

The Role of GLP Domains in Spreading of the G9a/GLP Complex and
Regulation of β -globin Gene Expression

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This thesis is submitted as a partial fulfillment of the M.Sc. program in
Cellular Molecular Medicine.

Submitted January 31st, 2012

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AUTHORIZATION

Figure 3: Authorization has been granted from Cold Spring Harbor Laboratory Press
(see APPENDIX I, page 106)

ABSTRACT

Marked by a defect in the production of the Beta (β)-globin chain that make-up hemoglobin, Beta (β)-thalassemia is the most prevalent form of inherited single-gene disorders in the world. To understand the molecular mechanisms that govern the expression of the β -globin polypeptide encoded by the β -globin locus, we examined closely the enzymes involved in the epigenetic regulation of gene expression through histone 3 lysine 9 mono and di-methylation (H3K9 me1/2). G9a-like protein (GLP), a mammalian methyltransferase involved in the establishment and maintenance of H3K9 me1/2 mark at euchromatin, regions was found to be critical for the full activation of the adult β -globin genes in vivo during Murine erythroleukemia cell line (MEL) differentiation. Though it was initially hypothesized that GLP binding to H3K9 me1/2 mark through its Ankyrin domain was key to its activating function, we found that Flag- GLP ankyrin mutants E817R and W791A unable to bind to the methyl mark, are able to activate β -globin genes as well as their wild-type counterpart. Additionally, this study found that the embryonic gene $\epsilon\gamma$, known to be re-activated after G9a KD at the mRNA level, was effectively transcribed at the protein level using Triton Urea Acetic acid (TAU) western blots, thereby identifying potential therapeutic applications for treatment for β -thalassemia patients.

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List of abbreviations

β	Beta, β -globin adult gene
ε	Epsilon, human α -globin embryonic gene
β^{h1}	Betah1, mouse β -globin embryonic gene
β^{maj}	Beta major, mouse β -globin adult gene
β^{min}	Beta minor, mouse β -globin adult gene
$\varepsilon\gamma$	Epsilon γ , mouse β -globin embryonic gene
Ank	Akyrin domain
$\text{A}\gamma$	Gamma A, human β -globin fetal gene
BFU-E	Burst-forming unit–erythroid progenitor cells
CDS	Coding sequence
ChIP	Chromatin immunoprecipitation
ddH ₂ O	Double-distilled water
DNA	Deoxyribonucleic acid
Dox	Doxycycline
DTT	Dithiothreitol
E. coli	Escherichia coli

ES cells	Embryonic stem cells
GATA-1	GATA-binding factor 1
G_{γ}	Gamma G, human β -globin fetal gene
H3K9me1/2	Histone 3 lysine 9 mono- and di- methyl
H3K9me3	Histone 3 lysine 9 trimethyl
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His-tag	Histidine tag
HS	DNase I hypersensitive
IPTG	Isopropyl β -D-1-thiogalactopyranoside
KD	Knockdown
KMTs	Lysine methyltransferases
KO	Knockout
LB	Luria-Bertani broth
MCS	Multiple cloning site
MEL	Murine erythroleukemia cell line
MEL-tr	MEL cells expressing the tetracycline repressor
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase

mRNA	Messenger ribonucleic acid
NF-E2	Nuclear factor erythroid-derived 2
PBS	Phosphate buffered saline
PIC	Pre-Initiation Complex
PIC	Protease Inhibitor Cocktail
PVDF	Polyvinylidene difluoride
RISC	RNA-induced silencing complex
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Real time quantitative polymerase chain reaction
SDS-Page	Sodium Dodecyl Sulfate Polyacrylamide
Set	Set domain
ShRNA	Small hairpin RNA
SOC	Super Optimal broth with Catabolite repression
Sv40 pA	SV40 early polyadenylation signal
TAU	Triton Urea acetic
TEB	Triton Extraction Buffer
Tet Repressor	tetracycline repressor gene

TetO2	tetracycline operator 2
TFIIH	Transcription factor II H
$\alpha_2 \beta_2$	Hemoglobin HbA
$\alpha_2 \gamma_2$	Hemoglobin fetal, HbF
$\alpha_2 \delta_2$	Hemoglobin HbA type 2
$\alpha_2 \epsilon_2$	Hemoglobin Gower type 2
α -MRE	α regulatory element
δ	Delta, human β -globin adult gene
ζ	Zeta, human α -globin embryonic gene
$\zeta_2 \gamma_2$	Hemoglobin Portland
$\zeta_2 \epsilon_2$	Hemoglobin Gower type 1

ACKNOWLEDGEMENTS

I dedicate this thesis to my family, especially my father and my mother who have supported me throughout these years. Thank you for the many sacrifices you undertook to financially support me during all these years. I love you.

To my friends who have also helped and encouraged me during this training time, I say thank you.

To my supervisor Dr. Marjorie Brand I would like to say thank you for all the support, guidance and patience you have demonstrated towards me, always pushing me to bring out the best in me. I really appreciated your trust in my abilities to learn and overtake the obstacles that rose before me during the time I spent in your lab.

To my committee members Dr. David Allan and Dr. Alexandre Blais, I would also like to thank you for your guidance and support.

To my labmates thanks for the bench space and time we have spent. It is truly timeless as we have shared the excitement of new results and the disappointment of failed experiments.

To you my readers thank you for believing that Science can change the World and that our research helps make the World a better place.

To God almighty be the glory forever.

STATEMENT OF CONTRIBUTIONS

All studies appearing in this manuscript were performed by Camilia Thieba under the supervision of Dr. Marjorie Brand with the exception of the following:

- The GLP knockdown clones were generated by Dr. Chandra-Prakash Chaturvedi.
- RT-qPCR analysis of embryonic globin chain ($\epsilon\gamma$) transcript in G9a knockout mice and control littermate was performed by Dr. Chandra-Prakash Chaturvedi.

