin gene and human fetal γ-globin gene.

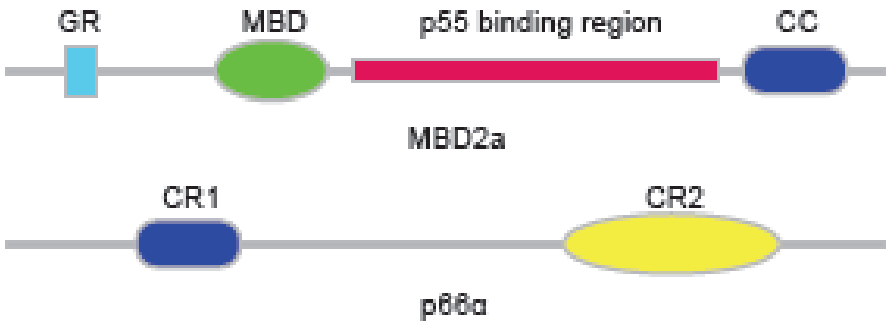

Figure 13. Domain organization of MBD2 and p66α: The top panel shows the domain organization of MBD2a. GR is a glycine-arginine repeat region; MBD is a methyl binding domain; p55 binding region was identified first in Drosophila as interacting with RbAp46/48 homologue p55; CC is a ~30 amino acid region predicted to form a coiled coil. The bottom panel shows the domain organization of p66α: CR1 is a coiled coil domain; CR2 includes a GATA zinc finger domain.

II. Methods

Protein preparation:

The coiled-coil regions of human MBD2b (amino acids 211-244) and p66α (amino acids 138-178) were cloned and expressed as thioredoxin fusion proteins in a modified pET32a vector (Cai et al. 2003). Smaller segments of the p66α coiled-coil region (N-terminal short:144-158; C-terminal short:152-168; and mid-region:144-168) were cloned and expressed in a similar manner. The proteins were purified by nickel sepharose affinity chromatography and preparative scale size exclusion chromatography. After combining at a 1:1 molar ratio, the thioredoxin fusion was removed by thrombin digestion. Isotopically labeled (^{15}N, ^{13}C) p66α (or MBD2) coiled-coil domain was expressed, purified as a monomer or as a heterodimer with unlabeled MBD2 (or p66α, respectively) coiled-coil domain. A stable complex was then isolated by ion exchange chromatography over a Mono S 5/50 GL column and buffer exchanged into NMR buffer (10mM sodium phosphate, pH 6.5, 0.01% sodium azide, 1 mM diothiothreitol, 10% $^{2}H_2O$).

Analytical ultracentrifugation and binding analysis:

The p66α and MBD2 coiled-coil domains with N-terminal thioredoxin fusions were analyzed by gel filtration chromatography using a Superdex75 10/300 (GEHealthcare) under physiologic conditions (20mM Tris pH 8.0, 150mM NaCl, 2mM □-mercaptoethanol). Analytical ultracentrifugation analysis was performed on an Beckman XL-I Analytical Ultracentrifuge in the same buffer at approximately 1 mg/mL protein

concentration (~50 μM). The sedimentation velocity was fit using a continuous size distribution (c(s)) and the effective molecular weight determined from the resulting sedimentation coefficients with the SEDFIT software (sedfitsedphat.nibib.nih.gov). Binding kinetics and affinity were analyzed by surface plasmon resonance on a Biacore T100 (Jason-Moller, Murphy & Bruno). A Sensor Chip CM5 was activated by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS). MBD2 coiled-coil thioredoxin fusion was coupled to the experimental channel and thioredoxin alone was coupled to the reference channel per the manufacturer's protocol. The p66α coiled-coil thioredox fusion was passed over the surface in concentrations ranging from 200 nM to 7 nM and the resulting sensorgram fit to a 1:1 binding model.

Expression constructs for the p66α and MBD2 coiled-coil domains were modified to incorporate N-terminal tyrosine residues and the individual peptides expressed and purified as described above. Circular dichroism spectra were collected on peptide samples (33.3 μg/mL total protein, 10mM sodium phosphate, pH 6.5) with a JASCO J-715 CD spectrometer (JASCO Corp) at 293 K, scanning from 190-260 nM (0.5 nM intervals), and analyzed for helical content with the DICHROWEB software using the k2d algorithm. Thermal unfolding was followed by measuring molar ellipticity at 222nm from 277 to 368 K at 1 K intervals. The data were fit to a simple two state thermodynamic model of unfolding as described by Koepf *et al* (Koepf et al. 1999).

NMR analysis and structure determination:

Samples containing either double (^{15}N, ^{13}C) or triple (^{2}H, ^{15}N, ^{13}C) labeled p66α or MBD2 and unlabeled p66α or MBD2 coiled-coil regions, respectively, were generated by expressing the protein in isotopically enriched minimal media. Standard NMR experiments for resonance assignments, distance and torsional angle restraints were measured on a Varian 500MHz Unity+ equipped with a triple resonance probe and pulse field gradient module. Residual dipolar couplings were measured by adding ~4% of PEG:hexanol ($C_{12}E_5$, r=0.85) to a triple labeled sample (both p66α and MBD2 labeled) and $^{1}D_{NH}$, $^{1}D_{NC'}$, and $^{1}D_{HNC'}$ couplings determined using standard IPAP and TROSY based experiments for both isotropic and partially aligned samples. The structure of the complex was calculated by simulated annealing using the Xplor-NIH software package. The minimized target function included the experimental NMR restraints (NOE-derived interproton distances, torsion angles restraints from J-coupling measurements and from chemical shifts using TALOS+, and residual dipolar couplings), hydrogen bond distance and angle restraints for α-helical residues (identified based on NH(i)-NH(i+1), NH(i)-Hα(i-3), and Hα(i)-Hβ(i-3) NOEs, backbone torsion angles and lack of NH-H_2O exchange crosspeaks), quartic van der Waals repulsion term for the nonbonded contacts, and a torsion angle data base potential of mean force.

Cell culture:

Chemical inducer of dimerization dependent β-YAC bone marrow cells, a kind gift from Dr. Kenneth R Peterson of University of Kansas Medical Center, were maintained in

Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat inactivated fetal bovine serum, non-essential amino acids, sodium bicarbonate, L-glutamine, 50 U/mL penicillin and streptomycin and 100 nmol/L AP20187 (Ariad Pharmaceuticals).[28] MELρ cells[12] were maintained in RPMI 1640 medium (with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.05 mM β-mercaptoethanol, 90%) containing 10% fetal bovine serum and 100 U/mL penicillin and 100 mg/mLstreptomycin. 293T cells were maintained in DMEM medium containing heat inactivated 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, and 100 U/mL penicillin and streptomycin. All cells were grown 37ºC and 5% CO_2.

RNA isolation:

Cells were spun down at 200g for 5 minutes at room temperature. To the cell pellet, 1 mL of TRIZOL (Invitrogen) reagent was used per 5-10x10^6 cells. 200 µL of chloroform was then added and mixed well. This was then spun down at 12,000xg for 15 minutes. The top aqueous phase was transferred to another tube and 0.5 mL of isopropanol was added and mixed well. RNA was precipitated by spinning down at 12,000xg for 10 minutes. The supernatant was discarded. The RNA pellet was washed twice with ice cold 70% ethanol prepared using DEPC water. Finally the RNA pellet was air dried and appropriate amount of DEPC water was added to dissolve it. RNA was then stored at -80ºC until use.

cDNA synthesis:

The residual DNA contaminated in the RNA was removed by DNAse digestion. 2 µg of RNA was mixed with 0.5 µL of DNAse (Ambion) along with DNAse buffer and 0.5 µL of Super-RNAse inhibitor (Ambion). The reaction mix was made up to 20 µL using DEPC water. It was then incubated at 37ºC for 30 minutes and then the enzyme was heat inactivated at 75 ºC for 10 minutes.

This DNAse treated RNA was then used for cDNA synthesis using iscript cDNA synthesis kit (Biorad). 1 µg of RNA and 1 µL of iScript Reverse Transcriptase enzyme was added to the 4 µL of the premade reaction mix. The reaction was made up to 20 µL using DEPC water. This mix was then incubated at 25 ºC for 5 minutes followed by 42 ºC for 30 minutes. Finally heat inactivation was performed at 85 ºC for 5 minutes. The cDNA was then stored at -20 ºC until further use.

Quantitative RT-PCR:

cDNA (20 ng) was amplified by Q-PCR 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) and relative quantification determined using the SDS 1000 software. The primers used for the Q-PCR were designed to specifically bind to the cDNA of interest and not any DNA that may be present. This was achieved by designing primers crossing the exon-exon boundaries using Perl Primer software.

Protein expression analysis (Western blot and Dot blot):

For protein analysis, 2-4x10^6 cells were lysed with 4.5% SDS buffer containing protease inhibitor cocktail (Pierce). The lysate was then sonicated using Biorupter sonication instrument (Diagenode) to fragment the genomic DNA. If there was debris still present in the lysate, the sample was spun down at 13,000xg for 5 minutes at 4ºC. The protein lysate was quantitated using DC protein assay (Biorad). Approximately 20 µg of the protein lysate resuspended in Laemelli sample buffer was boiled and then subjected to SDS-PAGE using Mini-PROTEAN II electrophoresis cell (Biorad). If the size of the protein of interest was above 15 kDa 10% separating and 4% stacking SDS-PAGE gel was used. The electrophoresis was run at 100 to 150 Volts for approximately 1 to 2 hours. Wet electrotransfer using PVDF membranes (Millipore) was performed at 100 V for 1 hour at 4 ºC. The membrane was then blocked using 5% milk in 1xPBST buffer for 1 hour at room temperature. The blot was then incubated in primary antibody diluted using the 5% milk blocking buffer overnight at 4ºC. The blot was then washed three times using 1xPBST buffer and then incubated with secondary HRP conjugated antibody diluted in 5% milk blocking buffer. The membrane was then washed at least thrice using 1xPBST buffer and then developed using SuperSignal West Pico or Dura substrate (Pierce).

In order to detect the expression of small molecular weight peptides, protein dot blot was performed. The protein lysate in Laemelli sample buffer was prepared as described in the previous paragraph. Methanol activated Immobilon-PSQ 0.2 µm membrane (Millipore) was used for dot blotting. Approximately 10 µg of the protein (per dot) was blotted onto the membrane using the HYBRI DOT® Manifold (Whattman

Biometra) which employs vaccum filtration method. The dot blotted membrane was then subjected to blocking, antibody incubation, and developing, as described in the previous paragraph.

RNAi knockdown studies:

Cells were transfected with 1μM of MBD2, p66α and Mi-2α and β gene specific siRNAs and AllStars siNeg (Qiagen) using Nucleofector kit V (Amaxa). The day prior to transfection the cells were plated at a density of $0.5x10^6$ cells/mL. On the day of transfection, $5x10^6$ cells were used per transfection. The cells were spun down at 100xg for 10 minutes and the supernatant was removed. 100 μL of the V solution (Amaxa) per transfection was added to the cell pellet and resuspended. 100 μL of the cell suspension was then mixed with the siRNA. The cells were then pulsed using Amaxa nucleofector program D-15. The cells were then transferred gently into a 1.5 ml tube containing pre-warmed medium and incubated at 37ºC. After 15 minutes of incubation, the cells were then transferred into a T-25 flask containing 5 mL of pre-warmed media. The cells were harvested at 24, 48, 72, and 96 hours for RNA and protein analyses.

For stable knockdown of MBD2, MELρ cells were transfected with 5 μg of shMBD2 (labeled as m90) and shScramble pSuperior vector (Kransdorf et al. 2006)using nucleofection (Amaxa) as described in the previous paragraph.

Murine shMBD2 sequence used was as follows:

5'Mlu1CCCC**AGGTAGCACTTACGTGAAA**TTCAAGAGA**TTTCACGTAAGTGCT ACCT**TTTTTGGAA Cla1 3'

Murine shScramble sequence used is as follows:

5'Mlu1**CCCCGCGCGCTATGTAGGATTCG**TTCAAGAGA**CGAATCCTACATAG CGCGC**TTTTTGGAA Cla1 3'

The sequence marked in red is the 19 nucleotide sense siRNA and the sequence marked in green is the 19 nucleotide antisense siRNA. The sequence between these two regions is the loop for the shRNA.

The nucleofection program used was T-016. The stable transfectants were selected using G418 (Invitrogen) at a dose of 800 µg/ mL of cells over a period of 15 days and then they were dilution cloned to obtain stable clones.

RNA was isolated using Trizol (Invitrogen), residual DNA digested with DNAse (Ambion), and the cDNA synthesize (Qiagen) per manufacturer's protocols. cDNA (20 ng) was amplified by Q-PCR (Sybr green 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) and relative quantification determined using the SDS 1000 software. For protein analysis, 2-4x10^6 cells were lysed with 4.5% SDS buffer containing protease inhibitor cocktail (Pierce) and expression levels determined by standard western blot analysis.

Overexpression studies:

The p66α coiled-coil domain (amino acids 138-178) was cloned into the pCMVTag2B (Stratagene) vector between BamH1 and Xho1 sites, in frame with an N-terminal Flag tag sequence. The sequence of the insert is as follows:

5'BamH1CCTGAAGAACGAGAAAGGATGATCAAGCAGCTGAAGGAAGAATTGA GGTTAGAAGAAGCAAAACTCGTGTTGTTGAAAAAGTTGCGGCAGAGTCAAAT ACAAAAGGAAGCCACCGCCCAGAAGTGAXho1 3'.

This p66α-pCMVTag2B plasmid and an empty vector control (5 μg) were transiently tranfected into CID β-YAC bone marrow cells using Nucleofector kit V (Amaxa, per manufacturer's protocol) and RNA and protein were isolated at 24, 48, 72 and 96 hours post transfection. The same vectors (5 μg ea.) were transfected into MELρ cells (Lipofectamine™ 2000, Invitrogen) and selected with Zeocin (Invitrogen) at 500 μg/mL.[12] RNA was isolated and cDNA synthesized, and Q-PCR performed as described previously. Oligonucleotide primers were designed to amplify only the flag-tagged transcript and not native transcript. The PCR products were evaluated by agarose gel electrophoresis (1.5% gel). Peptide expression was analyzed by dot blot analysis using HYBRI DOT® Manifold (Whattman Biometra) per the manufacturer's recommendations.

Immunoprecipitation:

293T cells were transfected with p66α-pCMV-Tag2B plasmid and empty vector control (10ug) by the calcium phosphate transfection method (as described earlier) and

harvested at 48 hours. Cells were lysed using 50 mM Tris HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 1% TRITON X-100 (Sigma Flag-IPT kit). The transfected cells were spun down and washed with PBS. 1 mL of the lysis buffer was added to $5x10^6$ cells. The tube was vortexed and incubated in a shaker at 4ºC. Then the lysate was centrifuged at 13,000xg for 10 minutes at 4ºC. The supernatant was collected for immunoprecipitation.

Flag M2 Agarose affinity gel (Sigma) and recombinant Protein G agarose beads (Invitrogen) were washed using wash buffer 50mM Tris, pH 7.4, 150mM NaCl (Sigma Flag-IPT kit) four times by spinning down at 5000xg for 30 seconds for 30 seconds and were resuspended in the wash buffer after the final wash. Cut tips were used when withdrawing the beads. The Flag M2 Agarose affinity gel contains the beads which have the anti-Flag antibody adhered to its surface.

In order to preclear the lysate of proteins those non-specifically bind to the Protein-G beads, 40 µL of the washed Protein G beads were added to the 1 mL of lysate and incubated in a rotator for two hours at 4ºC. They were then spun down at 5000xg at for 30 seconds at 4ºC. 30 µL of the supernatant was used for input sample. The remaining supernatant was divided equally into two cold tubes (approximately 480 µL into each tube). One tube will be used for the protein specific antibody pull down and the other tube will be used for non-specific IgG pull down. The IgG used for control was chosen to be of the same species as the protein-specific antibody. For anti-Flag pull downs, 40 µL the washed Flag M2 Agarose affinity gel beads were added. For non-specific IgG pull downs and for anti-MBD2 pull down, 40 µL of the washed protein G beads were added.

and immunoprecipitated with anti-Flag M2 Agarose affinity gel (Sigma), anti-MBD2 (Santacruz) and mouse and goat IgG (Santa Cruz) controls per manufacturer's protocol. The samples were incubated overnight at 4ºC. The next day, anti-MBD2 antibody and the IgG control antibodies were added to their respective tubes. The tubes were then incubated for another two hours at 4ºC in a rotator.

All the samples were then spun down at 5000xg for 30 seconds at 4ºC and were washed four times using wash buffer. After the washes, the beads were then suspended in 40 µL of 2x laemelli sample buffer and then boiled for 5 minutes. They were then spun down at 13,000 rpm for 30 seconds at room temperature and the supernatant which now contains the proteins bound to the beads were collected. The supernatant was once again spun down to remove any residual beads which may hinder with the SDS-PAGE. The samples were then loaded onto the SDS-PAGE and western analysis was performed to identify the protein of interest in the pull down.

Antibodies:

MBD2 (Abcam abD-15), MBD2/3 (Millipore 07-199), CHD4 antibody (Abcam ab54603, Millipore 06-878), RbAp48 antibody (Abcam ab1765), HDAC2 (05-814 Millipore), MTA2 (sc-28731, Santacruz), Anti-p66 (07-365 Millipore).

Primers: Primers were designed using PerlPrimer software. The sequences of the primers are tabulated below.

Primers for Q-RT PCR	Forward Primer	Reverse Primer	Taqman Probe
Human Gamma 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
Mouse Glycophorin A	GCC GAA TGA CAA AGA AAA GTT CA	TCA ATA GAA CTC AAA GGC ACA CTG T	FAM/TTGACATCCAATCT CCTGAGGGTGGTGA /BHQ
Mouse MBD2	TTT GAC TTC AGG ACC GGC AAG ATG	ATT GCT CGG GTG GTT CGT GAA TTT	
Mouse CyclophilinA	GAG CTG TTT GCA GAC AAA GTT C	CCC TGG CAC ATG AAT CCT GG	FAM/TTC GAG CTC TGA GCA CTG GAG AGA AA/BHQ
Mouse Gab1	GAC GAT CCA CAA GAC TAC CT	CAT TCA TTC CGT GTT TGC TC	
Mouse ZBTB32	GTA CTA GGA GGC AGC TAC AG	ATA TGG GCT GAT TAG TCT TGT G	
Human Gab1	TCA AGC AGT GAC TCA CAC GAC AGT	TCC TTT GGG CTT GAT CAT AGG GCT	FAM/TGT TCC CAT GAA CCC AAA CCT GTC CA/BHQ
Human ZBTB32	CTA TGC GTG CTC TGT CTG TGG AAA	TGA GGA CAA AGG CTA CAG GAG AAG	FAM/ACT CAA GCA TCA GAT GGA GAC GCA CT/BHQ
Mouse Ugt8 for ChIP	TTA GAG CTC TGA AGG GCA TGT GGA	TGT GCT AAT TCC TCC CGC AAC AGA	FAM/TGCTCTGCGTCTCC CAAACTCAAGAA/TAMRA
γ-globin promoter for ChIP	CCT TGC CTT GAC CAA TAG CCT TGA	TTC CAG AAG CGA GTG TGT GGA ACT	FAM/ACA AGG CTA CTA TCA CAA CC TGT GG/TAMRA

Table 3 Primers used for Q-RT PCR

Statistics:

Results are shown as mean (from three or more independent biological repeats) ± standard error. For comparison between data sets, unpaired two tailed t-tests were performed, p values are indicated as < 0.05 (*), <0.001 (**), and not significant (NS).

Accession codes:

The coordinates and NMR restraints for the p66α-MBD2 coiled-coil complex have been deposited in the RCSB Protein Data Bank (PDB ID: 2L2L); the NMR assignments have been deposited in the Biological Magnetic Resonance Bank (BMRB accession: 17138).