We obtained the following reagents from:

- GLP-cDNA by Dr. Y. Shinkai from the University of Kyoto in Japan
- G9a-cDNA by Dr. Y. Shinkai from the University of Kyoto in Japan
- Blood from different G9a conditional knockout mice from Dr. Fabio Rossi from University of British Columbia

1. INTRODUCTION

Thalassemia represents a group of single-gene blood disorders in which there is a decrease in the rate of synthesis of the polypeptides that make up hemoglobin, a molecular complex involved in oxygen transport within the red blood cells (erythrocytes). The clinical symptoms are often marked with hemolytic anemia (abnormal breakdown of erythrocytes) ranging from benign to severe manifestations and can be fatal depending on the genes involved and their interplay (Angastiniotis and Modell 1998; Lorey, Cunningham et al. 2001; Weatherall and Clegg 2001).

Thalassemia is one of the most common forms of inherited single-gene disorders with close to 15 million people affected and 60-80 million carriers worldwide. Although present in all races and ethnic groups, thalassemia affects mostly populations of Mediterranean, Western African and South Asian descents. With an expected annual incidence of symptomatic cases at 1 in 100,000 individuals and changing demographics, thalassemia is set to become a major global public health concern for many countries by putting a burden on their world's blood bank supplies (Angastiniotis and Modell 1998; Lorey 2000; Weatherall and Clegg 2001)

In order to unravel the origins of the many inherited mutations (approximately 1,000) known to cause thalassemia (Costagliola, Girot et al. 1992; Weatherall 2011), it is important to first understand the elements involved in the production of normal hemoglobin during erythropoiesis. It is in this prospect that research activities undertaken in Dr. Marjorie Brand's laboratory strives to comprehend the cellular and molecular mechanisms involved in the production of mature red blood cells, including hemoglobin synthesis.

1.1. Human Hemoglobin

Hemoglobin is a complex multi-subunit globular metalloprotein. It is made of two globin chains alpha (α) and Beta (β) that form a tetramer encompassing a prosthetic heme group (figure 1). Located on Chromosome 16, the human α -globin gene cluster (26 kilobase) encodes five developmentally specific α -like genes (Figure 2-A): one functional embryonic Zeta (ζ) gene, the earliest to be expressed during development, two non-functional closely related pseudogenes Psi-alpha 1 ($\psi\alpha 1$) and Psi-zeta ($\psi\zeta$) and two α genes ($\alpha 2$ and $\alpha 1$) synthesized as early as 7 weeks of gestation and throughout the individual's life. The α regulatory element (α -MRE) which is located 40 KB upstream of ζ , acts as a distal enhancer. The β -globin gene cluster (Figure 2-B) is located on Chromosome 11 and it contains four clustered β -like globin genes organized in the order in which they are expressed during development: one embryonic gene Epsilon (ϵ), two fetal genes Gamma ($^G\gamma$ and $^A\gamma$) and two adult genes Beta (β) and Delta (δ) (Jarman, Wood et al. 1991; Levings and Bungert 2002; Laa 2008). The Locus Control Region (LCR) located upstream is formed by five DNase I hypersensitive (HS) domains, this region is key to the high level of expression of β -globin genes. During the first week of embryogenesis in the yolk sac, the primitive erythroid cells produce three types of hemoglobin: embryonic hemoglobin Gower type 1 ($\zeta_2 \epsilon_2$) which is formed by the heterodimerization of two embryonic α -chains (ζ) with two embryonic β -globin chains (ϵ), embryonic hemoglobin Gower type 2 ($\alpha_2 \epsilon_2$) and hemoglobin Portland ($\zeta_2 \gamma_2$). As the site of hematopoiesis switches to the fetal liver, the fetal globin genes become active and the embryonic globin chains are progressively replaced with fetal hemoglobin HbF ($\alpha_2 \gamma_2$); this marks the first developmental switch. Following birth, a second switch takes place and the bone marrow becomes the main site of red blood cells production. In healthy adults,

hemoglobin HbA ($\alpha_2 \beta_2$) makes 98 % of total hemoglobin along with HbA2 ($\alpha_2 \delta_2$) and HbF, which continues to be produced at low level throughout life.

Complex coordinated regulatory events govern the globin genes switching. At the β -globin locus different trans-acting (erythroid specific and ubiquitous transcription factors and cofactors) dynamically interact with the cis-acting elements (promoters, enhancers, LCR,...) to control gene activation or repression through the recruitment and assembly of the basal transcription machinery along with chromatin remodeling complexes like the Histone deacetylase 1 (HDAC1) and SWItch/Sucrose Non-Fermenting (SWI/SNF) complex, activating complexes like the Basic leucine zipper family of transcription factors (NF-E2/p45) complex or repressor complexes like the Orphan nuclear transcription factor (COUP-TFII) and the Erythroid-specific Kruppel-like factor (EKLF) (Zhuma T et al. 1999; Gong QH et al. 1996; Filipe A et al. 1999). Despite the fact that numerous studies have been published concerning the expression of the globin genes, the critical events that govern the globin genes switching remain obscure. However, the vertebrate hemoglobin gene loci (especially the human and the mouse loci) serve as a good model for developmentally regulated multi-gene loci (Laat 2008; Wong, Winn et al. 2009). Indeed, cues on the synchronized transcription regulation of the human and mouse loci have provided information on how lineage specific programs are turned on during erythroid differentiation (Levings and Bungert 2002; Wong, Winn et al. 2009; Alison M. Hosey 2010).

Naturally occurring mutations on the globin genes loci lead to improper synthesis of hemoglobin's polypeptides and hence to hemoglobinopathies such as sickle-cell anemia (Allyson Cole-Strauss 1996) and thalassemia (M C Driscoll 1989).

While thalassemia patients exhibit a deficiency of α or β globin synthesis, sickle-cell anemia patients express aberrant hemoglobin. The most severe form of thalassemia is beta-thalassemia major (homozygous) which is characterized by the complete absence of the β -globin chain. This leads to the formation of abnormal hemoglobin thus causing severe anemia. Clinical data shows that 80% of untreated or partially treated individuals with β -thalassemia major die within 5 years of life due to complications due to anemia. Brought about by more than 200 point-mutations this disorder highlights the complexity and the necessity to understand the key elements governing the formation of the β -globin polypeptide. (Evangelia Yannaki, 2010). Thereby several attempts have been made to use pharmacological agents to treat thalassemia by increasing fetal hemoglobin content in adults by targeting Gamma (γ)-globin expression. Over-expressed γ -globin chains would equilibrate the deficit in β -globin and hence correct the shortfall in adult β -globin chains and consequently the anemia. Toward this goal different strategies have led to the development of several drugs.

From the observation that γ gene is hypomethylated in the urero, DNA demethylating agents like Azacitidine (International Nonproprietary Name) or 5-azacytidine have been used to trigger γ -globin gene hypomethylation. However, it was shown to be efficient only for short-term administration on patient with Beta-thalassemia (Ley TJ et al. 1982; Dunbar C, et al. 1989; Lowrey CH, Nienhuis AW 1993). Another strategy involved the use of cytostatic agents to alter erythropoiesis kinetics. Hydroxycarbamide (International Nonproprietary Name) or hydroxyurea, a drug used in clinical trial for short and long-term treatment of thalassemia patients was shown to promote the recruitment and accelerate the differentiation of primitive burst-forming unit–erythroid (BFU-E) progenitor cells, but led to a modest increase γ -globin level which did not always correlate with an overall increase in HbF as

expected.(Zeng YT, et al. 1995; Fucharoen S, et al. 1996; Hajjar FM et al 1994; Voskaridou E, et al.1995; Loukopoulos D, et al 1998;. Hoppe C, et al 1999; de Paula EV, et al 2003; Steinberg MH, Rodgers G 2001). The use of recombinant erythropoietin, a glycoprotein hormone involved in the regulation of erythropoiesis was also tested but the results were not promising as Fetal hemoglobin level remained steady as well as moderate to no changes to patient's overall well-being following treatments (Olivieri NF, et al. 1992; Rachmilewitz EA and Aker M 1999; Makis AC, et al 2001)

Deacetylase inhibitors have been used in an attempt to promote histone acetylation. A compound like Butyrate has been demonstrated to augment Hb F content in various animal model systems as well as in humans. Small clinical trials showed that treatment with Butyrate resulted in a significant increase γ -globin in some patients with no severe toxic side effects at small doses. However potential neurologic toxic effects could arise in case of high doses raising the question to the safety and efficacy of thalassemia therapy with butyrate.(Perrine SP, et al. 1989; Atweh GF, et al. 1999; Pawliuk R, et al. 2001). Though most of these therapies showed a substantial pharmacologic enhancement of fetal hemoglobin in early-phase clinical trials, several associated side-effects have been noted for most of them. Hence, new therapeutic strategies involving new pharmacological agents or new molecular targets remain to be developed to treat β -thalassemia patients through a better understanding of the regulation of the β globin genes during erythropoiesis.

One of the well characterized models for erythropoiesis is MEL (Murine Erythroleukemia) cell line which can be induced into terminal differentiation process in a mouse model. This cell line is blocked at the pro-erythroblast stage of erythroid

maturation (Levenson and Housman 1979). Terminal erythroid differentiation can be induced by adding 2% Dimethyl Sulfoxide (DMSO, Sigma-Aldrich) to the growth medium. These cells have been used extensively as a model system for erythroid differentiation because they can be consistently induced to express erythroid specific differentiation program characteristic of normal erythropoiesis (Volloch and Housman 1982; Pruzina, Antoniou et al. 1994; Sparatore, Melloni et al. 1996; Gu, Jiang et al. 2005; Kuykendall, Cox et al. 2007).

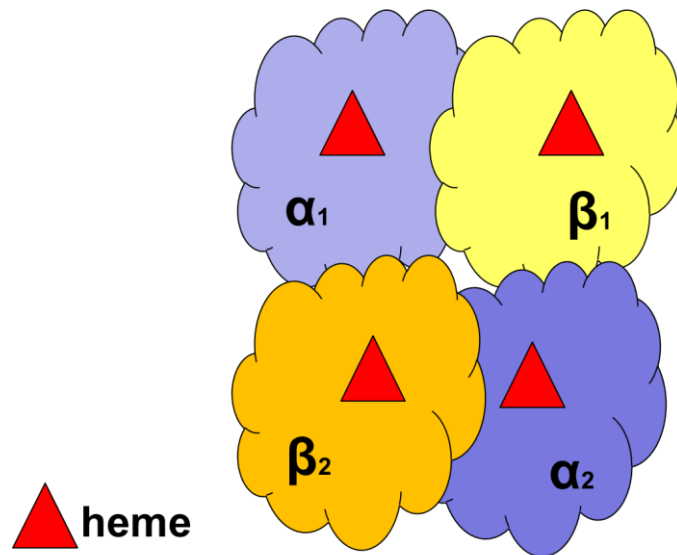


Figure 1: Structure of hemoglobin. The globular proteins α (α_1 and α_2) and β (β_1 and β_2) subunits are shown each enclosing a tightly bound iron-containing heme group *modified from ref (Bank 2005).*