III. Results:

A. The p66α and MBD2 coiled coil domains form a stable heterodimeric complex:

In order to study the biological consequence of the interaction between the CR1 coiled coil domain of p66α and the coiled coiled domain on globin gene regulation, we first investigated the interaction from a biophysical and structural perspective. For this we collaborated with structural biologist Dr. David Williams Jr. (Department of Pathology, VCU). We cloned and expressed the coiled coil domains of p66α (residues 137-178) and MBD2 (residues 360-393) in bacteria. We then asked whether these domains form a homomeric or a hetreomeric complex. For this, we mixed the peptides at a 1:1 molar ratio and performed gel filtration chromatography. As seen in **figure 14a**, the coiled coil domains of p66α and MBD2 elute as a single peak and this indicates that the two proteins form a stable equimolar complex.

In order to determine the stoichiometry of the interaction, the coiled coil peptides were subjected to analytical centrifugation studies. The following parameters for mass were obtained by sedimentation velocity analysis. MBD2 24.6 kDa observed (22.0 kDa

expected); p66α 24.1 kDa observed (23.0 kDa expected); MBD2-p66α 43.5 kDa observed (45.0 kDa expected). These studies showed that each peptide alone behaves as a monomer in solution, and when mixed together, they form a heterodimeric complex (**Figure 14b**).

Further, the affinity and kinetics of the interaction between the coiled coil domains of p66α and MBD2 were determined using surface plasmon resonance studies. MBD2 coiled coil peptide was coupled to a Sensor Chip CM5 and the binding kinetics of p66α coiled coil peptide to the chip was examined. The interaction fit quite well by a 1:1 binding model (**Figure 14c**). Moreover, the proteins bound with a relatively rapid on rate and a slow off rate which indicates a high affinity interaction. A nano molar dissociation constant (K_D= 12.4nM, k_a=2.4x10^{6}M^{-1}s^{-1}, k_d=0.031 s^{-1}) was documented for this interaction, indicating a tight interaction.

B. Solution structure of the MBD2-p66α coiled-coil complex:

Next, we investigated the solution structure of the MBD2-p66α coiled coil complex by NMR spectroscopy. NMR spectra of the individual domains showed that the isolated coiled coil domains are largely preformed monomeric helices. They interact with each other to form a stable heterodimeric complex. The various parameters of the solution structure have been listed in the **Table 4**.

The structural studies revealed that the heterodimeric complex adopts an anti-parallel coiled coil structure (**Figure 15a**). The two helices interact through a series of highly conserved hydrophobic side chains to form the classic "knobs" (p66α: *Ile145, Leu152, Leu159*; MBD2: Ile220, Val227, Leu234) that fit into "holes" (p66α: between

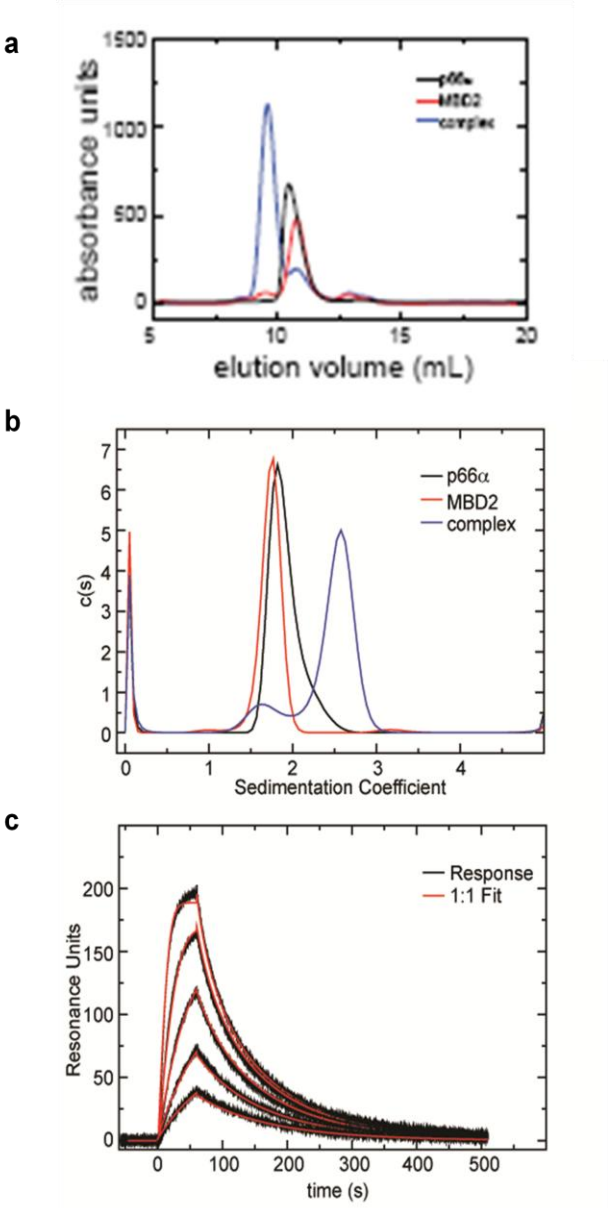

Figure 14 Coiled coil domains of p66α and MBD2 form a stable heterodimeric complex: a) Size exclusion chromatography profiles are shown for p66α and MBD2 coiled-coil domains (with N-terminal thioredoxin fusion polypeptides) in isolation and as a 1:1 molar mixture. Analytical ultracentrifugation was performed on p66α and MBD2 coiled-coil domains individually and as a 1:1 molar mixture. b) The sedimentation velocity was fit using a continuous size distribution (c(s)) and the effective molecular weight determined from the resulting sedimentation coefficients with SEDFIT software c) Binding kinetics were analyzed for p66α coiled-coil binding to MBD2 coiled-coil coupled to a Sensor Chip CM5 on a Biacore T100. The data was fit to a 1:1 binding model using Biacore evaluation software. (Performed by Dr. David C. Williams Jr.)

	Protein
NMR distance and dihedral constraints	
Distance constraints	
Total NOE	677
Intra-residue	223
Inter-residue	
Sequential ($\|i-j\|=1$)	203
Medium-range ($\|i-j\|\leq 4$)	145
Long-range ($\|i-j\|>5$)	0
Intermolecular	106
Hydrogen bonds	94
Total dihedral angle restraints	128
ϕ	58
ψ	58
Total RDCs	
NH	50
H^NC'	40
NC'	37
Q%	
NH	6.1
H^NC'	30.2
NC'	31.3
Structure statistics	
Violations (mean and s.d.)	
Distance constraints (Å)	0.017±0.004
Dihedral angle constraints (°)	0.27±0.16
Max. dihedral angle violation (°)	3.6
Max. distance constraint violation (Å)	0.39
Deviations from idealized geometry	
Bond lengths (Å)	0.0004±0.00005
Bond angles (°)	0.33±0.0005
Impropers (°)	0.27±0.02
Average pairwise r.m.s. deviation** (Å)	
Heavy	1.2
Backbone	0.5

**Pairwise r.m.s. deviation from the mean was calculated among 20 refined structures for ordered residues.

Table 4 NMR and refinement statistics
(Performed by Dr. David C. Williams Jr)

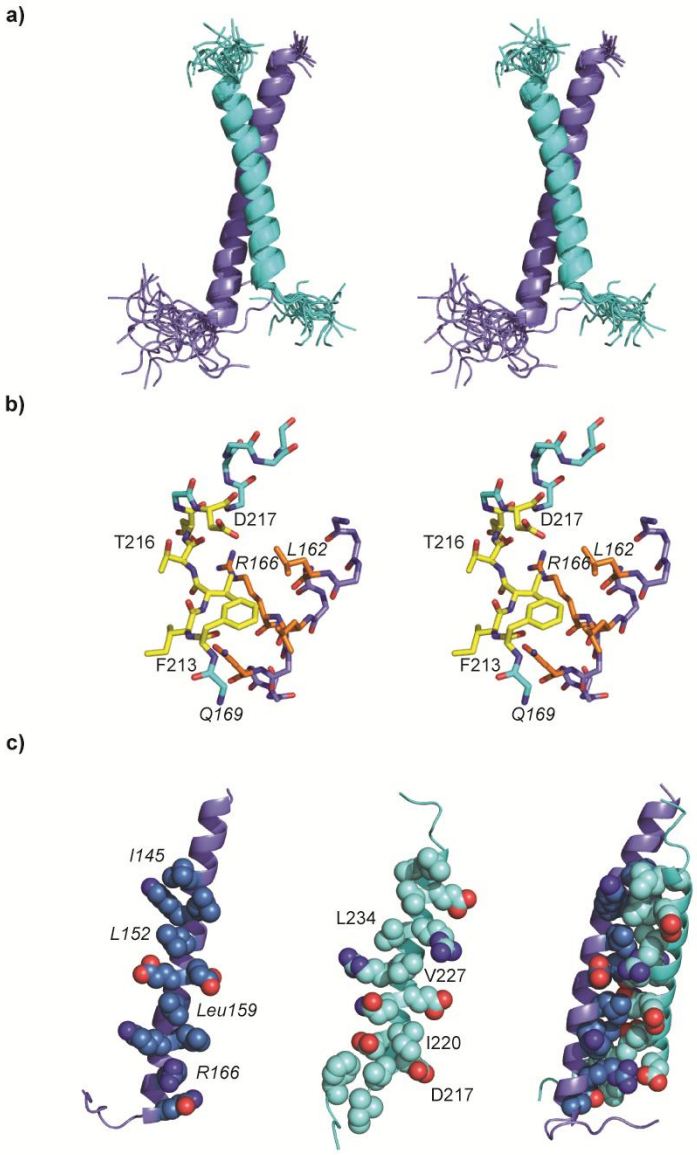

Figure 15 Solution structure: a) Stereo view of the aligned 20 lowest energy structures is shown as cartoon representations of p66α (blue) and MBD2 (cyan) coiled-coil domains. b) Stereo view stick representation is shown for the main chain atoms encompassing the N-terminal extended to helical transition of MBD2 and the contacting C-terminal region of p66α. Key residues from MBD2 (yellow) and p66α (orange) are shown and labeled (p66α in italics). c) The "knobs" and "holes" of the p66α and MBD2 coiled domain are shown using a sphere representation for each chain individually, with the contact surface facing the viewer, and as a complex. For reference, "knobs" and select residues are labeled. The figure was generated using the Pymol program (Perfromed by Dr. David C. Williams Jr).

residues *Leu148* and *Lys149*, *Glu155* and *Glu156*, *Leu162* and *Lys163*; MBD2: between residues Gln223 and Glu224, Val230 and Arg231, Ala237 and Leu238) (**Figure 15c**).

Several close ionic interactions are formed that likely contribute to the specificity of this interaction, in addition to the canonical hydrophobic packing. The hole formed between p66α *Glu155* and *Glu156* residues permits side chain ionic interactions with MBD2 Arg226 and Arg231, respectively. Another close ionic interaction forms between p66α *Lys163* and MBD2 Glu224. In addition a charged residue *Arg166* terminates the pattern of hydrophobic knobs protruding from p66α, and forms a close ionic interaction and a potential hydrogen bonding with Asp217 of MBD2. (**Figure 15c**).

Ionic interaction between *Arg166* on p66α and *Asp217* on MBD2 marks a transition between extended to helical secondary structure for MBD2. This transition is stabilized by a classic helix N-cap. The cap is formed by a side chain hydrogen bond between Thr216 and the backbone amide of Asp219, which in turn can form a side chain hydrogen bond with the backbone amide of Thr216 (**Figure 15b**). The extended N-terminal sequence of MBD2 allows Phe213 to interact with the hydrophobic *Leu162* and *Leu165* residues on the exposed side of p66α. The side chains of Val215 and *Leu162* interact while the side chain of *Gln169* can form hydrogen bonds with the backbone of *Val215* to stabilize this extended conformation of MBD2.

The relative importance of the different contacting residues were tested by generating a series of truncation and point mutants of the p66α coiled-coil domain, and by evaluating their binding to MBD2 coiled-coil using gel filtration chromatography. N-terminal (residues *144-158*) or C-terminal (residues *152-168*) truncated portions of p66α

did not form stable complexes with the MBD2 coiled-coil peptide. However, the portion containing the central 25 amino acids (residues *144-168*) of p66α, did bind to form a stable complex (K_D ~ 72 nM) (**Figure 16a).** This is likely due to the incorporation of most of the intermolecular contact residues in this peptide.

Point mutations were generated in order to replace *Arg166* with Glu or both *Glu155* and *Glu156* with Arg in the p66α peptide. These changes did not completely abolish binding as assayed by gel filtration chromatography (**Figure 16b**). Further, the affinity of these mutants was determined by surface plasmon resonance, which showed a marked decrease of almost three orders of magnitude for both *Arg166Glu* and *Glu155Arg/Glu156Arg* p66α mutations (*Arg166Glu* K_D ~ 8 μM, *Glu155Arg/Glu156Arg* K_D ~ 17 μM) (**Figure 16a**). On the other hand, mutating all three residues simultaneously to generate the *Arg166Glu/Glu155Arg/Glu156Arg* triple mutant, resulted ina failure to bind MBD2 coiled-coil even at a 30 μM protein concentration (**Figure 16b**). These findings taken together revealed the importance of the intact full-length coiled coil domain and ionic interactions in the stability of the complex. Moreover, these key contacting residues are highly conserved across species and among homologous proteins for both MBD2 and p66α.

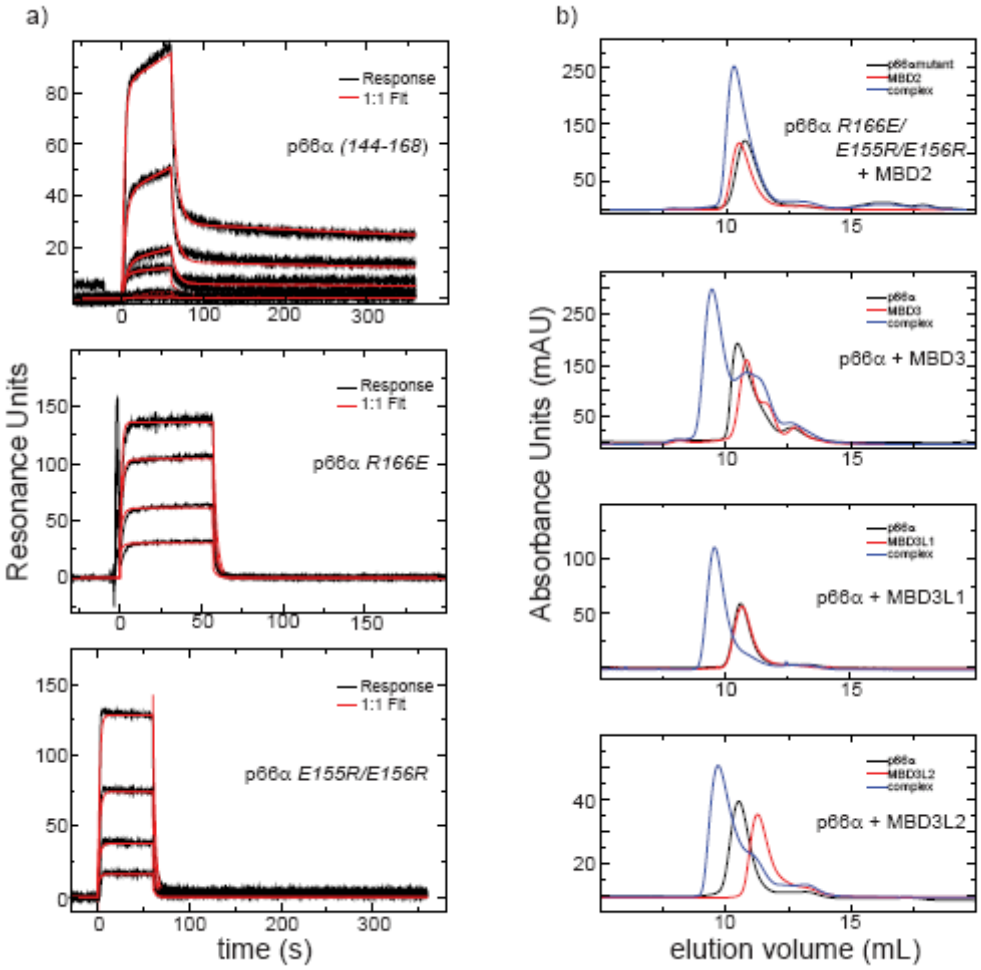

Figure 16 Binding analysis of the coiled coil domains of p66α mutants and MBD2 homologues: a) Binding kinetics were analyzed for p66α coiled-coil domain truncation (residues 144-168, KD ~ 72nM) and charge mutants (*Arg166Glu* K_D ~ 8 M, *Glu155Arg/Glu156Arg* K_D ~ 17 M *binding to*MBD2 coiled-coil coupled to a Sensor Chip CM5 on a Biacore T100 (GE Healthcare). The data were fit to a 1:1 binding model using Biacore evaluation software. b) Size exclusion chromatography elution profiles (Superdex75 10/300) show that the p66α coiled-coil domain *Arg166Glu/Glu155Arg/Glu156Arg* fails to bind MBD2 coiled-coil domain while MBD3, MBD3L1, and MBD3L2 coiled-coil domains can form stable complexes with the wild type p66α coiled-coil domain. (Performed by Dr. David C. Williams Jr.)

C. Biological Hypothesis:

Taken together, the biophysical and structural studies of the MBD2-p66α coiled coil complex showed that the interaction is highly stable and specific. Based on these observations, we hypothesized that the over-expression of the p66α coiled coil peptide in cells would bind stably to the endogenous MBD2 *in vivo* and interfere with the assembly of the MeCP1 complex, leading to the disruption of the function of MBD2. (**Figure 17**) We sought to study the effect of over-expression of the p66α coiled coil peptide in the MBD2 mediated silencing of chicken embryonic ρ-globin and human fetal γ-globin in adult erythroid cells. It is important to note that while MBD2 has been shown to bind to ρ-globin gene promoter and proximal transcribed region (exon 1 and intron 1) to regulate the gene, MBD2 has not been shown to directly bind to the γ-globin gene to cause its silencing.

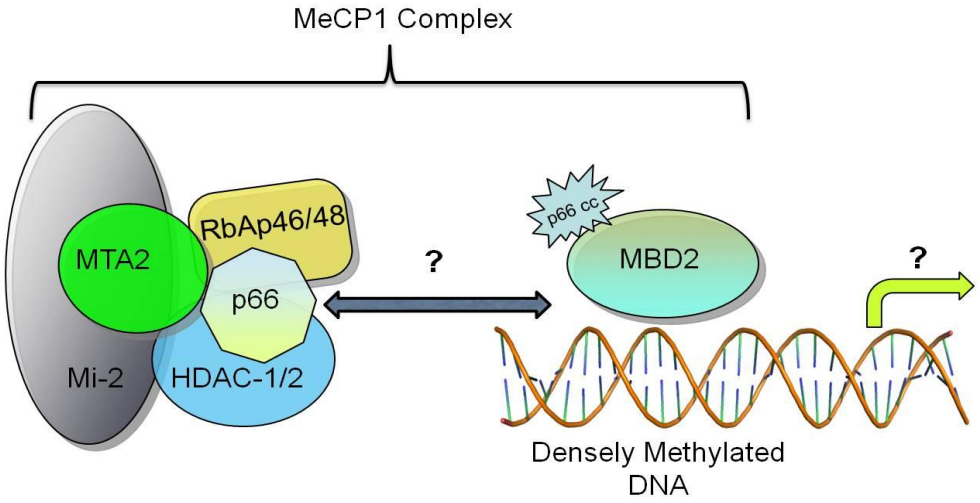

Figure 17 Biological hypothesis:

D. Knockdown of MBD2 causes the elevation of fetal/embryonic globin in adult erythroid cells:

In order to study the chicken embryonic ρ-globin gene, we used Mel-ρ cells. These are mouse erythroleukemia cells harboring a stably integrated methylated chicken ρ-globin mini-locus (chicken β-globin LCR and the ρ-globin gene) flanked by chicken HS-4 insulator elements (Kransdorf et al. 2006)).

In order to knockdown MBD2 in these cells, a pSuperior vector containing the sequences for either shMBD2 or shScramble control was transfected into MEL-ρ cells. Stably transfected clones were isolated using G418 antibiotic selection. Knocking down MBD2 stably in these cells caused a modest increase in the ρ-globin expression by approximately 2 fold compared to shScr control transfected cells. (**Figure 18**).