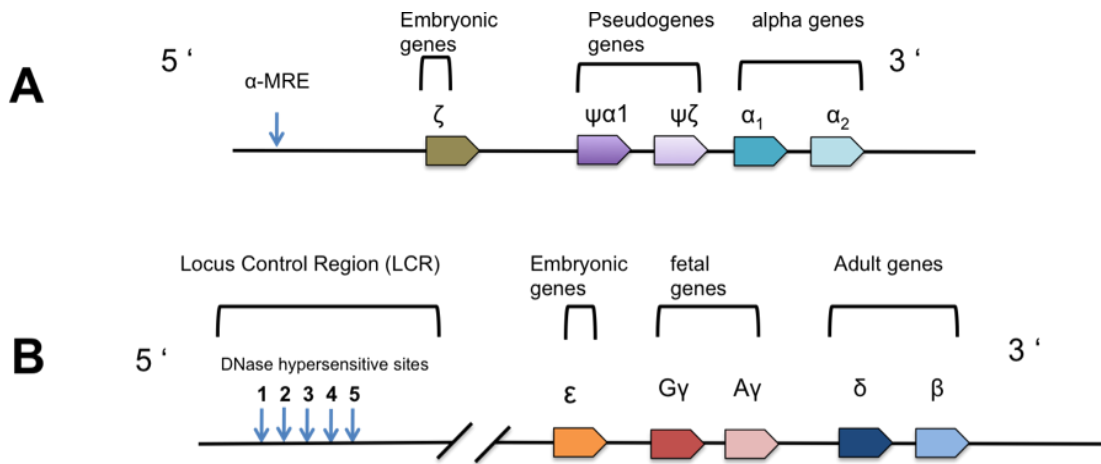


Figure 2: Schematic representation of the human alpha (A) and beta (B) globin genes cluster. Figures not drawn to scale. Vertical arrows indicate the location of DNaseI hypersensitive sites; boxes indicate the position of the globin genes. (A) α -globin gene cluster comprised of the embryonic genes ζ , the pseudogenes $\psi\alpha 1$ and $\psi\zeta$, and alpha genes $\alpha 1$ and $\alpha 2$. The major regulatory element (α -MRE) located far upstream is necessary for their high level of expression. (B) The genes of the β -globin cluster (ϵ , $G\gamma$, $A\gamma$, δ , and β) are located on chromosome 11. The β -locus control region (β -LCR) composed of five DNaseI sensitive sites is necessary for the high level of expression of those genes. *Modified from ref (Bank 2005)*

1.2. The Mouse β -globin Locus

The mouse β -globin locus (Figure 3), which encodes the hemoglobin β subunit is about 100 kb long and contains four clustered β -like globin genes: the embryonic genes, epsilon ($\epsilon\gamma$) and beta h1 ($\beta h1$), the adult genes beta major (β^{maj}) and beta minor (β^{min}). These genes are subjected to the same regulatory elements and they are differentially expressed throughout development. During embryonic development in mice, primitive erythrocytes produced in the yolk sac, express $\beta h1$ first at approximately embryonic day 7.5 (E7.5) then $\epsilon\gamma$, whose expression levels climax at E10.5. As embryogenesis proceeds around E11.5, these embryonic genes become progressively silenced and definitive erythrocytes that are produced in the fetal liver express exclusively the adult genes (β^{maj} and β^{min}) as the definitive developmental switch takes place (Laat 2008). A region located 6 to 22 kb upstream of these genes, which acts as a powerful distal enhancer is required for high-level of globin gene expression during development (Levings and Bungert 2002; Laat 2008). Similar to its human counterpart, the mouse LCR is comprised of five DNase I sensitive domains called hypersensitive (HS) sites and binding sites for erythrocyte-specific transcription factors such as GATA-1, and NF-E2 complex. These are necessary for the tissue, spatial and temporal specific expression of the β -globin genes. Tight regulation of such multi-gene locus involves epigenetic elements.

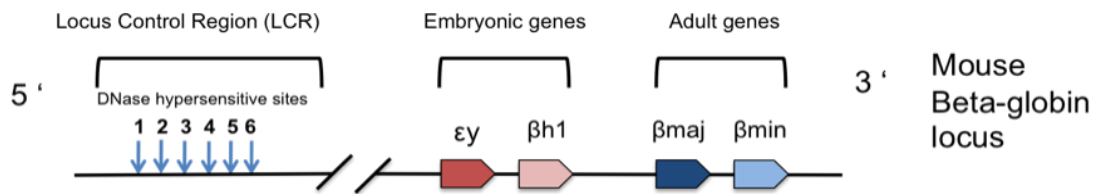


Figure 3: Schematic representation of the Mouse beta globin genes cluster.

Figure not drawn to scale. Vertical arrows indicate the location of DNaseI hypersensitive sites; boxes indicate the position of the globin genes. The genes of the β -globin gene cluster ($\epsilon\gamma$, $\beta h1$, βmaj and βmin) are located on chromosome 11. The β -locus control region (β -LCR) composed of six DNaseI sensitive sites is necessary for the high level of expression of those genes. *Modified from ref (Frenette and Atweh 2007)*

1.3. Histones and Genes Regulation

Introduced in 1942 by Conrad Waddington, epigenetics is the collective heritable change in gene expression or phenotype due to processes independent of changes in the primary DNA sequence. In eukaryotes, DNA is packed into small structures called nucleosomes. These are made of 166 base pairs of chromosomal DNA (negatively charged) tied around eight small positively charged proteins called histones (Karolin Luger, 1997). Primarily, 146 DNA base pairs are wrapped around a histone octamer, made of two copies of each histone H2A, H2B, H3, and H4. The structure is further folded by the incorporation of one H1 protein, which wraps the remaining 20 base pairs making nucleosomes, the basic unit of chromatin (DNA-protein complex). "String-of-beads" formed by the assembly of thousands of these nucleosomes linked together by linker DNA are further coiled to form higher-order structures; chromosomes (Kornberg 1974; Kornberg and Thomas 1974; Bednar, Horowitz et al. 1998; Fischle, Wang et al. 2003) . This level of DNA compaction restricts DNA-templated processes such as transcription, replication and repair as it prevents the regulatory factors and polymerase from having access to the DNA template. Therefore, chromatin spatial organization has to be highly dynamic to allow gene precise temporal and tissue-specific expression. Histone tails that protrude from the nucleosome can be post-translationally modified, thereby altering histones interaction with DNA and nuclear proteins. In fact, H2A, H2B, H3 and H4 N-terminal tails are subjected to a wide range of covalent modifications such as acetylation, methylation, ubiquitination, ADP-ribosylation, and phosphorylation whose combinations are thought to make up the histone code (Kimura, Tada et al. 2004; Lau and Cheung 2011). Although the histone code is not fully understood, it suggests that

specific or combinations of histone modifications can be translated within the cell into distinct nuclear processes (Strahl and Allis 2000).

Lysine methyltransferases (KMTs) mediate histones lysine methylation. This modification is one of the key chromatin-regulatory mechanisms involved in governing DNA-templated programs like gene transcription or DNA repair (Albert and Helin 2010; Levy, Kuo et al. 2011). Trimethylation of lysine 9 on histone 3 (H3K9me3) is central in the initiation, propagation and maintenance of the highly condensed chromatin heterochromatin. In fact, chromatin can be categorized into two different types based on the level of condensation: heterochromatin and euchromatin. Heterochromatin is the most stable form of the nuclear chromatin, mainly made of transcriptionally silent genes (facultative heterochromatin) and repetitive DNA elements (constitutive heterochromatin) (Gilmour and Lis 1984; Solomon and Varshavsky 1985). While euchromatin is generally considered to have a lower degree of compaction and it is comprised of constitutive or inducible active genes.

1.4. G9a and GLP

The H3K9 methyltransferase, G9a (Histone-lysine N-methyltransferase EHMT 2) catalyzes the addition of one or two methyl group to histone H3 lysine 9 residues, contributing to the establishment of transcriptional repressed environment at euchromatic regions. Ubiquitously expressed, G9a is made of two functional large N-terminal domains: the catalytic SET domain (about 130 amino acids long) and the Ankyrin (ANK) domain made of seven Ankyrin repeats which binds to H3K9me1 and H3K9me2 (Collins, Northrop et al. 2008; Shinkai and Tachibana 2011).

On the β -globin locus, G9a is responsible for a large H3K9me2 differentiation-dependent domain covering almost 100 Kb (Jorgensen and Fisher 2009). Previous work done by a post-doctoral fellow (Chandra-Prakash Chaturvedi) in our laboratory on Murine ErythroLeukemia cells (MEL) showed that G9a is involved in the differential regulation of the β -globin genes during differentiation. Through the erythroid specific transcription factors NF-E2/p45, G9a is first recruited to the LCR then spreads across the locus, establishing an environment enriched in H3K9me2 and H3K27me2 while orchestrating β^{maj} activation (Figure 5 A and B). Interestingly, G9a knockdown was found to lead not only to the impaired activation of the adult genes (β^{maj} and β^{min}) but also to the aberrant reactivation of the embryonic gene $\epsilon\gamma$ at the RNA level (figure 2 C).

GLP (G9a like protein) which is a homolog of G9a is another mammalian lysine methyltransferase. It forms a stoichiometric heteromeric complex with G9a via its SET domain (Figure 4). G9a and GLP dimerization is essential for euchromatic H3K9 methylation in vivo (Ueda, Tachibana et al. 2006; Collins, Northrop et al. 2008). The resolution of G9a and GLP crystal structures followed by *in vitro* binding experiments have identified several amino acids (within the Ankyrin domain), that mediate G9a and GLP specific binding to dimethylated histone H3 lysine 9 (H3K9me2); the product of their own enzymatic activity (Collins, Northrop et al. 2008; Collins and Cheng 2010). These results suggest a possible mechanism through which spreading of the G9a/GLP complex would be mediated via methylation of adjacent nucleosomes followed by a transfer of the complex onto the modified histones. The cyclic repetition of this process would allow the mark to propagate over great distances (4.9 Mb). (Figure 5) (Collins and Cheng 2010).

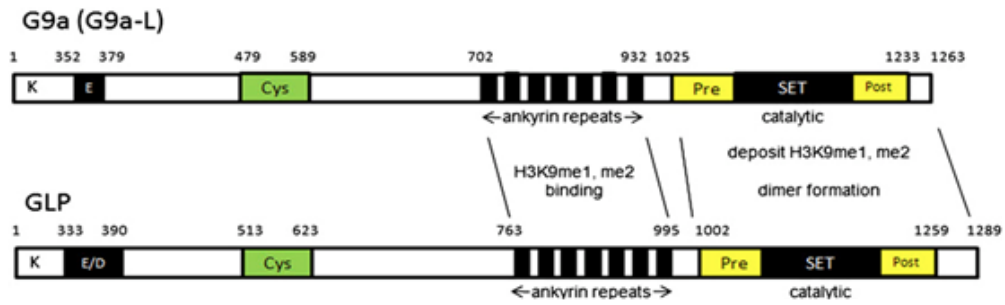


Figure 4: Domain organization of the core G9a and GLP.