CID dependent β-YAC bone marrow cells were used to study human γ-globin regulation. These cells are derived from the bone marrow of β-YAC transgenic mice and can be propagated in culture by virtue of an artificial proliferation signal comprised of the thrombopoietin (mpl) signaling domain fused to FKBP (FK506 binding protein) binding domains responsive to a chemical inducer of dimerization (CID) (Blau et al. 2005). In the presence of a CID, homodimers are generated, and the resultant growth signal maintains the BMC population indefinitely and also causes spontaneous differentiation into the myeloid lineage. These cells predominantly express human adult β-globin and very low levels of γ-globin (<1% of β-globin), similar to human adult erythroid cells (Blau et al. 2005). Since none of the human erythroleukemia cell lines have the adult β-globin gene

expression profile, these cells provide for a valuable in vitro model to study β-globin regulation (**Figure 19a**).

We first knocked down MBD2 in these cells to determine if this relieved γ-globin silencing as observed in β-YAC transgenic mice. For this, we nucleofected these cells with siMBD2, and siNeg control. At 48 hours after transfection, approximately 80% knockdown of MBD2 was obtained (**Figure 19b**). This knockdown caused six fold induction of γ-globin at 72 hours post-transfection (**Figure 19b)**, similar to the levels obtained in the β-YAC in vivo mouse model, indicating that this cell line is a valid model to study MBD2 mediated human β-globin gene regulation.

Based on these results, we used the Mel-ρ cells and CID β-YAC cells as appropriate cell culture models to study the effect of the overexpression of the p66α coiled coil peptide on MBD2 mediated silencing of the chicken embryonic ρ-globin gene and human fetal γ-globin gene.

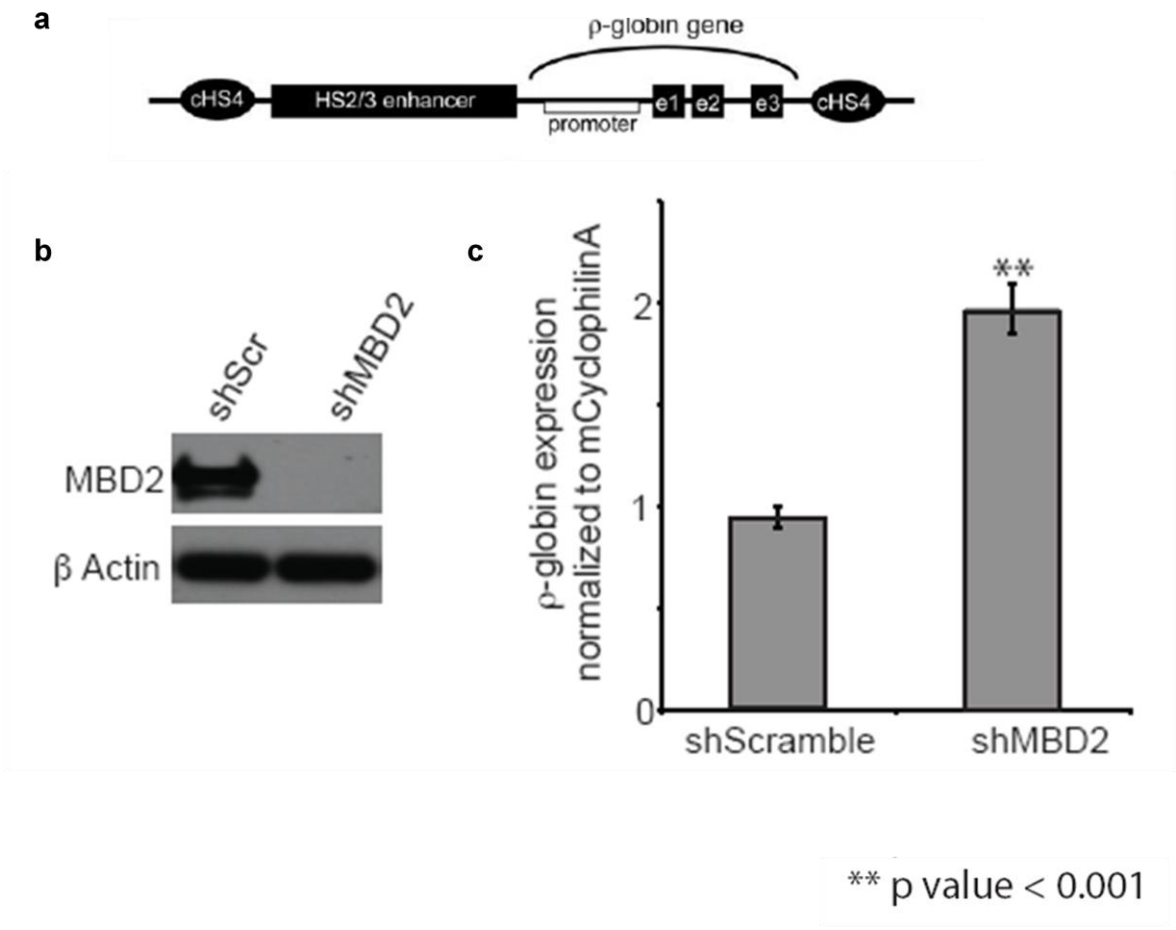

Figure 18 Knockdown of MBD2 in Mel-ρ cells: Mouse erythroleukemia cell line that harbors methylated chicken ρ-globin mini-locus (Mel-ρ) was stably transfected with shMBD2 pSuperior vector (n=6) and shScramble (n=3). a) Schematic of the chicken ρ-globin mini locus b) MBD2 protein knockdown levels were studied by western analysis and the c) γ-globin mRNA levels in the MBD2 knocked down cells were studied by Q-RT PCR analysis. Error bars indicate mean ± SE

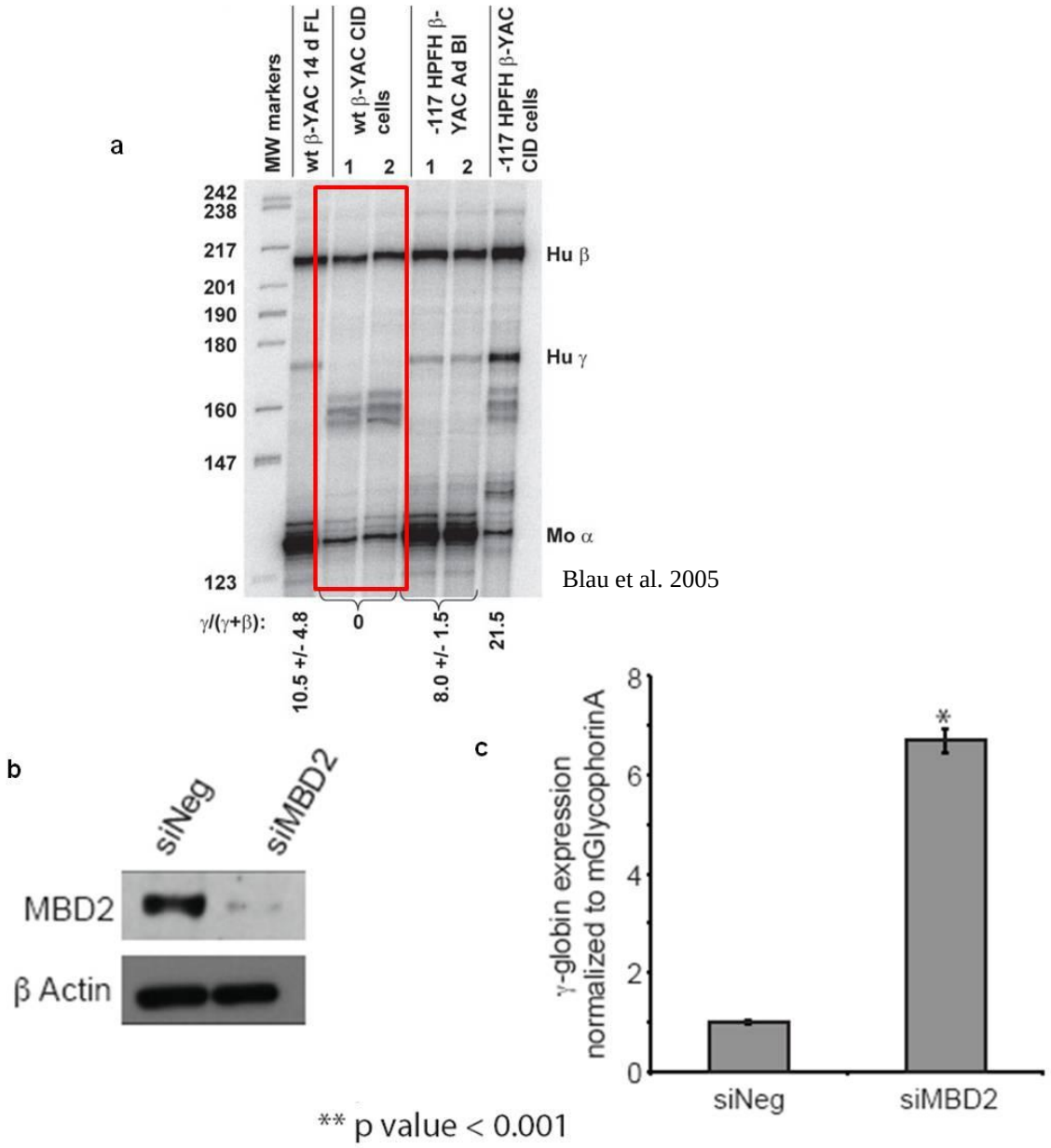

Figure 19 MBD2 knockdown in CID β-YAC cells: a) The human β-globin transcripts expressed in CID-dependent β-YAC bone marrow cells detected by RNAse protection analysis (Blau et al. 2005). The CID β-YAC cells were transiently transfected with siMBD2 and siNeg control (n=3). b) MBD2 knockdown levels were studied by western analysis. c) γ-globin mRNA levels were studied by Q-RT PCR analysis. Error bars indicate mean ± SE

E. Expression of the p66α coiled coil peptide augments chicken embryonic ρ-globin expression in an erythroleukemia cell line:

We investigated the effect of the overexpression of the p66α coiled coil peptide on chicken embryonic ρ-globin gene using the Mel-ρ cells. A pCMV-Tag2B vector containing the coding sequence for a N terminal Flag tagged p66α coiled-coil domain (residues 137-178) and an empty vector control expressing just the Flag peptide, were transfected into MEL-ρ cells. Stably transfected clones were isolated using Zeocin antibiotic selection.

In order to detect the overexpression of the p66α coiled-coil peptide in these cells protein dot blot was performed. Although western blotting is the standard technique to detect protein expression, we were unable to detect the peptide using this method. This might be due to the small size of our peptide (4kDa) and the reduced sensitivity of western analysis for small peptides (Duchesne, Fernig 2007). On the other hand, dot blot has been shown to be 4000 times more sensitive for small peptides compared to western blot (Duchesne, Fernig 2007). We were able to detect the expression of the peptide in the transfected cells using the dot blot technique (**Figure 20**).

In the presence of p66α coiled-coil peptide, embryonic ρ-globin gene expression was increased approximately 3 fold compared to clones that expressed the vector derived Flag peptide alone (**Figure 20**). Thus the degree of ρ-globin gene de-repression in the presence of the p66α coiled-coil domain peptide was similar to that in cells in which MBD2 was stably knocked down.

F. Expression of the p66α coiled coil peptide augments human fetal γ-globin expression in adult erythroid cells:

We then evaluated the effect of the overexpression of the p66α coiled coil peptide on human γ-globin expression using CID β-YAC cells. These cells were transiently transfected with pCMV-Tag2B vector containing the coding sequence for a Flag tagged p66α coiled-coil domain and an empty vector control. The expression of the p66a coiled-coil domain was monitored using semi-quantitative PCR and western dot blot (**Figure 21**). The overexpression of this peptide led to an approximate 3 fold increase in γ-globin expression (**Figure 21**). Although the increase in γ-globin expression is lower than that obtained by MBD2 knockdown in these cells, these results demonstrate that the isolated p66α coiled-coil domain has the ability to disrupt the silencing of the human fetal γ-globin gene.

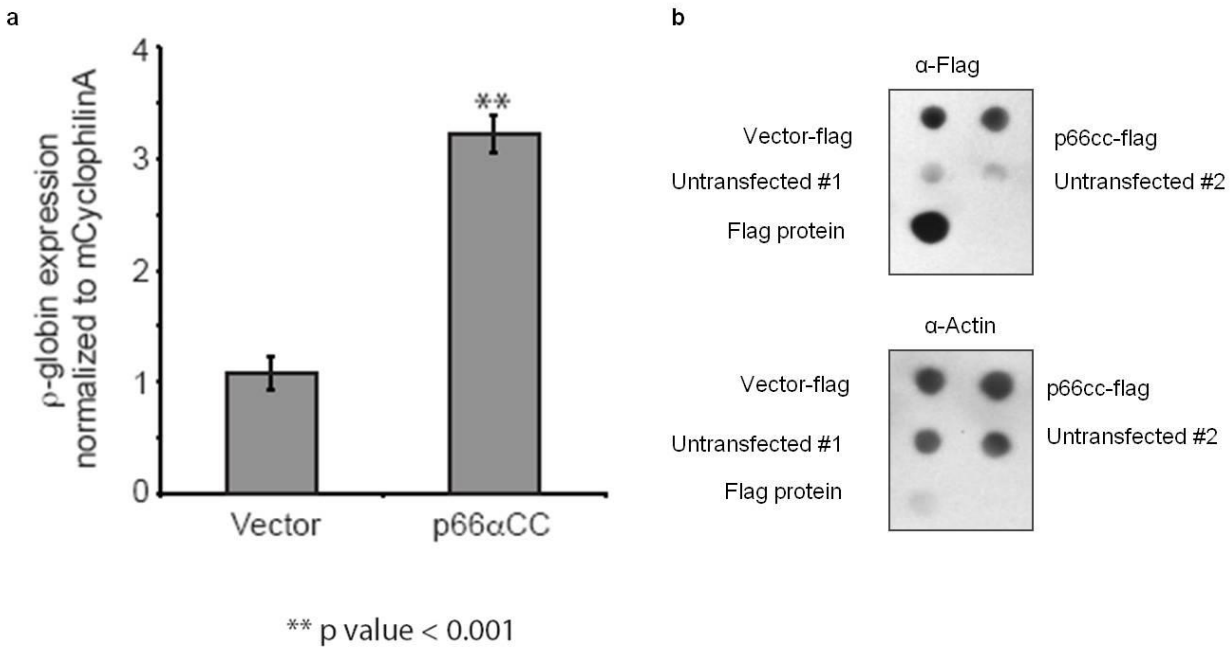

Figure 20 Expression of p66α coiled coil peptide in Mel-ρ cells: Mouse erythroleukemia cell line that harbors methylated chicken ρ-globin mini-locus (Mel-ρ), was stably transfected with Flag tagged p66α coiled-coil domain in the pCMV-Tag2B vector (n=3) and the corresponding empty vector control (n=3). a) γ-globin mRNA levels in the p66 alpha coiled coil peptide overexpressing cells were studied by Q-RT PCR analysis. b) Protein dot blot analysis using anti-Flag and anti-actin antibodies shows that both the p66α-pCMV-Tag2B plasmid and empty vector express Flag-tagged peptide while two different samples of untransfected MEL-ρ cells do not. Purified recombinant Flag protein shows appropriate positive reactivity. Error bars indicate mean ± SE

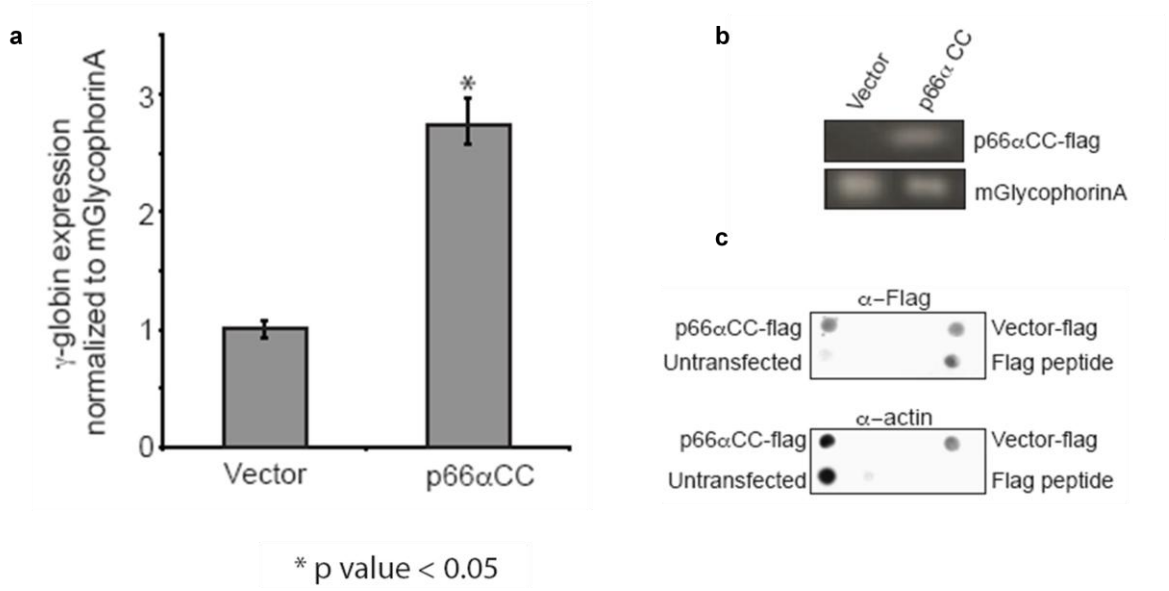

Figure 21 CID dependent β-YAC bone marrow cells were transiently transfected with Flag tagged p66 alpha coiled coiled domain and empty vector control (n=3). a) γ-globin mRNA levels in the p66 alpha coiled coil peptide overexpressing cells were studied by Q-RT PCR analysis. b) RT-PCR analysis using primers specific for Flag-tagged p66α coiledcoil domain cDNA show mRNA expression. c) Protein dot blot analysis using anti-Flag and anti-actin antibodies shows that both the p66α-pCMV-Tag2B plasmid and empty vector express Flag-tagged peptides while untransfected cells do not. Error bars indicate mean ± SE.

G. p66α coiled coil peptide interacts with MBD2 in vivo:

We next examined if the p66α coiled coil peptide overexpressed in cells interacted with endogenous MBD2 in vivo as hypothesized. For this high transfection efficiency 293T cells were transiently transfected with pCMV-Tag2B vector containing Flag tagged p66α coiled-coil sequence and the vector control expressing just the Flag peptide. Immunoprecipitation was performed with anti-Flag antibody. As expected the endogenous MBD2 coimmunoprecipitated with the Flag antibody in the cells containing the p66α coiled coil peptide and not in the vector control cells (**Figure 22**).

H. p66α coiled coil peptide disrupts the assembly of the MeCP1 complex:

Since endogenous MBD2 co-precipitated with the Flag tagged p66α coiled coil peptide, we next examined which other components of MeCP1 complex were pulled down by the antibody. While MTA2, HDAC2, and Rbap48 were present in the pull down, interestingly p66α/β and Mi-2α/β were absent (**Figure 22**). As a positive control, we performed immunoprecipitation using anti-MBD2 antibody in untransfected cells and confirmed that all the components of the MeCP1 complex were pulled down.