Amino acid sequences of mouse (m) G9a-L (9QZ148), mGLP (A2AIS4). (K) Potential methylation sites by G9a or GLP; (E) Glu-rich region; (E/D) Glu/Asprich region; (Cys) Cys-rich region; (Pre) pre-SET domain; (SET) SET domain; (Post) post-SET domain; (Z) zinc finger motif are shown. From (Shinkai, Y. & Tachibana, 2011)

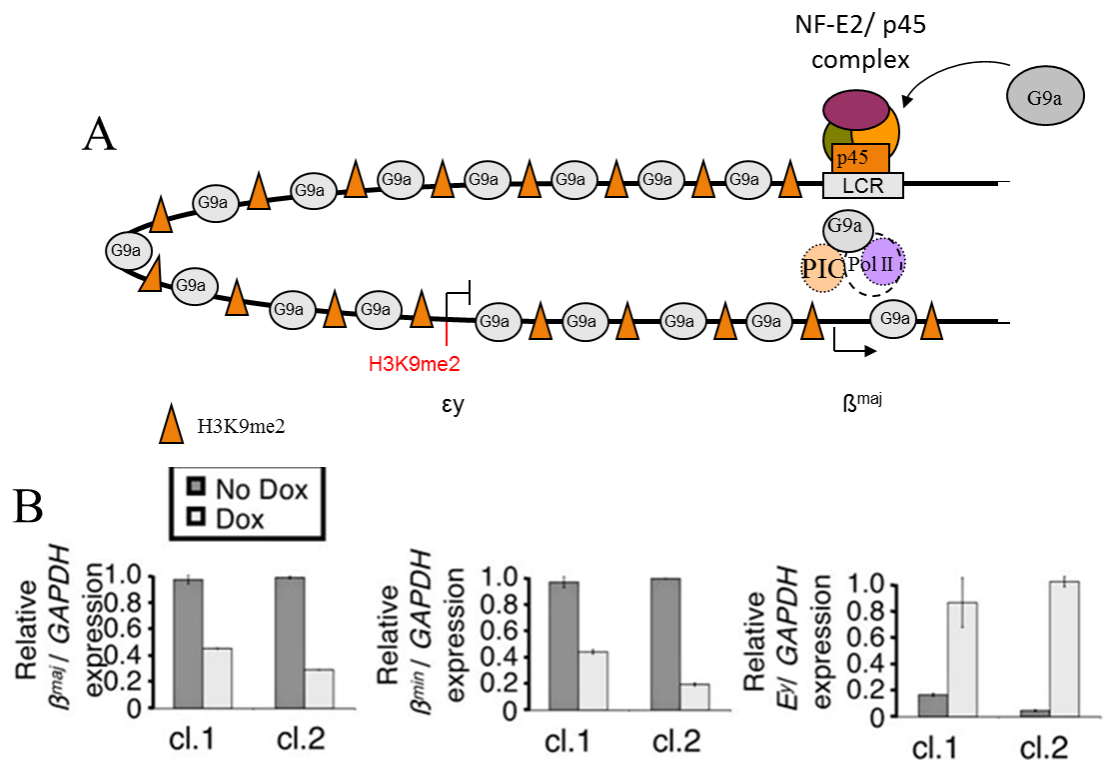


Figure 5: G9a has a dual role in regulating expression of β -globin genes (A) Schematic representation of the murine β -globin locus. During differentiation, G9a is recruited to the LCR in a NF-E2/p45 dependent manner and spreads across the locus thereby mediating β^{maj} activation and $\epsilon\gamma$ repression. (B) Transcription of β^{maj} , β^{min} and $\epsilon\gamma$ genes was assessed by RT-qPCR after differentiation in G9a KD (Dox) vs. normal (No Dox) MEL cells. Transcripts are expressed relative to GAPDH with the highest ratio set to 1. Modified from (Chaturvedi, Hosey et al. 2009).

1.5. Project Rationale and Overview

In an effort to understand the molecular implications that post-transcriptional modifications have on the production hemoglobin during erythropoiesis we propose to investigate G9a and GLP spreading mechanism and their role in the regulation of mouse the β -globin locus genes expression. Chronatin Immunoprecipitation (ChIP) results accumulated so far have shown that the methyltransferase G9a spreads across the β -globin locus and is necessary for the proper regulation of the β -globin genes during erythroid differentiation. Though Makoto Tachibana *et al* in 2005 showed its interacting partner GLP was critical for G9a stability and that their heterodimerization was essential for the complex methyltransferase activity *in vivo* not much is known about GLP's role and how its domain could mediate the G9a/GLP complex spreading.

1.6. Objectives

The main objective of this thesis was to investigate the role of GLP in regulating developmental-specific β -globin genes during erythropoeisis. The specific goals to meet this objective were:

- a) Investigate the role of GLP Ankyrin (ANK) domain and SET domain in regulating G9a/GLP complex spreading and β -globin genes expression during erythroid differentiation
- b) Determine the presence of the embryonic epsilon globin chain (protein) in various samples of G9a KD using Triton urea acetic gel.

1.7. Hypothesis

GLP binding to the methyl mark H3K9me1/2 mediates G9a/GLP complex spreading across the chromatin. To test this hypothesis, experiments were designed to meet 2 specific working hypotheses to meet the goals previously stated.

- a) GLP activates the adult and represses the embryonic β -globin genes transcription through its binding to the H3K9me1/2 mark.
- b) GLP Ankyrin and Set domain mutants are enable to activate and repress the β -globin genes.
- c) Embryonic $\epsilon\gamma$ protein is present in G9a KD MEL cells.

2. MATERIALS AND METHODS

2.1. MEL GLP Knockdown Screening

The MEL (Murine Erythroleukemia) cell line was used as a model system for erythropoiesis in the studies discussed in this thesis. Stable doxycycline (dox) dependent MEL GLP knockdown (KD) clones were generated from MEL, Murine erythroleukemia (745A) cells expressing tetracycline repressor by Dr. Chandra-Prakash Chaturvedi. These clones were screened to identify the best knockdown clone by growing each one of them at 37°C and 5% CO₂ in tetracycline-free RPMI-1640 media (Sigma-Aldrich) supplemented with 10% foetal bovine serum (Tet-free FBS, Wisent Inc.), 20 µg/ml of Blastidone (Wisent Inc.), 1% penicillin/streptomycin (Gibco®), G418 Geneticin® (400ug/ml, Wisent Inc.), and 2% DMSO in Corning® flasks for 5 days with or without 5 µg/ml doxycycline (Wisent Inc.). Nuclear proteins were extracted and western blot was performed to determine the clones that had the greatest reduction in GLP content after dox treatment. The best clone was thereby selected and used to generate Flag-GLP clones.

2.2. Flag-GLP Wild Type and Mutants Clones

In order to make GLP wild type and mutant fused to a Flag tag, GLP cDNA, that was provided by Y. Shinkai (Kyoto University, Japan), was cloned into the mammalian vector 3x Flag-pcDNA 5-to (Invitrogen) at BamHI-XhoI sites using T4 DNA ligase (Invitrogen). The ligation product was used to transform One Shot® TOP10 Chemically Competent E. coli (Invitrogen) by heat shock in Super Optimal broth with catabolite repression (SOC) media (2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂ and 20mM glucose) and subsequently plated onto Ampicillin (50 µg/ml, Wisent Inc.) Luria-Bertani broth

plates (LB, Fisher Scientific). The transformed bacterial colonies were allowed to grow overnight followed by plasmid extraction by QIAprep Spin Miniprep Kit (Qiagen) and subjected to double restriction digestion using the enzymes BamHI and XhoI (New England Biolabs) to determine the ones that had the correct insert (Flag-GLP-pcDNA5). Successful sub-cloning was further confirmed by sequencing at StemCore Laboratories (Ottawa Hospital Research Institute (OHRI)). This vector served as Flag-GLP construct backbones; it was amplified and purified by GenElute™ HP Plasmid Maxiprep (Sigma-Aldrich) transformed DH5 α E. coli. Using the purified Flag-GLP-pcDNA5 plasmid as a template, a silent mutagenesis reaction was performed with QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) to substitute 6 sequence specific nucleotides to make exogenous Flag-GLP wild type construct resistant to GLP shRNA 1 target sequence (see Table 1 for primers sequences and Figure 30 for mutagenesis strategy in Appendix I). The point mutations were validated by sequencing and the vector was further modified by non-silent mutagenesis reaction to engineer Flag-GLP W791A, E817R, Δ NHHC mutants. Ankyrin mutants, Flag-GLP W791A construct consisted of replacing tryptophan 791 (W) to alanine (A) while Flag-GLP E817R of substituting glutamic acid 817 (E) to arginine (R). Set domain mutant Flag-GLP Δ NHHC was designed by deleting four amino acid residues (1112-1115) asparagine, 2X histidine and cysteine (See Table 1 for mutagenesis primers and Figure 30 for mutagenesis strategy in Appendix I). Successful mutagenesis was confirmed once again by sequencing. These Flag GLP constructs were used to electroporate GLP KD clone 1.1 in 4 mm cuvettes using Biorad Gene pulser X (260 V; 960 μ F; Q = ∞ , Bio-Rad Laboratories Inc). Either 15 μ g of supercoiled plasmid DNA was used to electroporate 15 million cells or 50 μ g of linearized (NruI, New England Biolabs) plasmid DNA was used to electroporate 30

million cells. The cells were cultured following electroporation for two days in tetracycline-free RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum (Tet-free FBS, Wisent Inc.), 20 µg/ml of Blastcidine (Wisent Inc.), 1% penicillin/streptomycin (Gibco®) and G418 Geneticin® (400ug/ml, Wisent Inc.). Subsequently, the media was supplemented with Hygromycin B (800 ug/ml, Wisent Inc.), and selection antibiotic for two weeks to select stable clones (batch culture). Each batch culture was subjected to 96 well plate dilutions to isolate single clones. These were screened by growing the cells in 10 ml culture media for 5 days with 5 µg/ml dox and 2% DMSO. For each clone, nuclear proteins were extracted and western blot was performed to analyze Flag levels within the Flag-GLP cell line after dox treatment.

2.3. Cells Culture Conditions

MEL cells expressing tetracycline repressor were maintained at 37°C and 5% CO₂ in tetracycline-free RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% tetracycline free fetal bovine serum (Tet-free FBS, Wisent Inc.), 20 µg/ml of Blastcidine (Wisent Inc.), and 1% penicillin/streptomycin (Gibco®) in Corning® flasks .

MEL-tr GLP KD clones were similarly cultured at 37°C and 5% CO₂ in tetracycline-free RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Tet-free FBS, Wisent Inc), 20 µg/ml of Blastcidine (Wisent Inc.), 1% penicillin/streptomycin (Gibco®), and G418 Geneticin® (Wisent Inc) in Corning® flasks .

Flag Mel-tr GLP KD clones engineered from GLP KD clone 1 (parent cell line) were similarly cultured at 37°C and 5% CO₂ in tetracycline-free RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Tet-free FBS, Wisent Inc), 20 µg/ml of Blastidone (Wisent Inc), 1% penicillin/streptomycin (Gibco®) G418 Geneticin® (Wisent Inc), and Hygromycin B (Wisent Inc) in Corning® flasks .