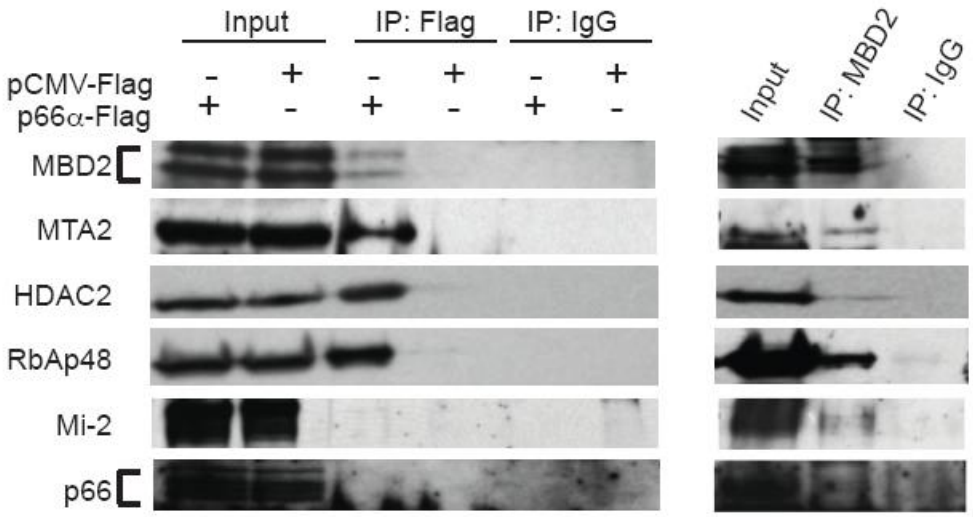

Figure 22 Co-immunoprecipitation studies: High transfection efficiency 293T cells were transiently transfected with the p66α coiled-coil pCMV-Tag2B construct and empty vector control. Immunoprecipitation with anti-Flag antibody co-precipitated native MBD2a, MTA2, HDAC2, and Rbap48 detected by western analysis. Neither p66α/β nor Mi-2 was detected in the western blot analysis. Immunoprecipitation with anti-MBD2 antibody in untransfected cells co-precipitated all the components of the MeCP1 complex.

I. p66α and Mi-2β are involved in human γ-globin silencing:

These immunoprecipitation studies indicated that the disruption of MBD2 mediated silencing by the p66α coiled coil peptide could be due to the absence of endogenous p66 and Mi-2 in the MeCP1 complex. Thus, we investigated the role of p66α/β and Mi-2α/β in the silencing of human fetal γ-globin gene using CID- β-YAC cells. We knocked down p66α, p66β, Mi-2α and Mi-2β in CID dependent β-YAC cells using siRNAs. Transient knockdown of p66α by ~80% was accompanied by a six-fold increase in γ-globin mRNA (**Figure 23**) and transient knockdown of Mi-2β by ~80% resulted in an eight fold induction of γ-globin mRNA in these cells (**Figure 23**). On the other hand knockdown of p66β or Mi-2α did not have a similar effect on γ-globin (**Figure 24**). It is noteworthy that the level of endogenous expression of Mi-2β in the CID β-YAC cells was very low. These results taken together, indicate that the specific isoforms of p66 and Mi-2 have important roles in the regulation of γ-globin silencing.

J. MBD3 is not involved in the human γ-globin silencing:

Immunoprecipitation of the p66α coiled-coil domain also co-precipitated MBD3, a homologue of MBD2 that does not bind to methylated DNA (**Figure 25**). This interaction was not surprising due to the high degree of homology between MBD2 and MBD3 coiled coil domains. This suggests that the p66α coiled coil peptide might also have an effect on MBD3-NuRD assembly. In order to verify if MBD3 may have any role in γ-globin silencing, we knocked down MBD3 using siRNAs in CID β-YAC cells. Knockdown of MBD3 by ~75% did not augment γ-globin expression in CID-dependent β-YAC bone

marrow cells (**Figure 25**), suggesting this interaction is not functionally relevant to γ-globin gene silencing in this model system.

Together, these results demonstrate that the isolated p66α coiled-coil domain can disrupt globin gene silencing by disrupting the recruitment of p66α/β and Mi-2α/β to the MBD2 containing MeCP1 complex.

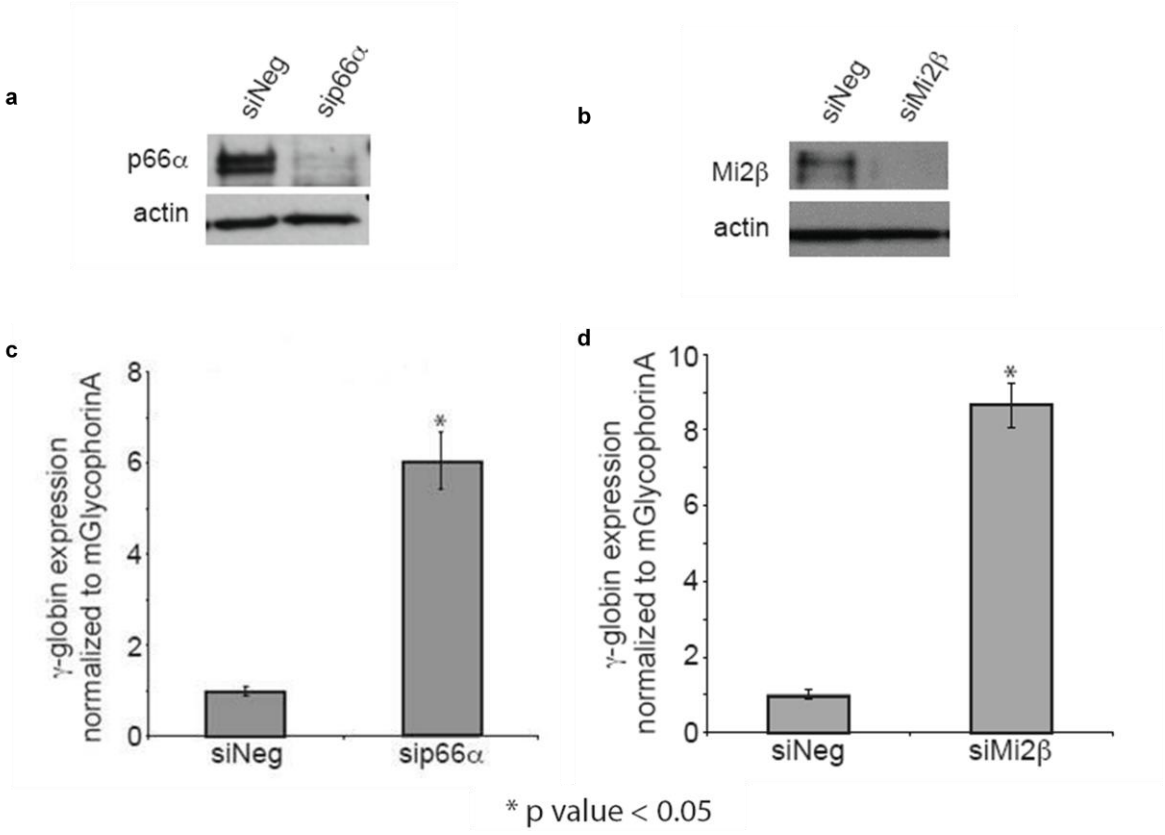

Figure 23 Knockdown of p66α and Mi-2β in CID β-YAC cells: CID dependent β-YAC bone marrow cells were transiently transfected with si p66 α, siMi2beta and siNeg (n=3). Protein knockdown levels of a) p66 α- and b) Mi-2 beta were studied by western analysis. γ-globin mRNA levels in c) p66 alpha knockdown cells and d) Mi-2 beta knockdown cells were studied by Q-RT PCR analysis. Error bars indicate mean ± SE. (p66α knockdown experiments were performed by Maria L Amaya, VCU)

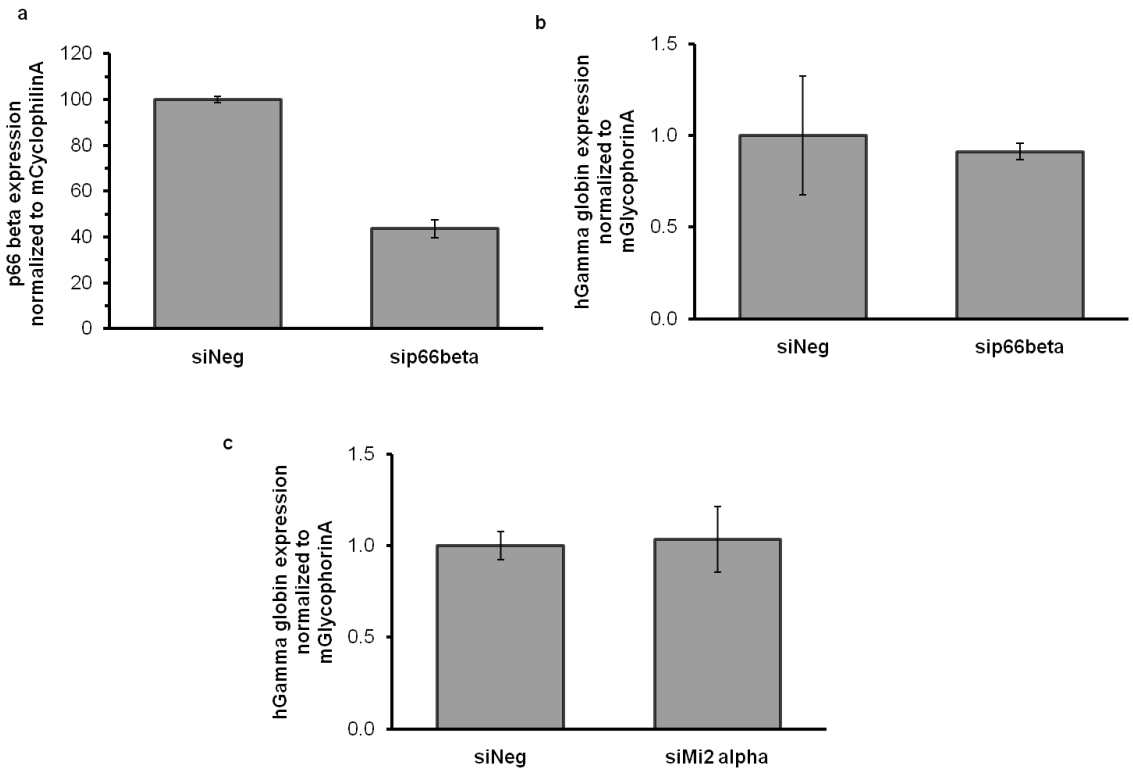

Figure 24 Knockdown of p66β and Mi-2α in CID β-YAC cells: CID dependent β-YAC bone marrow cells were transiently transfected with si p66β, siMi2α and siNeg (n=3). a) mRNA knockdown levels p66β quantified by Q-RT PCR. γ-globin mRNA levels in b) p66β, knockdown cells and c) Mi-2 α knockdown cells were studied by Q-RT PCR analysis. The Mi-2α endogenous expression levels in these cells were very low and hence we were unable to quantify the knock down levels of the mRNA. Error bars indicate mean ± SE. (Performed by Maria L Amaya, VCU)

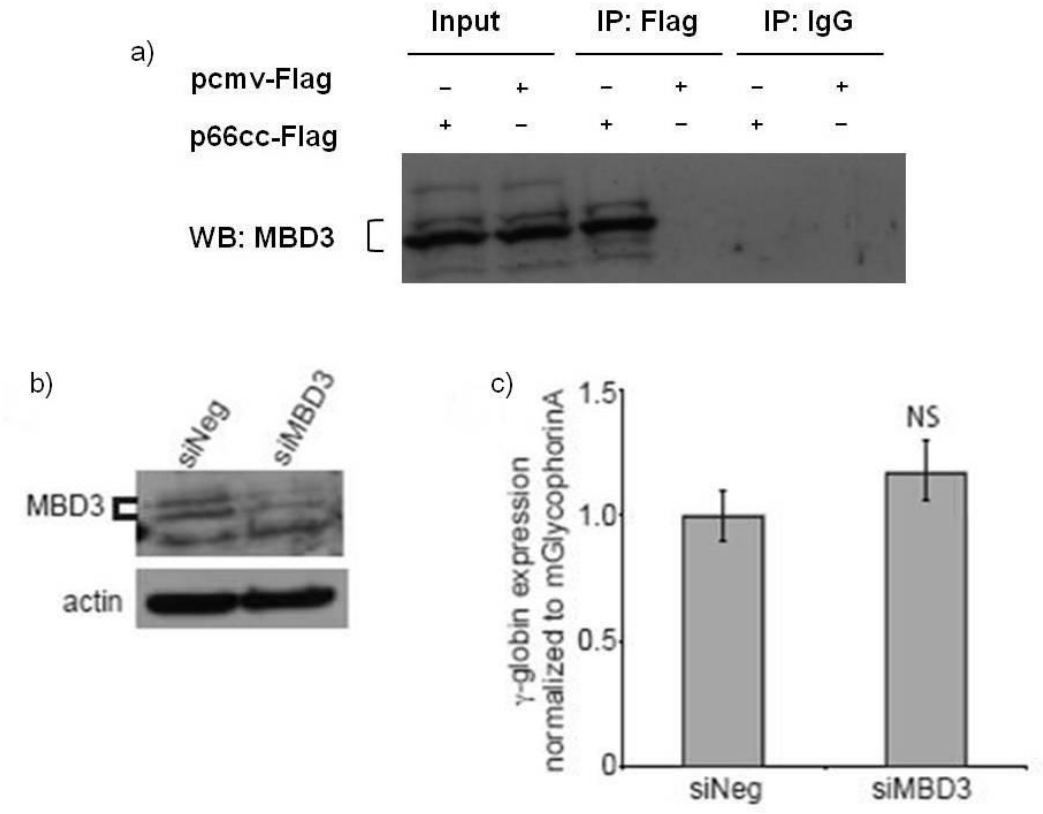

Figure 25 MBD3 and γ-globin gene regulation: Flag tagged p66α coiled coiled domain-pCMV-Tag2B plasmid and the empty vector control were transiently transfected into high efficiency 293T cells. a) Immunoprecipitation with anti-Flag, anti-MBD2, and IgG control antibodies show that native MBD3 co-precipitates with Flag-p66α coiled-coil domain but not with native MBD2, as shown in the western blot. siMBD3 and siNeg control (n=3) were transiently transfected into CID β-YAC bone marrow cells. b) Western blot analysis shows efficient knockdown of MBD3 protein. c) q-PCR analysis shows that γ-globin mRNA levels were not affected by MBD3 knockdown. Error bars represent mean ± SE.

IV. Discussion:

Methyl CpG binding domain protein2 (MBD2) belongs to a family of proteins that contains the MBD domain which enables it to bind preferentially to the methylated CpGs. These proteins can cause transcriptional silencing by recruiting co-repressor complexes to the methylated DNA. MBD2 recruits the nucleosome remodeling and histone deacetylase complex (NuRD) to mediate its silencing (Meehan et al. 1989, Wade et al. 1999). MBD2-/- mice are viable and fertile and exhibit only a subtle phenotype (Hendrich et al. 2001).

We had previously shown that MBD2 plays a critical role in the silencing of human fetal γ-globin and chicken embryonic ρ-globin in adult erythrocytes (Rupon et al. 2006, Kransdorf et al. 2006). We then characterized the MBD2 containing MeCP1 complex in primary chicken erythroid cells (Kransdorf et al. 2006). The canonical components such as MBD2, p66, Rbap48, MTA2, HDAC2 and Mi-2 were present in the complex.

How the different components in the MeCP1 complex interact with each other to assemble the complex and how the individual components contribute to the transcriptional repression of the complex is not clearly understood. In this study, we have probed the interaction between the transcriptional repressor p66 and MBD2. These experiments have provided molecular details of the highly stable and specific interaction between the coiled-coil domains of MBD2 and p66α. They have further unveiled the biological importance of the interaction in the MBD2 mediated transcriptional silencing of chicken embryonic ρ-globin and human fetal γ-globin. These results allude to the potential therapeutic use for a peptidomimetic of p66α coiled-coil peptide as an inhibitor of MBD2 function.

We have investigated the interaction between the coiled coil domains of p66α and MBD2 from a biophysical and structural perspective. Gel filtration chromatography, ultracentrifugation, and surface plasmon resonance imaging studies showed that the coiled coil domains of p66 and MBD2 interact with very high affinity. NMR analyses of MBD2-p66α coiled coil complex revealed that each protein adopts a regular helical structure to form a canonical anti-parallel coiled-coil interface with an observed disassociation constant in the low nanomolar region (12.4 nM). While the canonical hydrophobic packing gives stability to this anti-parallel coiled coil complex, a series of highly conserved close ionic interactions further stabilize the p66α-MBD2 interaction and likely contribute to the specificity of binding to an otherwise fairly common binding motif.

The p66α and MBD2 coiled-coil domains behave as largely helical monomers in solution only to form a stable heterodimer when mixed. These observations argue against the idea that MBD2 and MBD3 proteins form homodimeric complexes and/or bind to one another through their coiled-coil domains. Instead the lack of homodimeric interaction supports the emerging view that MBD2 and MBD3 form mutually exclusive NuRD complexes with unique functions (Le Guezennec et al. 2006).

Recent investigations have revealed that the interactions that involve intrinsically unstructured domains that adopt a locally defined regular structure upon binding a target, can be disrupted by small peptides and peptide analogs based on the native sequence, and thus provide potential therapeutic targets (Russell, Gibson 2008). Because p66α and MBD2 bind through a small peptide complex involving two stable helices, we postulated that the p66α coiled-coil domain in isolation could bind native MBD2 and function as a

competitive inhibitor. As predicted, over-expressing this domain in two different cell models of globin gene regulation augmented fetal/embryonic globin gene expression to a degree similar to knockdown of MBD2.

Further, immunoprecipitation studies showed that the peptide in vivo indeed interacts with MBD2 and other components of the MeCP1 complex such as MTA2, HDAC2 and Rbap48. However the peptide did not pull down the endogenous p66α/β and Mi-2α/β. These results indicate that the p66 coiled coil peptide can competitively disrupt the interaction of the endogenous proteins with MBD2.

In order to examine the roles of p66 and Mi2 in the silencing of human γ-globin gene, these proteins were knocked down in CID β-YAC cells. Knocking down p66α augments human γ-globin mRNA to levels similar to MBD2 knockdown in CID dependent β-YAC bone marrow cells. This observation is consistent with previous studies which showed that p66 knockdown abrogates MBD2 mediated transcriptional repression (Brackertz et al. 2006). However, those studies were performed by expressing MBD2 fused with Gal4 DNA binding domain and measuring activity of a luciferase reporter containing Gal4 DNA binding sites (Brackertz et al. 2006). Thus, this is the first time the contribution of the interaction between p66 and MBD2 on MBD2 mediated silencing of endogenous genes have been examined. Likewise knocking down Mi-2β in the CID β-YAC cells led to an elevation in γ-globin expression. These observations point to important roles for p66 and Mi-2 in MBD2 mediated silencing of the globin genes. Moreover, these studies also raise the possibility that the recruitment of Mi-2 to the MeCP1 complex may be through the domains other than the coiled coil domain of p66.

Unlike p66α and Mi-2β, knocking down the levels of their isoforms p66β or Mi-2α did not affect γ-globin expression in CID β-YAC cells. This demonstrates the specificity for particular isoforms of these proteins in mediating the repressor activity of the MeCP1 complex. It could be speculated that the preference for a particular isoform in the assembly of the MeCP1 complex varies depending on the gene that is silenced or the cell type; due to the high expression of one isoform compared to another depending on the cell type. In fact, we observed that Mi-2α was expressed at very low levels in CID β-YAC cells. It was particularly difficult to analyze the knockdown levels by Q-RT PCR because the transcript was detected at very late cycle numbers.

The decrease in the transcriptional repression of the complex lacking p66 and Mi-2 despite the presence of HDAC complements previous work which has shown that treatment with HDAC inhibitors does not relieve the silencing of chicken embryonic ρ-globin both *in vivo* and in cell culture models, and the human γ-globin gene in β-YAC transgenic mice (Ginder 1984, Singal et al. 2002, Pace et al. 1994). These observations demonstrate that DNA methylation mediated gene silencing does not always depend on the enzymatic function of the HDAC proteins in the MeCP1 complex.

The key ionic contact residues that contribute to the specificity of the interaction between the coiled coil residues of p66α and MBD2 are conserved across species and among different homologues of MBD2 (MBD3, MBD3L1, or MBD3L2) and p66 (p66α or p66β) in the same species. Thus, we expect that similar high affinity complexes could form between any of the MBD2 homologues and p66 homologues. As expected, MBD3 was pulled down by the peptide by immunoprecipitation in vivo. However, knockdown of

MBD3 does not cause to any increase in γ-globin in the CID β-YAC cells. This indicates that the effect of p66α coiled coil peptide on γ-globin expression is through MBD2-NuRD and not MBD3-NuRD. This further reiterates the emerging view that MBD2-NuRD and MBD3-NuRD are two separate complexes with distinct functions (Le Guezennec et al. 2006).

These studies together have led us to propose the following model for how p66α coiled coil domain inhibits the function of MeCP1 complex (**Figure 26**). The isolated coiled-coil domain of p66α competitively binds to MBD2 preventing the recruitment of the endogenous p66α/β and Mi-2α/β into the MeCP1 complex. This p66 and Mi-2 deficient complex may still bind methylated DNA by competing with the intact functional MeCP1 complex for the binding to the DNA. In this manner it could act as a dominant negative inhibitor for the binding of a functional MeCP1 complex to the DNA. Alternatively, the p66α coiled coil peptide may sequester the majority of MBD2 present in the cell into the p66 and Mi-2 deficient complex. In this manner it could prevent the binding of sufficient amounts of the functional MeCP1 complex to methylated DNA.

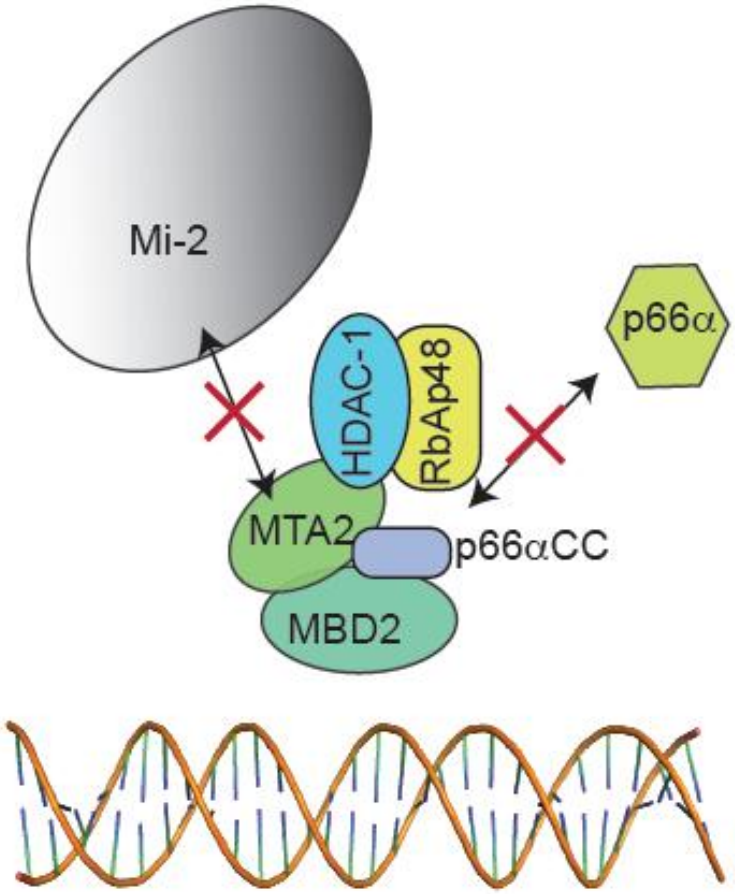

Figure 26 Model for inhibition of the MeCP1 complex by the p66α coiled-coil peptide. p66α coiled-coil peptide binds to four of the six core components from MeCP1 (MTA2, RbAp48, HDAC-1, and MBD2) yet prevents recruitment of native p66α/β and Mi-2α/ β proteins.

CHAPTER 3: MBD2 regulated candidate genes for the modulation of human γ-globin expression

I. **Introduction:**

Methyl CpG binding domain protein 2 (MBD2) belongs to the family of proteins characterized by the presence of MBD domain. This domain enables these proteins to preferentially bind to methylated DNA. MBD2 binds to methylated CpG islands and recruits Nucleosomal Remodeling and Histone Deacetylase (NuRD) co-repressor complex, to cause chromatin compaction and transcriptional silencing (Meehan et al. 1989, Wade et al. 1999, Wade 2001).

We have previously demonstrated that MBD2 knockout in β-YAC transgenic mice leads to at least a tenfold induction of human fetal γ-globin in adult erythroid cells (Rupon et al. 2006). This level of induction is very similar to that which is obtained with the treatment of a DNA methylation inhibitor, 5-Azactydine. During the switch to definitive erythropoiesis in the β-YAC transgenic mice embryos, the absence of MBD2 causes an inability to fully silence the fetal γ-globin.

Although MBD2 was shown to have a role in the silencing of the γ-globin gene, it did not bind to the gene to mediate its silencing (Rupon et al. 2006). This was not entirely surprising because there are no CpG islands within 6 kb of the γ-globin gene. In fact, no

region in the promoter contains more than 4 CpGs in a span of 70 bp DNA. While MBD2 has been shown to bind to a sequence containing as few as three CpGs in vitro (Fraga et al. 2003), MeCP1 complex as a whole seems to require at least 15 CpGs per the complex in vitro (Meehan et al. 1989, Wade 2001). Moreover, in vivo, MBD2 has only been shown to bind to methylated CpG island sequences (Ginder, Gnanapragasam & Mian 2008).

Since MBD2 does not bind to the γ-globin gene to cause its silencing in adult erythrocytes, we postulate that the loss of MBD2 results in transcriptional activation of a gene or genes that are normally silent in adult erythroid cells. The product of this gene(s) could in turn result in the transcriptional activation of the γ-globin gene (**Figure 27**). We have tested this hypothesis in the studies presented here.

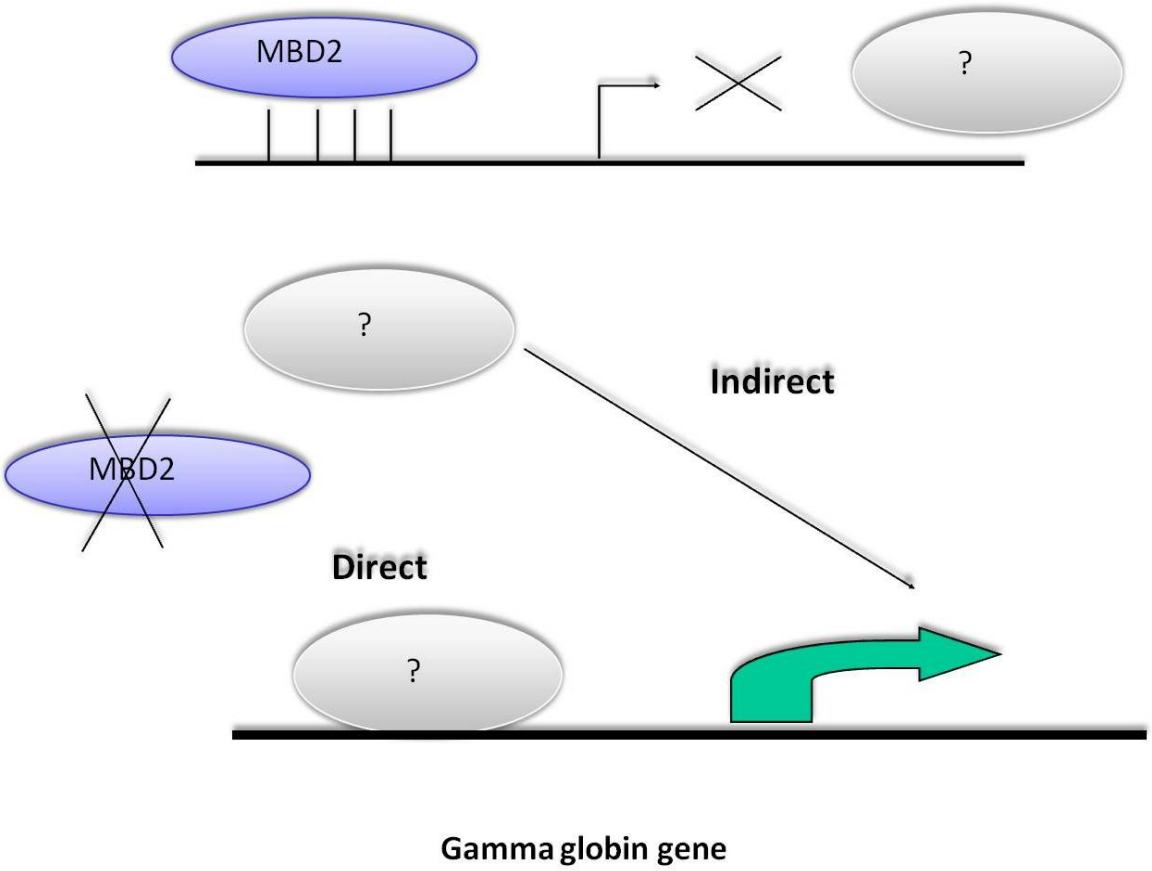

Figure 27 Hypothesis for the indirect silencing of γ-globin by MBD2: Since MBD2 does not bind to the γ-globin gene, it may suppress γ-globin gene transcriptoin by an indirect mechanism ie., by binding to and repressing transcription of intermediary gene/s which may be involved in γ globin gene regulation.

II. Methods:

Microarray analysis:

MBD2-/- mice and wild type mice were made anemic by treatment for two days with intra-peritonial injection of 1-acetyl-2-phenylhydrazine (10 mg/ml; Sigma) at a dose of 0.4 mg/10 g weight of mice. This was done so that spleen becomes greater than 90% erythroid. On the fifth day the spleens from four MBD2-/- and wild type mice were harvested and RNA was extracted using Trizol (Sigma) as explained earlier. DNA contamination in the RNA was removed using DNAse1 treatment using the Turbo DNAse kit (Ambion). The samples were then treated with the RNeasy MinElute Cleanup kit (Qiagen) in order to further clean up and concentrate the RNA. The RNA was eluted in DEPC treated water. 1ul of SUPERase In (Ambion) was added to 40ul of the eluted RNA in order to inhibit any RNAse in the sample. These RNA samples were sent to Dr. Catherine Dumur in the Department of Pathology at Virginia Commonwealth University for further processing.

RNA quality for microarray was judged by capillary electrophoresis performed with an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) on a 6000 Pico LabChip. 5ug of RNA was then reverse transcribed to cDNA using Superscript cDNA Synthesis Systems (Life Technologies). A 24-mer oligodeoxythymidylic acid primer with a T7 RNA polymerase promoter site added at the 3' end of the T7-oligo(dT) was used for cDNA synthesis. In vitro transcription was performed to produce biotin-labeled cRNA using the Enzo Bioarray High Yield RNA Transcript Labeling kit (Enzo).

20ug of cRNA was fragmented for each sample and hybridized for 18-20h on Affymetrix Genechip 430A 2.0 microarray (Affymetrix). A single microarray chip contains 45,000 probe sets analyzing the expression of over 39,000 different transcripts. One transcript in generally interrogated by more than one probe set. Each probe set contains probes that are either perfectly matched (PM) to the sequence of the target gene or contain a single mismatched (MM) nucleotide in the middle position of the corresponding perfectly matched probe. The difference in the intensity of the perfectly match (PM) and mismatched (MM) probes was used to determine whether a gene transcript is detected or not.

Each microarray was washed and stained with streptavidin-phycoerythrin and scanned at a 6um resolution with the Agilent G2500A Technologies Gene Array scanner (Agilent Technologies) according to the GeneChip Expression Analysis Technical Manual procedures (Affymetrix). After the scanning, different parameters such as scaling factor, the noise (RawQ) value for a given probe array hybridization, the percentage of probe sets declared "Present" (%P) by the detection call algorithm, the 3'/5' ratios of the signal intensity values for two house-keeping genes (GAPDH and β-Actin), and the presence or absence call for the ribosomal RNAs 18s and 28s for quality control purposes were determined.

Normalization, background subtraction, and expression summaries were calculated for every probe set using three different methods. 1) The Microarray Suite 5.0 method was used to obtain probe set summaries. 2) Model based expression indices were calculated by use of a multiplicative model to account for probe affinity effects in calculating probe set

expression summaries. 3) Lastly, the multiarray average method which uses quantile normalization followed by a median polish to remove probe affinity effects when calculating probe set summaries was used. The genes which were significantly changed in all the three methods were used for further analyses.

Pathway Analysis:

The differentially expressed gene list obtained from the microarray was analyzed using Ingenuity Pathway Analysis (IPA) program (Ingenuity Systems). IPA dynamically computes a large "global" molecular network based on published physical interactions (direct and indirect) as well as functional interactions between orthologous genes using an extensive database. This analysis was used to identify the genes which have been documented to be involved in hematopoiesis. The program then uses Ingenuity Pathways Knowledge Bank (IPKB) to identify the different molecular networks, thereby providing biological insight into the observed gene expression changes. The affymetrix identifiers such as probe set IDs were imported into the IPA analysis. The input to this application was a dataset file consisting of the differentially expressed genes between MBD2 knockout and wild type mice ($p<0.01$). The biological networks were then ranked by score, which corresponds to the likelihood that the set of genes occurred in the network by chance.

CpG island determination:

Mouse Genome informatics website (http://www.informatics.jax.org/) was used to obtain the genomic DNA sequence of the genes upregulated in MBD2 knockout mice compared

to wild type mice in the microarray. These genes were then investigated for the presence of CpG island using The CpG Island Searcher program with the default program parameters for CpG island.

Chromatin Immunoprecipitation (ChIP) assay:

Both wild type and β-YAC transgenic were treated were made anemic by treatment for two days with intra-peritonial injection of 1-acetyl-2-phenylhydrazine (10 mg/ml; Sigma) at a dose of 0.4 mg/10 g weight of mice. On the fifth day the spleens were harvested and single cell suspensions were obtained by gentle brushing. ChIP assay was performed as described by Rupon et al., (Rupon et al. 2006) with the following modifications. For the crosslinking of chromatin 1% formaldehyde was used for 10 minutes at room temperature. The chromatin was sonicated eight times instead of six times. Chromatin was washed three times with low salt buffer and one time with medium salt buffer, which comprises of equal parts high salt and low salt buffer. Immunoprecipitation was performed using protein G salmon sperm agarose beads, anti-MBD2 (Upstate), goat IgG (Santa Cruz) as a negative control. DNA that was pulled down was analyzed using real-time PCR using Power Sybr green PCR master mix (ABI) as explained earlier. Primers used for real-time PCR analysis are tabulated below.

Since lower stringency washes were used, in order to account for increases non-specific pull down, the enrichment was determined as follows: The value for (gene bound/input)/(amylase bound/input) was calculated for anti-MBD2 sample and was divided by the (gene bound/input)/(amylase bound/input) value obtained for IgG sample.

Knockdown studies:

Knockdown of MBD2 was performed in the CID β-YAC cells and MEL cells exactly as described in the previous chapter. Knockdown of MBD2 in human CD34+ cells has been explained below under lentivirus transduction.

Overexpression studies:

CID β-YAC cells were overexpressed exactly as described earlier in the previous chapter. In order to overexpress Gab1, the coding sequence of the human Gab1 cDNA was cloned into pcDNA4/TO vector at the HindIII restriction site. To overexpress ZBTB32, pCEP Flag FAZF plasmid, a generous gift from Dr. Maureen Hoatlin (Oregan Health and Science University, Portland, Oregan, USA) was used (Dai et al. 2002, Hoatlin et al. 1999).

Primary human CD34+ hematopoietic progenitor cell culture:

We have obtained access to de-identified and discarded human adult mobilized peripheral blood samples through Massey cancer center (VCU, Richmond, Virginia) stem cell transplant program. The aphaeresis packs were thawed at 37°C and the cells were diluted with an equal volume of PBS containing 2%FBS. The mononuclear cells were isolated using Ficol Paque Plus (Stem Cell Technologies). For this, cells were loaded on Ficol Paque Plus and spun at 400x G for 30 minutes with no brake. The volume is Ficol Paque Plus used was half the volume of the cells loaded on top of it. The middle

mononuclear layer was withdrawn and diluted with PBS containing 2% FBS and 1mM EDTA. It was filtered using a 70μM nylon filter (BD Biosciences). The cells were then washed twice with PBS containing 2% FBS and 1mM EDTA and were diluted to $4x10^8$ cells/mL in a 5mL Falcon Polystyrene tube.

The CD34+ hematopoietic progenitor cells were enriched by magnetic sorting. For this we used a CD34+ positive selection cocktail which contains magnetic nanoparticles coated with CD34+ antibody coated onto magnetic nanoparticles(Stem Cell Technologies). 100 μL of the cocktail was added to 1 mL of cells and incubated at room temperature for 10 minutes. The volume of cells was increased to 2.5 mL per tube using PBS containing 2% FBS and 1mM EDTA. The tube was then placed in an EasySep Magnet (Stem Cell Technologies) for 5 minutes and then the tube was removed and the supernatant was discarded immediately. The cells were again resuspended with 2.5 mL of the buffer and the magnetic separation process was repeated four times.

After the final wash the cells were resuspended at approximately $1x10^6$ cells /mL of StemSpan® Serum-Free Expansion Medium (SFEM) manufactured by Stem Cell Technologies. This medium was supplemented with StemSpan® Cytokine Cocktails (CC100 which consists of recombinant cytokines such as Flt3 ligand, SCF, IL-3 and IL-6), low density lipoproteins and Penicillin-Streptomycin.

After about 1 week of culture in the amplification medium, the cells were washed off of the amplification medium and were grown in erythroid differentiation medium (SFEM medium containing 20ng/mL SCF, 1u/mL Epo, 5ng/mL IL-3, 2 μM Dexamethason, 1 μM Estradiol and Penicillin-Streptomycin). The cells were allowed to

differentiate for approximately 10 days. The cell pellets turn pink as an indication of successful erythroid differentiation. Moreover, while the progenitor cells have a high γ- to β-globin ratio, the differentiated cells have very low γ-globin and high β-globin mRNA expression (γ-globin mRNA is less than 1% of the total β-globin mRNA).

Lentivirus generation:

Lentiviruses were produced in 293T cells which were transfected with packaging plasmid (pCMV R DNA), envelop plasmid (pMD DNA) and the Transfer vector (pLVTHM containing shMBD2 or shScramble) using calcium phosphate method.

Human shMBD2 sequence used:

5'Mlu1CCCC**GGGTAAACCAGACTTGAAT**TTCAAGAGA**ATTCAAGTCTGGTTT ACCC** TTTTTGGAA Cla1 3'

The sequence marked in red is the 19 nucleotide sense siRNA and the sequence marked in green is the 19 nucleotide antisense siRNA. The sequence between these two regions is the loop for the shRNA.

The 293T cells were grown using DMEM (Gibco) medium containing 20% FBS (Hyclone) in 37ºC, 5% CO_2 incubator. The day prior to the calcium phosphate transfection, $6x10^6$ cells were plated evenly with 10 mL media in a 10 cm dish such that they will be 80% confluent on the day of transfection. The next day, the old medium was removed and 9ml of fresh medium was added to the cells at least 2 to 3 hours prior to transfection.

For calcium phosphate transfection, a mixture of 7.5 µg of pCMV R DNA (packaging plasmid0, 4 µg of pMD DNA (envelope plasmid) and 10 µg of pLVTHM containing shMBD2 or shScramble was made up to 437 µL using dH_2O in a 2 mL tube. Then 63 µL of 2M $CaCl_2$ was added drop wise to the tube. The above 500 µL mix was added one drop at a time to 500 µL of 2xHBS buffer slowly. The solution was then distributed evenly over the cells drop by drop. After incubating for 5 minutes at room temperature, the plates were transferred to the 37°C incubator. After approximately 18 hours, the medium was removed and fresh media was added to the cells.

48 hours after transfection, the medium which now contains the virus was harvested and then spun down at 3000rpm for 5 minutes at room temperature. It was then filtered through 0.45µm filter (Nalgene). The viral supernatants were stored at -70°C in cryovials.

While working with viruses, although replication incompetent, we used the BSL 2+ recommended guidelines.

Lentiviral transduction of the primary human hematopoietic progenitor cells:

The human primary erythroid progenitors being cultured in the amplification medium were transduced with the lentivirus carrying the shMBD2 and shScramble constructs. For this we plated $1x10^5$ cells using 100 µL of the amplification medium in a 24 well plate. 250-300 µL of the virus was added to the well. 1 µL of polybrene per ml of virus was added to increase the transduction efficiency. The plate was then placed in a shaker in 37ºC, 5% CO_2 incubator for at least 2 hours. The cells were then investigated for

GFP expression after 3 days since the transfer vector carries the GFP marker. GFP positive cells were flow sorted. This population of transduced cells was then subjected to erythroid differentiation as described above.

Primers: The sequences of the primers used in chapter 3 are tabulated below.

Primers for Q-RT PCR	Forward Primer	Reverse Primer	Taqman Probe
Human Gamma 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
Chicken Rho globin	CAG AGG TTC TTT GAT AAC TTC GG	ACG ATG ATG AGG ATG TTC CC	
Mouse Glycophorin A	GCC GAA TGA CAA AGA AAA GTT CA	TCA ATA GAA CTC AAA GGC ACA CTG T	FAM/TTGACATCCAATCT CCTGAGGGTGGTGA /BHQ
Mouse MBD2	TTT GAC TTC AGG ACC GGC AAG ATG	ATT GCT CGG GTG GTT CGT GAA TTT	
Mouse p66 alpha	AAT AAC GGG TCC TCA CTA CAG	GTA TTC TCG CTG TCG ATC CA	
Mouse p66 beta	GCA GTG TAT GAC TTC CAA CC	CTG TAA TCG CTG TTC AAT TTC C	
Mouse MBD3	CGC TAT GAT TCT TCC AAC CAG	GTC AAA GGC ACT CAA TCC AC	
Mouse Mi2 beta	GAA CCA CAG GGA GTT AAT GAG	CTT ATA GAG GGA GTA GAG GAA GAC	
flag tagged p66alpha coiled coil peptide pcmv Tag2B	ACA AGG ATG ACG ACG ATA AGA GCC	TGT ATT TGA CTC TGC CGC AAC T	FAM/CGA GAA AGG ATG ATC AAG CAG CTG AAG G/BHQ
Mouse CyclophilinA	GAG CTG TTT GCA GAC AAA GTT C	CCC TGG CAC ATG AAT CCT GG	FAM/TTC GAG CTC TGA GCA CTG GAG AGA AA/BHQ

Table 5 Primers used in Chapter 3

Statistics:

Results are shown as mean (from three or more independent biological repeats) ± standard error. For comparison between data sets, unpaired two tailed t-tests were performed, p values are indicated as < 0.05 (*), <0.001 (**), and not significant (NS).

III. Results:

A. MBD2 does not bind to the β-globin locus:

MBD2 is involved in the human γ-globin silencing in adult erythrocytes without directly binding to the γ-globin gene. The γ-globin gene is very CpG sparse. In fact, the promoter does not contain more than 4 CpGs spanning a 70 bp region. Thus, it is not surprising that MBD2 does not bind to the γ-globin gene. In order to determine if MBD2 binds anywhere else in the β-globin locus, ChIP assays were performed in adult erythrocytes obtained from spleens of anemic wild type and MBD2-/- β-YAC transgenic mice (**Figure 28**). The mice were made anemic by 1-acetyl-2-phenylhydrazine treatment. ChIP assays were performed using anti-MBD2 antibody and sheep IgG, which was used as a negative control. The whole of the β-globin locus is CpG sparse. MBD2 binding was probed in the human ε-globin promoter, a weak CpG island region between γ-globin and δ-globin genes, the human β-globin coding region, and the CpG island of the Ugt8 gene. Ugt8 gene served as a positive control for MBD2 binding. As shown in figure, MBD2 did not bind to any of the regions probed in the β-globin locus. This result was likely since the β-globin locus lacks the presence of CpG islands. MBD2 has been shown to bind only to CpG islands, in vivo (Ginder, Gnanapragasam & Mian 2008, Wade 2001)

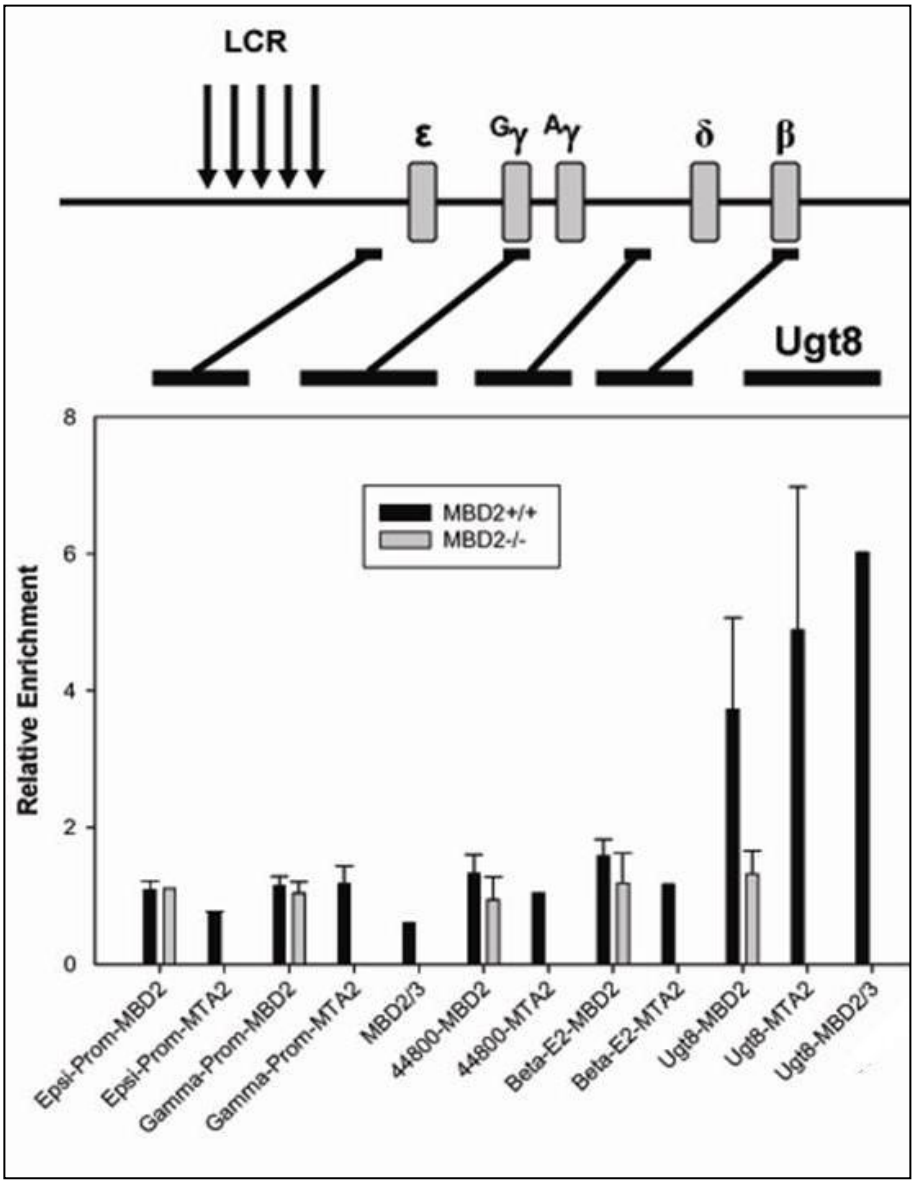

Figure 28 Chromatin Immunoprecipitation of MBD2 and MTA2 in the β-globin locus. ChIP assays were performed using anti-MBD2, anti-MTA2, antiMBD2/3, and sheep and goat IgG control antibodies in in adult erythrocytes from MBD2 wild type and knockout mice. DNA was analyzed using real-time PCR at the human ε- and γ-globin promoters, a weak CpG island between the γ-globin and δ-globin genes, the human β-globin coding region, and the Ugt8 gene CpG island. Data was normalized to endogenous amylase gene levels to account for loading errors and IgG controls to account for non-specific pull-downs. Error bars indicate ±SD. (Performed by Dr. Jeremy W Rupon)

B. Hypothesis and Strategy:

Because MBD2 does not bind to the β-globin locus, we hypothesized that MBD2 silences the γ-globin gene indirectly. We postulated that during the switching to definitive erythropoiesis, MBD2 silences another gene(s) that is necessary for the expression of γ-globin gene. In the absence of MBD2 (MBD2-/- mice), this gene(s) is expressed and its product either directly or indirectly causes the incomplete silencing of the γ-globin gene during the switch to definitive erythropoiesis, leading to the elevated expression of the γ-globin gene in adult erythrocytes.

In order to identify this putative gene(s) involved in MBD2 mediated silencing of γ-globin gene, we adopted the following strategy (**Figure 29**). We obtained the list of genes differentially expressed between MBD2-/- and wild type mice by microarray analyses. Using this list of genes obtained from microarray analyses, we prioritized the genes based on two criteria: their relevance in hematological pathways (IPA analyses) and their expression during primitive erythropoiesis (biological filter). Once this list of priority genes was validated we performed functional studies to identify their role in γ-globin silencing.

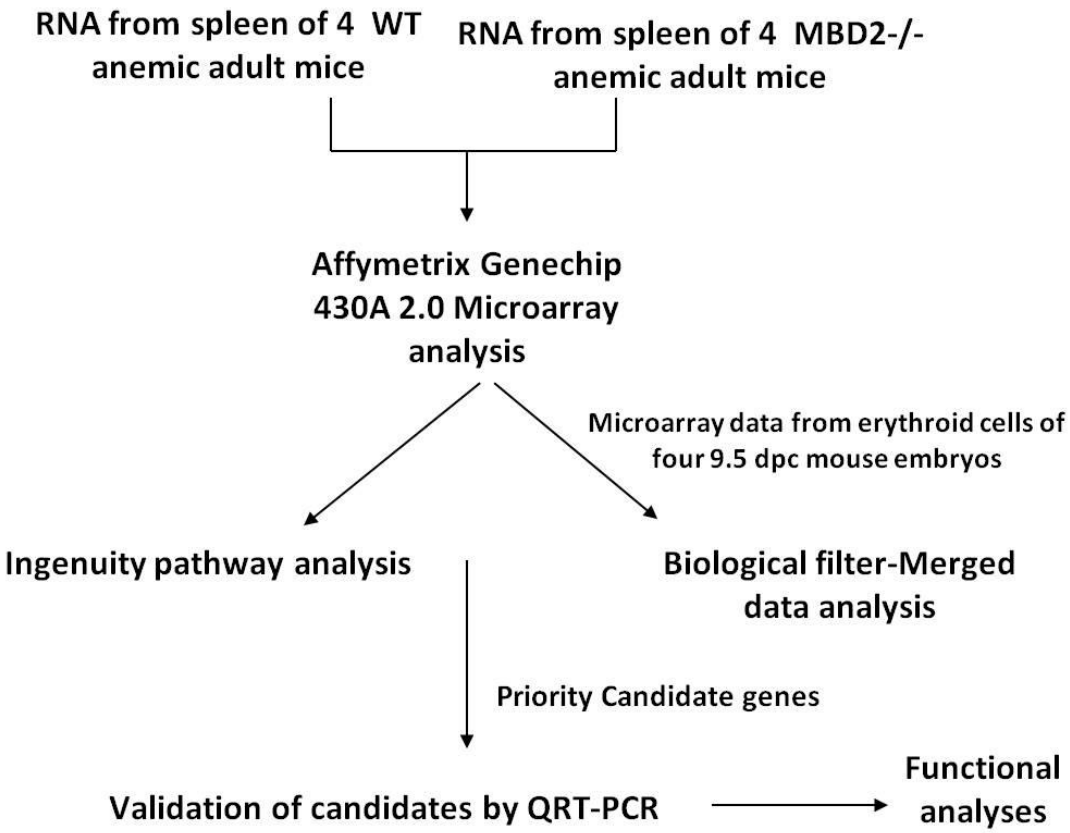

Figure 29 Methodology flow chart

C. Genetic profile of MBD2 knockdown mouse:

Four wild type and four MBD2-/- mice were made anemic by 1-acetyl-2-phenylhydrazine treatment in order to make their spleens almost 90% erythroid. RNA was isolated from the spleens and was sent for microarray analyses to our collaborator Dr. Catherine Dumur (Departement of Pathology, VCU). The quality of the RNA was checked by capillary electrophoresis. RNA was converted to cRNA and was then hybridized to Affymetrix 430A2.0 mouse microarray chips. This chip contains over 22,600 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes. The difference in the intensity of hybridization signal between the perfectly matched probe and mismatched probe is used to determine whether a gene transcript is detected or not.

For every probe set, normalization, background subtraction, and expression summaries were calculated using the Microarray Suite 5.0 method, the Li and Wong multiplicative model-based expression index (MBEI), and robust multiarray average (RMA) method. To generate the list of genes which are differentially expressed between MBD2-/- and wild type adult mouse erythroid cells, a statistical cut off p value of ≤ 0.01 was used. 93 genes were upregulated and 50 genes were downregulated in MBD2-/- mice compared to wild type mice adult erythroid cells. For the statistical analyses, we collaborated with Dr. Kellie J. Archer (Department of Biostatistics, VCU).

D. Prioritization of Candidate genes for the indirect effect of MBD2:

We focused on the genes that are upregulated in MBD2-/- compared to wild type adult erythroid cells. This is because, MBD2 has been shown to be primarily involved in

silencing genes by recruiting NuRD corepressor complex to the gene. In order to identify the candidate genes for the indirect effect of MBD2, two filters were used to prioritize the candidate genes.

For the first filter, Ingenuity Pathway Analysis (IPA) software was used. IPA dynamically computes a large "global" molecular network based on published physical interactions (direct and indirect) as well as functional interactions between orthologous genes using an extensive database. We identified the differentially expressed genes that belonged to hematological pathways and focused on the genes with CpG islands for prioritization. This is because the MBD2 containing MeCP1 complex has been shown to require densely methylated DNA for its binding (Ginder, Gnanapragasam & Mian 2008, Meehan et al. 1989). Zinc finger and BTB domain containing 32 (Zbtb32), YY1-associated factor 2 (YAF2), BRG1-associated factor 57 (Baf57), and Itchy E3 ubiquitin protein ligase homolog (ITCH) were chosen as priority genes based on this criteria. YAF2 (YY1-associated factor 2), was chosen as a priority candidate gene despite the absence of CpG island since it has been shown in previous studies to be involved in γ- and ε-globin regulation by its association with YY1 (Wandersee, Ferris & Ginder 1996).

In an alternative approach to identify a gene(s) that is both a positive regulator of γ-globin gene transcription and is repressed by MBD2, a second filter which we termed "biological filter" was used. For this, we merged our wild type and MBD2 knock out comparison data with microarray data from RNA of erythroid cells obtained by laser capture microdissection from four frozen sections of embryonic day 9.5 yolk sac, published by our collaborator Dr. Joyce Lloyd (Department of Human Genetics, VCU)

(Redmond et al. 2008). Within each dataset, probe sets were compared using appropriate two-class comparison method such as two-sample t-test and two datasets were then merged by probe set ID. For each gene, the resulting p values from the two independent studies were combined using Fisher's procedure. Using a correction for multiple hypotheses testing, the combined p-values were examined to identify a list of significant genes.

This analysis yielded a small list of 13 genes that were significantly up-regulated in erythroid cells from both 9 dpc wild type mouse embryo and MBD2 knock out adult mice, compared to wild type mice. Of this list GRB2-associated binding protein 1 (Gab1) and YY1 associated factor 2 (YAF2) were chosen as our priority candidate genes. Gab1 was chosen because of the presence of CpG island and based on its critical role in erythropoietin signalin. Activation of the MAPK pathway in the erythropoietin signaling cascade has been associated with the elevation in γ-globin expression (Bhanu et al. 2004). YAF2 was chosen despite the absence of a CpG island due to its functional relevance to γ-globin regulation by its association with YY1 (Wandersee, Ferris & Ginder 1996) as discussed earlier.

E. Validation of Candidate genes:

i. Adult erythrocytes from β-YAC transgenic mice:

It is important to confirm the results obtained by microarray analyses by Q-RT PCR, due to the possibility of false positive in microarrays results and since the gene expression analyses obtained from microarrays are not quantitative. We verified if the

candidate genes on our priority list were elevated in expression in MBD2-/- compared to wild type mice using Q-RT PCR. For this we isolated RNA from spleens of three anemic MBD2-/- and wild type mice. The mice used were different from those used for microarray studies. The mice used in our studies were of mixed background. The strength in using mice of mixed background rather than inbred strains is that it is more representative of the variability observed in human populations. However, the modifier effects arising due to mixed background could potentially contribute to variability in our observations. Thus, it was particularly important to verify the microarray results in independent samples.

After verification by Q-PCR, ZBTB32 and Gab1 were the two genes which got validated for increased expression in MBD2-/- mice. UDP galactoyltransferase 8A (Ugt8) gene was used as a positive control for Q-PCR since this was one of the genes which showed the highest fold difference in the microarray. As shown in **figure 30,** Gab1 was elevated by ~2.5 fold and ZBTB32 was elevated by ~4 fold in adult erythrocytes from MBD2-/- mice compared to wild type mice.

ii. Mouse erythroleukemia cells:

In order to further verify these two candidate genes in an in vitro system, MBD2 was knocked down in mouse erythroleukemia (MEL) cell line using shRNA. MBD2 was stably knocked down by about 90% both at the mRNA and protein level (**Figure 31**). Knock down of MBD2 caused an elevation in the expression of both Gab1 and ZBTB32 compared to shScramble control cells (**Figure 31**).

iii. Primary human CD34+ hematopoietic progenitor cells:

Encouraged by these results, we went on to verify whether knocking down MBD2 can lead to upregulation of Gab1 and ZBTB32 in primary human CD34+ hematopoietic progenitors. MBD2 was knocked down by transducing these cells with shMBD2 and shScramble lentiviruses. MBD2 mRNA was knocked down by approximately 70%.(**Figure 32a**) After the transduction with lentiviruses, these cells were differentiated to erythroid lineage. After approximately 10 days of differentiation, γ-globin mRNA levels were measure. MBD2 knockdown lead to an induction of γ-globin mRNA levels by approximately 4 fold compared to shScramble controls(**Figure 32b**). The knockdown of MBD2 also caused a modest increase in both Gab1 (~2.5 fold) and ZBTB32 (~2.5 fold) expression at the mRNA level (**Figure 32c,d**).

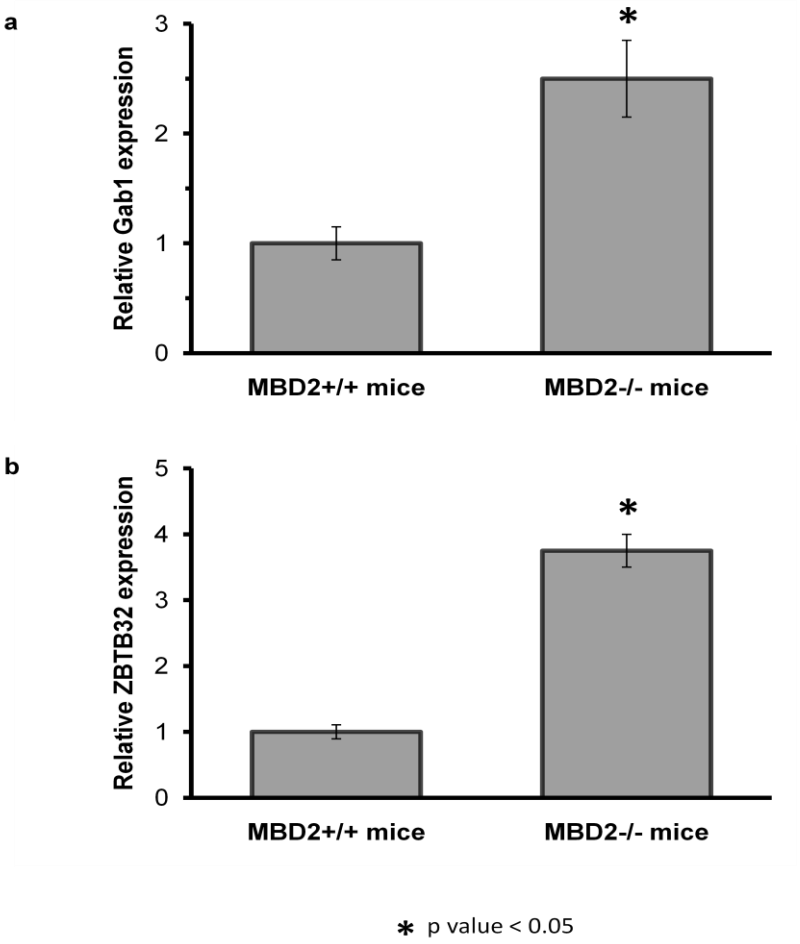

Figure 30 Validation of candidate genes in MBD2 knockout mice by Q-RT PCR: RNA from spleen of three anemic MBD2 knock down (MBD2-/-) and wild type (MBD2+/+) mice was analyzed by Q-RT PCR to verify the differential expression of Gab1 and Zbtb32; n=3, Error bars indicate mean ±SD.

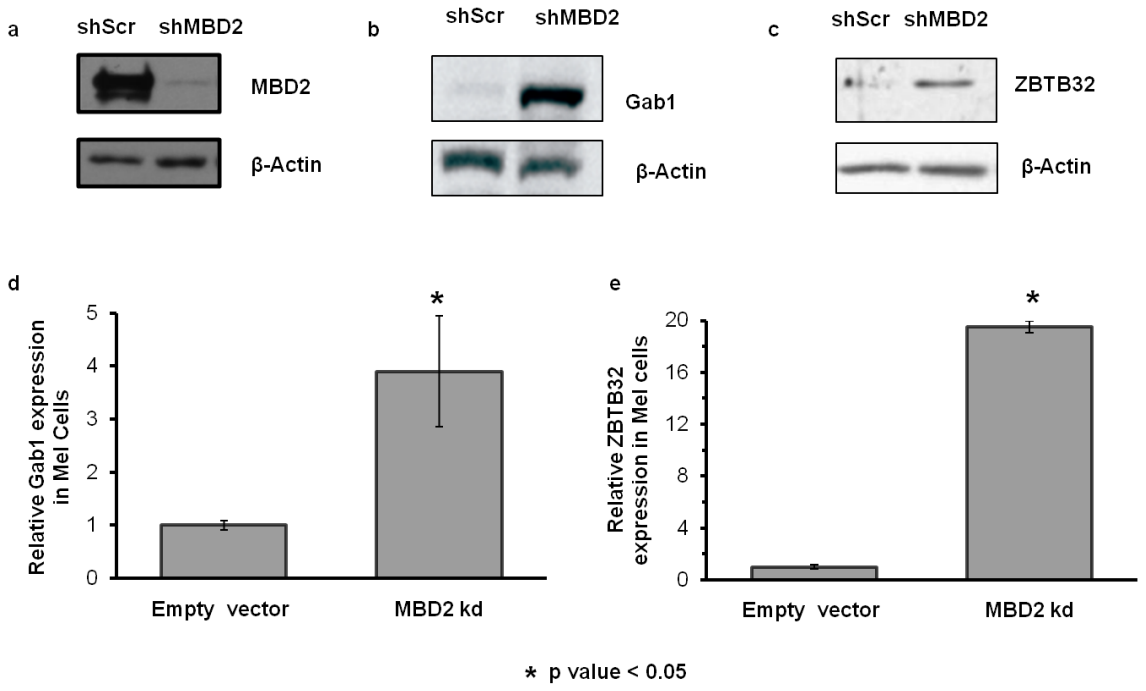

Figure 31 Validation of candidate genes in MEL cell line: Mouse erythroleukemia cell line was transfected with pSuperior-MBD2 shRNA and empty vector control. Western analysis was performed to determine of a) MBD2 knockdown levels; b) Gab1 and c) ZBTB32 protein levels in the MBD2 knockdown cells. Q-RT PCR was performed to determine d) Gab1 and e) Zbtb32 mRNA levels in the MBD2 knockdown cells. CyclophilinA was used for normalization in Q-RT PCR. Error bars indicate mean ±SE

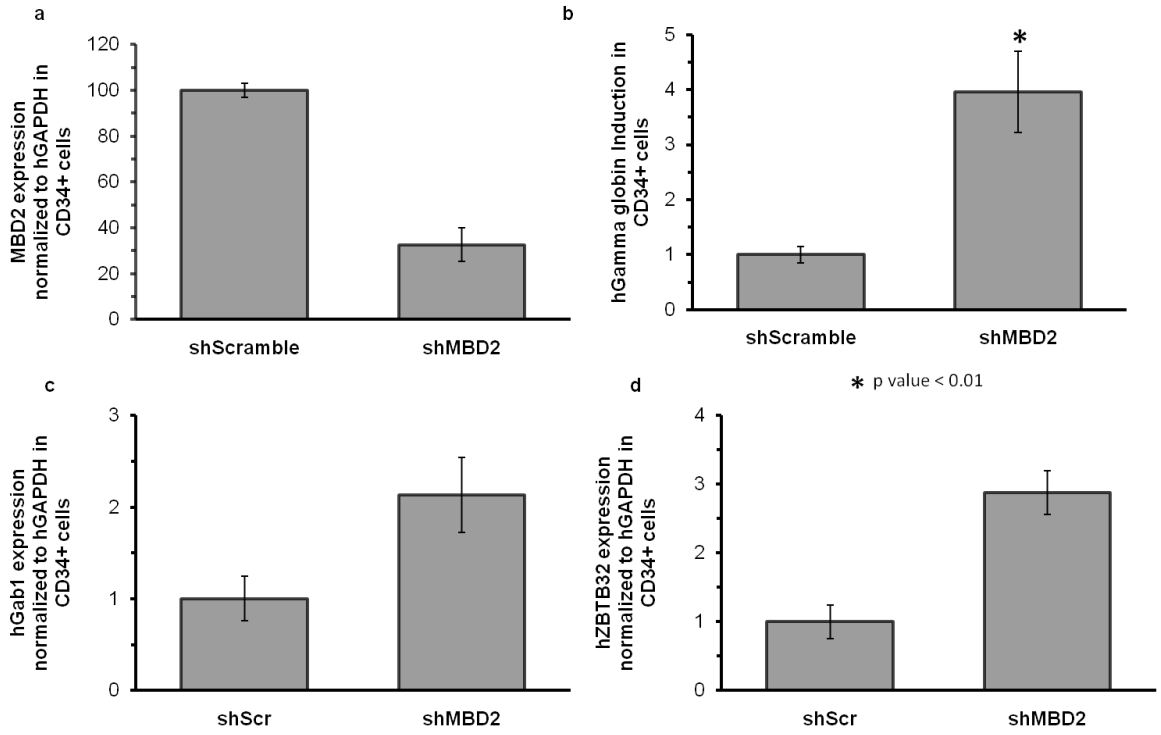

Figure 32 Validation of the candidate genes in primary human erythroid cells: Primary human CD34+ hematopoietic progenitor cells were lentivirally transduced with shMBD2 and shScramble containing viruses . These cells were then differentiated towards erythroid lineage. Q-RT PCR was performed to determine the levels of a) MBD2 knockdown, b) γ-globin mRNA (n=4), c) Gab1 mRNA (n=1) and d) ZBTB32 mRNA expression (n=1). MBD2, Gab1 and ZBTB32 expression were normalized to CyclophilinA and gamma globin expression was normalized to GlycophorinA .Error bars indicate mean ± SE (Experiments in Figure 32 a and b were performed by Shou Zhen Wang, VCU)

F. MBD2 binds to ZBTB32 CpG island:

Growth factor receptor bound protein 2- associated binding protein 1 (Gab1) is an adapter protein that has been implicated in erythropoietin signaling. The balance between growth and differentiation is critical for erythropoiesis. The MAPK pathway is involved in the growth arm in erythropoietin signaling. Increased activation of growth related signaling such as the MAPK pathway has been implicated in increased γ-globin levels (Bhanu et al. 2004). Gab1 adapter protein plays a critical role in MAPK signaling pathway and we can speculate that increased levels of Gab1 could activate the MAPK pathway leading to an increase in γ-globin levels. Although this gene has a CpG island in its promoter, it is ubiquitously expressed and it has not been documented as a differentially methylated region. Thus, we speculate that the knockdown of MBD2 can induce the expression of Gab1 through indirect mechanisms.

Zinc finger and BTB domain containing 32 **(**ZBTB32) interacts with GATA2, which upregulates γ-globin expression in K562 human erythroleukemia cells (Tsuzuki, Enver 2002, Ikonomi et al. 2000). It contains a CpG island in the body of the gene. We investigated whether MBD2 binds to this CpG island. ChIP assays were performed using adult erythroid cells from spleen of anemic MBD2-/- and wild type mice. Anti-MBD2 antibody and rabbit IgG control was used for the immunoprecipitation. The CpG island in the promoter of the Ugt8 gene was used as a positive control for the binding of MBD2. MBD2 showed enrichment at the CpG islands of the ZBTB32 and Ugt8 genes in wild type mice but not in control MBD2-/- mice (**Figure 33**). This observation demonstrated that MBD2 binds directly to the ZBTB32 gene in adult erythrocytes.

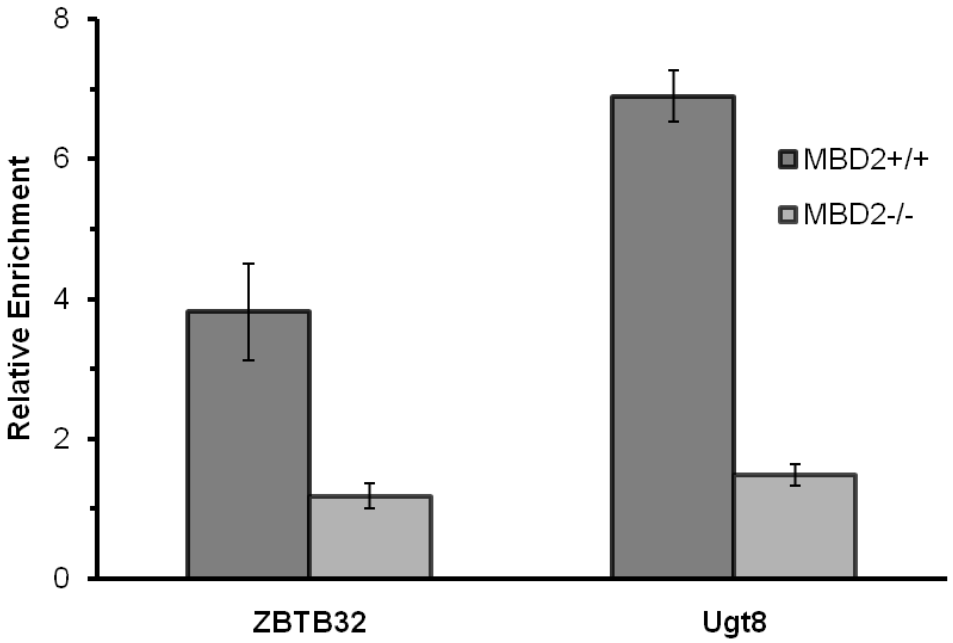

Figure 33 Chromatin Immunoprecipitation of MBD2 in ZBTB32 gene. ChIP assays were performed using anti-MBD2, anti-MTA2, and sheep and goat IgG control antibodies in adult erythrocytes from MBD2 wild type and knockout mice. DNA was analyzed using real-time PCR the Ugt8 gene CpG island and ZBTB32 gene CpG island. Data was normalized to endogenous amylase gene levels to account for loading errors and IgG controls to account for non-specific pull-downs. Error bars indicate mean ±SD. (Performed by Dr. Jeremy W Rupon)

F. Functional studies:

We investigated if the validated candidate genes had a functional role in regulation of the γ-globin gene. We were unable to use human erythroid cell lines to study this because they display a abnormal globin gene expression pattern. We have used chemical inducer of dimerization dependent multipotential β-YAC bone marrow cells (BMCs). These cells are derived from the bone marrow of β-YAC transgenic mice and can be propagated in culture by virtue of an artificial proliferation signal comprised of the thrombopoietin signaling domain fused to FKBP (FK506 binding protein) binding domain, which are responsive to a chemical inducer of dimerization (CID) (Blau et al. 2005). In the presence of a CID, homodimers are generated, and the resultant growth signal maintains the BMC population indefinitely and also causes spontaneous differentiation into the myeloid lineage. These cells predominantly express human adult β-globin and very low levels of γ-globin (<1% of β-globin), similar to human adult erythroid cells (Blau et al. 2005).

First, we aimed to determine if knocking down MBD2 leads to elevation of γ-globin expression in these cells as observed in β-YAC transgenic mice. MBD2 was knocked down with siRNAs (Qiagen) using amaxa nucleofection method. All stars negative siRNA (Qiagen), a highly validated non-silencing siRNA was used as a negative control. At 48 hours after transfection, MBD2 was knocked down by ~80 % (**Figure 34a**) and this knockdown caused ~ six fold induction of γ-globin at 72 hours post-transfection, similar to the levels obtained in the β-YAC in vivo mouse model, indicating that this cell line is a valid model for the study of MBD2 mediated γ-globin regulation. (**Figure34b**).

We then studied the effect of MBD2 knockdown on the candidate genes of interest; Gab1 and ZBTB32. As observed in mice and other cell culture models, the expression of these genes was elevated in MBD2 knockdown cells compared to control siNeg cells (**Figure 34c,d**).

In order to perform functional studies, plasmids containing Gab1 and ZBTB32 cDNA were overexpressed in the CID β-YAC cells using nucleofection. Empty vector was used as a negative control. On overexpressing Gab1, a modest elevation of γ-globin by ~ 2.5 fold was observed at 72 hours post-transfection (**Figure 35a**). Likewise, the overexpression of ZBTB32, caused the elevation of the γ-globin level by ~ 2.5 fold compared to empty vector control (**Figure 35b**). Moreover, preliminary studies have shown that co-expression of Gab1 and ZBTB32 causes an additive increase in γ-globin expression (**Figure 35c**).

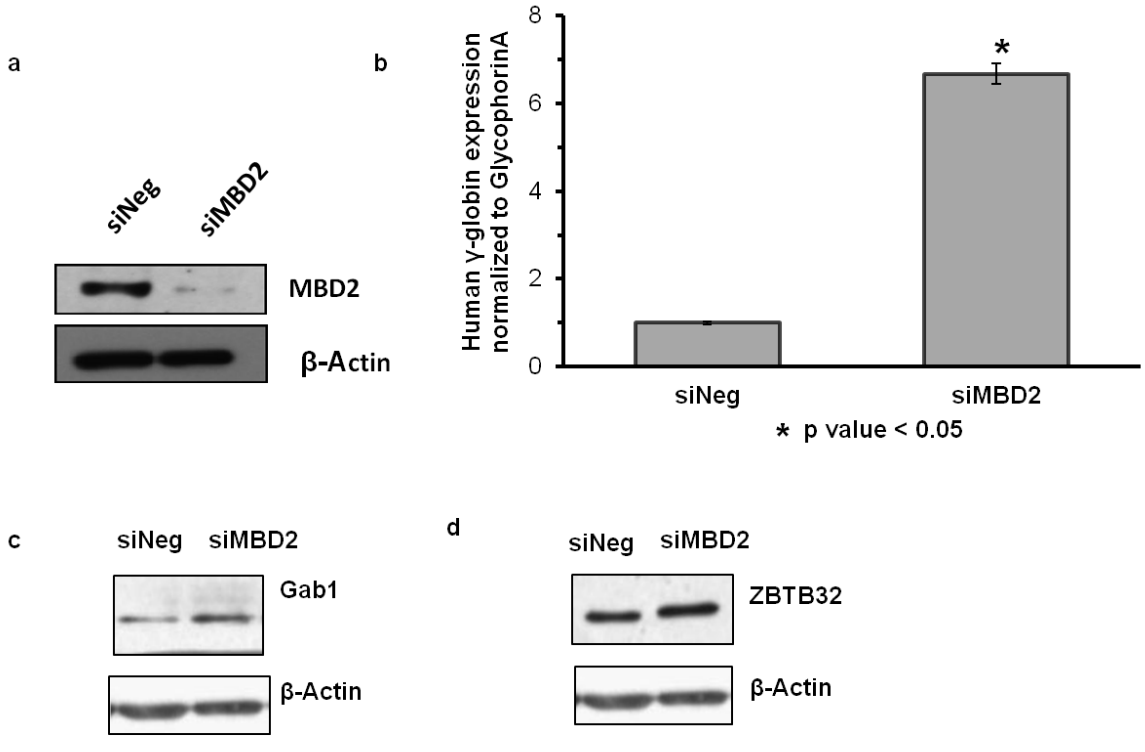

Figure 34 Validation of candidate genes in CID β-YAC cells: CID dependent β-YAC bone marrow cells were transiently transfected with siMBD2 and siNeg control (n=3). They were analyzed for a) MBD2 protein levels by western analysis; b) γ-globin mRNA level by Q-RT PCR; and c) Gab1 and e) ZBTB32 protein levels by western analysis. All the experiments were performed at 72h post transfection. Error bars represent mean ±SE.

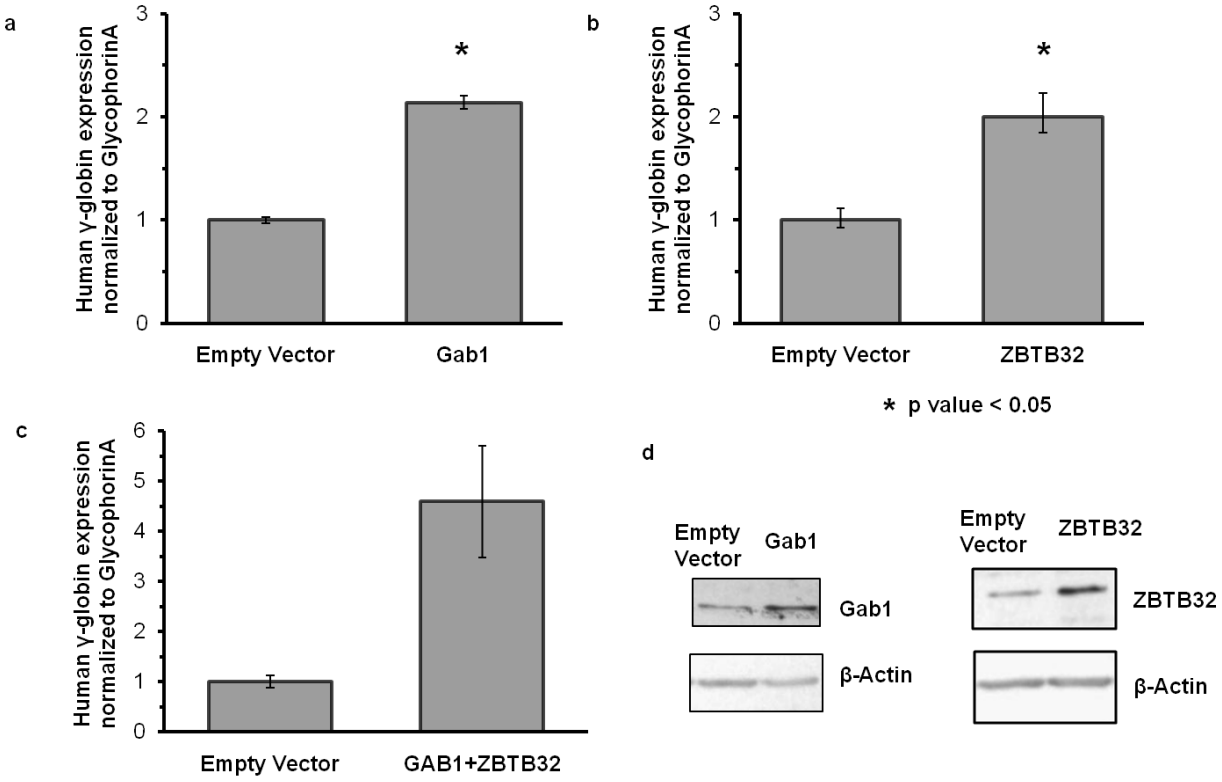

Figure 35 Functional studies in CID β-YAC cells: CID dependent β-YAC bone marrow cells were transiently transfected with the coding regions of Gab1 (n=3), ZBTB32 (n=3) and both Gab1 and ZBTB32 (n=1) along with their corresponding empty vector controls. mRNA levels of γ-globin was calculated using Q-RT PCR in the following samples a) Gab1 overexpressing cells b) ZBTB32 overexpressing cells c) both Gab1 and ZBTB32 overexpressing cells. Gamma globin expression was normalized to GlycophorinA. d) The overexpression Gab1 and ZBTB32 was verified using western analysis. Error bars indicate mean ± SE

IV. Discussion:

In the current study, we sought to understand how the Methylated CpG Binding Domain Protein 2 (MBD2) mediates silencing of the γ-globin gene without binding directly to the gene. Because MBD2 does not bind to the γ-globin gene, we examined if it bound to the other regions of the β-globin locus such as the human ε-globin promoter, the human β-globin coding region, and the weak CpG island region between γ-globin and δ-globin genes. None of these regions were bound by MBD2 when examined by ChIP assays. The MBD2 containing MeCP1 complex requires at least 15 methylated CpGs per complex for binding in vitro (Ginder, Gnanapragasam & Mian 2008, Meehan et al. 1989, Wade 2001). In vivo, MBD2 has only been shown to bind to methylated CpG islands (Ginder, Gnanapragasam & Mian 2008). Thus, it is not surprising that MBD2 does not bind to the β-globin locus since it lacks CpG islands.

We hypothesized that MBD2 silences the human γ-globin gene in definitive erythrocytes by an indirect mechanism: Loss of MBD2 results in transcriptional activation of a gene or genes that are normally silent in adult erythroid cells. The elevated expression of this gene(s) could in turn result in the transcriptional activation of the γ-globin gene. In order to test this hypothesis, we first have identified the genes which are differentially regulated in adult erythroid cells of MBD2-/- compared to wild type mice. We performed microarray analyses and identified 93 genes which were upregulated and 50 genes downregulated in MBD2-/- mice compared to wild type mice.

Thus far, MBD2 has been shown to regulate only a handful of genes in normal cells. In mice, MBD2 knock out causes disruption of the expression patterns of the

interleukin 4 and interferon γ genes during T cell differentiation (Kersh 2006, Hutchins et al. 2002), abnormal expression of digestive enzyme genes in the colon (Berger et al. 2007), dysregulation of the adult olfactory epithelial progenitor-driven neurogenesis (Macdonald et al. 2010) and dysregulated expression of the Xist gene (Barr et al. 2007). Moreover, loss of MBD2 suppresses the formation of intestinal adenoma in $Apc^{min/+}$ mice by attenuating the Wnt signaling pathway (Berger, Bird 2005a, Sansom et al. 2003, Phesse et al. 2008). More extensive molecular studies on different tissues of MBD2-/- mice are necessary to dissect the role of MBD2 in gene regulation in normal cells. Currently, there is no information on what genes, other than, γ-globin in humans (Rupon et al. 2006) and ρ-globin in chicken (Kransdorf et al. 2006) , are regulated by MBD2 either directly or indirectly in erythroid cells. Our studies indicate that MBD2 knock out disrupts only a limited set (hundred and not thousands) of protein coding genes.

We have used two filters to prioritize candidate genes for their potential role in the MBD2 mediated silencing of the γ-globin gene. In order to prioritize the genes from a list of 93 genes which were found to be upregulated in MBD2 knockout mice compared to wild type mice, two filters were used. IPA software analysis was used to identify genes functionally involved in hematological pathways. We also used a novel biological filter, to help us identify the genes which are expressed highly in primitive erythrocytes and in MBD2-/- adult erythrocytes. For this, we merged our list of upregulated genes in MBD2 knockout mice with genes which are expressed higher in erythroid cells from the yolk sac of 9.5 dpc embryos. For this purpose, we used the microarray data from RNA of erythroid cells obtained by laser capture microdissection from four frozen sections of embryonic day

9.5 yolk sac, published by our collaborator Dr. Joyce Lloyd (Department of Human Genetics, VCU) (Redmond et al. 2008). This filter helped us narrow down the list of potential candidate genes from 93 to 13. This outcome demonstrates that similar biological filters could be employed to prioritize candidate genes from the long list of differentially expressed genes usually obtained from microarray analyses.

We narrowed down a list of six genes based on the two filters as candidate genes: Zinc finger and BTB domain containing 32 (Zbtb32), YY1-associated factor 2 (YAF2), BRG1-associated factor 57 (Baf57), Itchy E3 ubiquitin protein ligase homolog (ITCH),and GRB2-associated binding protein 1 (Gab1). Among these genes, only ZBTB32 and Gab1 were validated for their elevated expression in MBD2-/- adult erythroctyes by Q-RT PCR. These two genes were further validated for their elevated expression in MBD2 knocked down mouse erythroleukemia cell line, human primary CD34+ hematopoietic progenitor cells, and CID β-YAC bone marrow cells. A high number of false positives could be attributed to the variability caused by modifier genes in the mixed background of mice used for this study. Increase in sample size for microarrays when using samples from mixed background mice may help rectify this issue. Despite this limitation, mixed background may be a better model than inbred mice strains, because it better reflects the variation present in human populations.

Gab1 and ZBTB32 were functionally verified for their role in γ-globin regulation. Because there are no human erythroleukemia cell lines that have an adult β-globin gene expression profile, we used CID dependent β-YAC bone marrow cells. These cells predominantly express the adult β-globin and very low levels of the fetal γ-globin. The

functional studies revealed that both these genes have an effect on γ-globin silencing. When these genes were overexpressed in these cells, γ-globin expression showed a modest increase. Moreover, preliminary studies in which two genes were co-expressed in these cells seem to suggest an additive increase of γ-globin.

The mechanism by which Gab1 and ZBTB32 have a role in γ-globin silencing in adult erythrocytes is speculative at this stage. No evidence for their direct involvement in γ-globin silencing has so far been documented. Gab1, which is an adapter protein in erythropoietin signaling, possibly acts indirectly on γ-globin gene regulation by activating the MAPK pathway. The balance between growth and differentiation is critical for erythropoiesis and globin gene regulation. Increased activation of growth related signaling by the MAPK pathway has been implicated in increased γ-globin levels (Bhanu et al. 2004). Thus, elevated Gab1 expression could upset the fine balance between growth and differentiation by activating the MAPK pathway of erythropoietin signaling, which could then lead to an increase in γ-globin levels. Although Gab1 has a CpG island, it is a ubiquitously expressed gene, and its CpG island has not been documented to be a differentially methylated region. Thus, we do not predict that MBD2 binds to this gene to regulate its expression.

We have shown that the CpG island in the ZBTB32 gene is bound by MBD2 directly. Although ZBTB32 has not previously been shown to be involved in γ-globin regulation, it has been reported to interact with GATA2 which has been shown to be involved in γ-globin gene regulation in K562 cells (Tsuzuki, Enver 2002, Ikonomi et al. 2000).

Our studies have identified a novel role for Gab1 and ZBTB32 in the MBD2 mediated silencing of the γ-globin gene in adult erythrocytes. These functional studies were conducted in CID dependent β-YAC bone marrow cells. It is important to perform the these studies in primary human CD34+ hematopoietic progenitor cells to validate the functional importance of these genes, because these cells serve as better *in vitro* models for human erythropoiesis.

CHAPTER 4: Summary and Future directions

The human β-globin locus is one of the most extensively studied loci in the mammalian genome. The genes in the locus are located in the order of their expression during development: 5' ε, γ, δ, and β 3'. Two switches in globin expression occur during development. The first occurs during the switch from embryonic to definitive hematopoiesis at 5 weeks post- conception. At this time, γ-globin replaces ε-globin as the major β chain subunit. This time also marks a change in the site of hematopoiesis form the yolk sac to fetal liver. At the time of birth, the second switch takes place whereby β globin and a small amount of δ-globin replaces γ- globin. By birth, bone marrow has become the primary site of hematopoiesis. This process, by which red cells in the blood stream containing predominantly one hemoglobin are gradually replaced by cells containing predominantly another hemoglobin was termed "hemoglobin switching" by Nienhuis and Stamatoyannopoulos (Nienhuis, Stamatoyannopoulos 1978).

The primitive to definitive and the fetal to adult hemoglobin switching have served as useful models to understand gene regulation during development and differentiation. The mechanism of gene regulation in this locus is still not entirely understood despite numerous studies. From a therapeutic standpoint, re-expressing the silent fetal γ-globin in the erythrocytes of individuals with β-hemoglobinopathies has disease ameliorating effects

(Stamatoyannopoulos G., Grosveld F. 2001, Nathan et al. 2003). Various cis elements, trans factors and DNA methylation are implicated in the γ-globin silencing in adult erythrocytes. The role of DNA methylation has been a focus of our laboratory.

DNA methylation in vertebrates occurs at position 5 in the cytosine ring, and almost exclusively in the CpG dinucleotide (DOSKOCIL, SORM 1962). DNA methylation is carried out by DNA methyltransferase (DNMT) enzymes, which catalyze the transfer of a methyl group from S-adenosyl-methionine to cytosine. The predominant mode of repression by DNA methylation involves methyl cytosine- binding proteins (MCBPs) that are attracted to the methyl cytosine signal (Ginder, Gnanapragasam & Mian 2008). MCBPs, along with their associated corepressor factors and complexes mediate the repressive effect of DNA methylation through enzymatic modification of adjacent histones, recruitment of chromatin remodeling proteins, or direct inhibition of transcription initiation (Ginder, Gnanapragasam & Mian 2008).

Two major classes of the methyl cytosine- binding proteins (MCBP) which are capable of mediating transcriptional silencing by binding to methylated CpGs have been characterized: the methyl binding domain (MBD) proteins which share a related 80 amino-acid methyl-CpG binding domain (MBD) (Ginder, Gnanapragasam & Mian 2008, Klose, Bird 2006), and the Kaiso-like proteins which bind DNA methylated at CpGs via a conserved zinc finger motif (Prokhortchouk et al. 2001, Filion et al. 2006).

Methyl CpG binding domain protein 2 (MBD2) belongs to the MBD family of methyl CpG binding proteins. MBD2-/- mice are viable and fertile (Hendrich et al. 2001).

We recently isolated an MBD2-NuRD remodeling complex (MeCP1) from primary chicken erythroid cells and also showed that MBD2 contributes to the silencing of the chicken embryonic ρ-globin and human fetal γ-globin gene (Kransdorf et al. 2006). These observations suggest that the disruption of the interaction of MBD2 with the NuRD co-repressor complex could augment the fetal/embryonic globin genes.

In the studies presented in chapter 2 of this thesis, we have pursued a structural and biophysical analysis of a coiled-coil interaction between MBD2 and p66α. We have shown that the individual coiled coil regions from MBD2 and a subunit of the NuRD complex, p66α, form a stable heterodimeric complex. Solving the structure of this coiled coil complex by NMR reveals that the interaction involves a combination of hydrophobic and ionic interactions typical of coiled coil complexes as well as a unique charge interaction involving a pair of highly conserved glutamates residues from p66α and arginine residues from MBD2.

In order to explore this interaction as a potential therapeutic target, we hypothesized that over-expressing the p66α coiled coil region in erythroid cells would disrupt the formation of a normal MeCP1 complex and thereby block the MBD2 mediated silencing of embryonic/fetal globin genes. In MEL-ρ cells and human CID dependent βYAC bone marrow cells, expressing the p66α coiled coil domain augments the expression of embryonic and fetal β-type globin genes, respectively. Furthermore, this region interacts with MBD2 and prevents the recruitment of p66α/β and Mi-2α/β proteins to the MeCP1 complex. In addition, knock-down of p66α and Mi-2β induces human fetal γ-globin gene expression to a degree similar to knockdown of MBD2. These studies suggest a model in

which the p66α coiled coil peptide can bind MBD2 and block recruitment of native p66α/β and Mi-2α/ β to the NuRD complex, thereby acting in a dominant-negative manner to disrupt MBD2 function. These studies suggest that a peptidomimetic of the p66α coiled coil region could be used therapeutically to augment fetal hemoglobin expression.

Effective therapeutic disruption of the protein-protein interactions presents many challenges which are being vigorously addressed in recent years. There is great interest in this area since most transcriptional repressors mediate their effect by protein-protein interactions with co-repressor complexes. Exogenous peptides designed to incorporate the protein interaction domain have been used to disrupt the endogenous protein-protein interaction by competing with the endogenous protein in the cells. However, peptide inhibitors are not viable options for therapeutics since they lack the physiological stability and the ability to cross cell membranes unlike the small molecule inhibitors (Arkin, Whitty 2009). In order to overcome many of these problems, "peptide stapling" was developed by Verdine lab and has been successfully applied to inhibiting Hdm2-p53 protein-protein helical interactions (Bernal et al. 2007, Wilder, Charpentier & Weber 2007). In order to staple the p53 peptide that has a helical interaction with Hdm2, non-natural α, α – disubstituted amino acids containing olefinic side chains were strategically incorporated into the peptide and then the alkyl side chains were cross-linked using ruthenium-catalyzed ring-closing olefin metathesis. By "stapling" the peptide, it is forced adopt a single α helical secondary structure which could otherwise adopt numerous conformations in solution. A similar approach was also used to develop peptomimetic inhibitor for BCL6

oncogene which could be delivered into cells both in vitro and in vivo to block the biological functions of BCL6 (Cerchietti et al. 2009).

Most drugs are small molecule inhibitors and have been designed to target enzymes and receptors. However, there are very few examples of small molecule inhibitors targeting protein-protein interactions. This is because most PPIs are flat and lack a cleft or cavity suitable for the binding of a small molecule with high affinity. Recent studies have shown that when the PPIs can be inhibited by a fairly short peptide derived from one of the binding partners in the interaction, it implies the presence of a cleft or a groove suitable for the binding of small molecule inhibitors (Arkin, Whitty 2009). Considering the functional importance of the protein-protein interaction between MBD2 and p66α in the globin gene regulation and the observation that the interaction can be disrupted by the overexpression of p66α coiled coil peptide, it is worth evaluating the potential for the use of small molecules to disrupt this interaction.

One of the long term goals for our lab is to delineate how the different components of the MeCP1 complex are recruited and how they interact with each other to mediate the repressive effects of the complex, specifically with respect to embryonic and fetal globin genes. In the studies described in chapter 2 we have focused on the interaction between p66 and MBD2 have identified the importance of this interaction in the regulation of embryonic/fetal globin genes. Similarly, understanding how the rest of the components such as Mi-2, Rbabp46/48 and HDAC2 are assembled into the complex and how those interactions contribute to the repressor activity of the complex will be highly useful to devise strategies to disrupt this complex.

In chapter 3 we have focused on delineating the mechanism by which MBD2 regulates γ-globin induction. We had previously shown that knock out of methyl CpG binding domain protein 2 (MBD2) in transgenic mice carrying the human beta globin gene cluster (ß-YAC mice), results in de-repression of gamma globin gene expression in adult erythrocytes. However, MBD2 does not directly bind to the gamma globin gene to mediate its silencing. We hypothesized that MBD2 may suppress human gamma globin gene transcription in adult erythrocytes by an indirect mechanism ie., by binding to and repressing transcription of intermediary gene/s which may be involved in gamma globin gene regulation. Microarray assays were performed on Affymetrix GeneChip® 430A 2.0 array for protein coding genes using RNA from four MBD2-/- and wild type mice adult erythroid cells. Ingenuity pathway analysis (IPA) and 'biological filter' were used to prioritize candidate genes for their role in MBD2 mediated silencing of γ-globin genes. For the biological filter, we merged our wild type and MBD2 knock out adult erythroid cells comparison data with microarray data from RNA of erythroid cells from embryonic day 9.5 yolk sacs (Redmond et al. 2008). ZBTB32, Gab1, ITCH, PACAF, BAF57 and YAF2 were identified as potential candidate genes. Among them, ZBTB32 and Gab1 were validated for their elevated expression in MBD2 knock down adult erythrocytes by Q-RT PCR. Functional studies in CID dependent β-YAC bone marrow cells showed that the overexpression of these candidate genes can cause elevated expression of γ-globin.

MBD2, thus far has been shown to directly regulate only a handful of genes in normal cells. Extensive molecular studies on different tissues of MBD2-/- mice are necessary to further our understanding of the function of MBD2 in normal cells. The role

of MBD2 in erythroid cells apart from the silencing of the embryonic and fetal globin genes has not been examined in detail. Our study shows that the knockout of MBD2 disrupts only a limited set (hundred and not thousands) of protein coding genes.

Apart from the protein coding genes, MBD2 could potentially regulate microRNAs. DNA methylation has been found to have significant effects on miRNA expression. So far, none of the studies have focused on the miRNAs regulated by MBD2. In a DNA methyltransferase1 and 3b (DNMT1 and DNMT3b) double knockout cell line, 10% (13/135) of miRNAs were found to be upregulated by at least 3 fold compared to that of its parental HCT116 cell line (Han et al. 2007, Lujambio et al. 2007). Thus, it is likely that MBD2 could be involved in the regulation of miRNAs in erythroid cells. Since only 13 miRNAs were differentially expressed in DNMT knock out cell lines (Han et al. 2007, Lujambio et al. 2007), we can expect that only a handful of microRNAs will be differentially expressed between MBD2-/- compared to wild type mouse erythroid cells. It is possible that miRNAs regulated by MBD2 could also be involved in the MBD2 mediated silencing of γ-globin.

Most of the studies presented in this thesis have been performed in CID dependent β-YAC bone marrow cells. This is because, none of the human erythroid lines display a normal adult pattern of globin gene expression and thus cannot be used for the studies on globin regulation. These CID β-YAC cells are derived from the bone marrow of β-YAC transgenic mice and can be propagated in culture by virtue of an artificial proliferation signal comprised of the thrombopoietin signaling domain fused to FKBP (FK506 binding protein) drug binding domains which is responsive to a chemical inducer of dimerization

(CID) (Blau et al. 2005). In the presence of a CID chemical, homodimers of the thrombopoietin receptors are generated, and the resultant growth signal maintains the progenitor population indefinitely and also causes spontaneous differentiation into the myeloid lineage. These cells predominantly express human adult β-globin and very low levels of γ-globin (<1% of β-globin), similar to human adult erythroid cells (Blau et al. 2005).

Primary human CD34+ hematopoietic cells can serve as better *in vitro* models for studying human γ-globin regulation, compared to the murine CID dependent β-YAC bone marrow cells. This is because they provide the opportunity to study the human β-globin gene in the context of its endogenous location. Upon erythroid differentiation of these cells in vitro, they express predominantly the adult β-globin gene and less than 1% of the fetal γ-globin gene. MBD2 knockdown in these cells leads to an approximately four fold elevation of fetal γ-globin mRNA levels. It is important to validate the observations obtained using CID β-YAC bone marrow cells in human primary erythroid cells.

In vivo differentiation conditions for the primary human CD34+ hematopoietic progenitor cells have also been proposed since they can better recapitulate human erythropoiesis. Although such models have been extensively studied for leukemia related studies (Cashman et al. 1997, Piacibello et al. 2002), they have not been validated for studying the regulation of globin genes. In vivo differentiation conditions can be obtained by injecting CD34+ hematopoietic progenitor cells into sub-lethally irradiated NOD-SCID mice (Cashman et al. 1997, Piacibello et al. 2002). NOD/SCID mice serve as superior hosts of transplantation since the DNA repair gene defect of SCID mice that severely

impairs B- and T-cell development has been combined with the reduced natural killer cell activity, absence of complement activity and the defect in phagocyte function of the NOD mouse. These human primary cells, prior to transplantation into the NOD-SCID mice, could be lentivirally transduced to knock down or overexpress the gene(s) of interest in order to study the role of these genes in erythropoiesis and globin gene regulation. If validated, these in vivo models could provide a great avenue to study β-globin gene switching in primary human erythroid cells differentiated in vivo.

The observations presented in this thesis can contribute towards the long term goal of devising strategies to disrupt the function of MBD2 and the MBD2 containing MeCP1 repressor complex. Many compounds currently used to treat patients with β-hemoglobinopathies carry short- or long-term risks of toxicity and the responses to the agents are variable (Stamatoyannopoulos G., Grosveld F. 2001). Most of the genes currently identified to be involved in γ-globin silencing such as GATA1, KLF1, and BCL11A are indispensible for embryonic development. On the other hand, MBD2 is dispensable for normal mammalian embryonic development and only confers a subtle phenotype in knockout mice. Moreover, the induction of γ-globin obtained when knocking down MBD2 in primary human erythroid cells is similar to that obtained by knocking down other proteins involved in γ-globin silencing such as BCL11A. Thus MBD2 could serve as an excellent therapeutic target in the treatment of β-hemoglobinopathies. Since MBD2 plays a prominent role in silencing tumor suppressor genes in cancers, disrupting its function could also be potentially beneficial in the treatment of cancer.

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