2.4. β -Globin Genes Real-Time Reverse Transcription-Polymerase Chain Reaction

RNA extraction was performed using 10 million cells with RNA-stat 60 (AMS Biotechnology Ltd) according to the manufacture instructions. cDNA was reverse transcribed from 1 µg of RNA by M-MLV Reverse Transcriptase (Promega) and was further diluted to 200 ng/µl. The diluted cDNA, 5 µl was used to quantify the transcripts in a 25 µl reaction volume with Taqman probes (ABI) and primers (see Appendix I for sequences) in a two-step quantitative real-time RTqPCR reaction performed using the Rotorgene instrument 6000 (Corbett Research). The copy numbers were calculated using standard curves generated from genomic DNA and the relative gene expression levels were calculated using the comparative cycle threshold (CT) method by normalizing against the housekeeping genes; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 expression, DDX5.

2.5. Nuclear Extraction

Nuclear proteins were extracted from 80 million cells and harvested by centrifugation at 1500 RPM for 5min. The pelleted cells were washed twice with phosphate buffered saline (PBS), centrifuged at 1500 RPM for 5min at 4°C, and washed once with hypotonic buffer A (HEPES-10 mM (pH 7.9 at 25°C) , MgCl₂-1.5 mM, KCL-10 mM,

DTT-0.5 mM, PIC-1X). Cells were allowed to swell in 200 μ l buffer A and disrupted with 12 strokes of 1ml syringe, 27 gauge 1/2-Inch needle on ice. The cytoplasmic fraction was removed and the nuclear proteins were released from the nuclei with 50 μ l buffer C (HEPES-20 mM (pH 7.9 at 25°C), MgCl₂-1.5 mM, KCL-0.6 mM, Glycerol-25% (v/v), EDTA-0.2 mM, DTT-0.5 mM, PIC-1X) on ice, and equilibrated with 50 μ l buffer D (HEPES-20 mM (pH 8 at 25°C) , MgCl₂-5 mM, KCL-0.6 mM, Glycerol-20% (v/v), DTT-0.3 mM, PIC-1X). Nuclear extracts were subjected to Bradford protein assay (Bio-Rad Laboratories) to determine protein concentrations and stored at -80°C until used.

2.6. Histones Acid Extraction

20 million pelleted cells were centrifuged at 1500 RPM for 5 min and used for histones extraction according to abcam® protocol. The pelleted cells were washed twice in ice cold PBS and the cells were lysed in 1 ml Triton Extraction Buffer (TEB: PBS containing 0.5% Triton X 100 (v/v), 2mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) Sodium azide) using 12 strokes of 1ml syringe, 27 gauge 1/2-Inch needle on ice. Nucleus were collected by centrifugation at 6,500 g for 10 minutes at 4°C and further washed in 0.5 ml of TEB. The nucleus were treated with 0.5 ml 0.2N hydrochloric acid overnight at 4°C on a rotator to release the acid soluble histones and these were quantified using Bradford protein assay (Bio-Rad Laboratories), and stored at -80°C until used.

2.7. Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page) and western blotting were utilized throughout this study to assay proteins from MEL nuclear and histone extracts, bacterial whole cells and purified lysates. For each sample, 5 μ g of

proteins were mixed with SDS-loading buffer. The proteins were boiled at 95°C for 5 minutes (to denature them) and loaded on 10% SDS-polyacrylamide gel with precision-Plus protein standards (Bio-Rad Laboratories Inc.) as molecular weight markers. The gel was resolved at 30 mA (constant current) using BioRad Mini-PROTEAN 3 Electrophoresis Cell with running buffer. The proteins were transferred onto nitrocellulose membrane (Bio-Rad Laboratories Inc.) using the Mini-Protean II Electrophoresis Cell kit system (Bio-Rad Laboratories Inc.) at 150 V for 1 hour in a cooled transfer buffer (0.06% (w/v) Tris base, 0.29% (w/v) glycine). Nitrocellulose membrane blots were blocked for 1 hour in 2% skim milk powder in PBS. Primary antibody (listed below) incubation (1:1000) was carried out in 2% skim milk powder in PBS overnight at 4°C. The membrane was washed 3X in PBS for five minutes at room temperature, followed by 1 hour of secondary antibody incubation (1:5000) in PBS. Three final washes of five minutes each were performed in PBS supplemented with 0.05% Tween. Pierce® ECL Western Blotting Substrate (Thermo Scientific) was used to reveal and visualize the proteins on Bioflex econo film 8×10 (Clonex corporation). The following primary antibodies were used for incubation: GLP (Perseus Proteomics, PP-B0422-00), G9a (Perseus Proteomics, PP-A8620A-00), TFIIH p89 (S-19) (Santa Cruz biotechnology, sc-293), H3K9me1 (Abcam, ab9045), H3K9me2 (Abcam, ab1220), and Flag (Sigma-Aldrich). The corresponding anti-mouse or anti-rabbit conjugated to horseradish peroxidase (BioRad) were used as secondary antibodies.

2.8. G9a Recombinant Protein Production

G9a cDNA that was provided by Y. Shinkai (Kyoto University, Japan) was cloned into the mammalian expression vector pET28a at BamHI-XhoI sites using T4 DNA

ligase (Invitrogen), and transformed by heat shock using One Shot® TOP10 Chemically Competent E. coli (Invitrogen) in Super Optimal broth with Catabolite repression (SOC) media (2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂ and 20mM glucose) and plated onto Kanamycin (50 µg/ml, Wiscent Inc.) Luria-Bertani broth (LB, Fisher Scientific) plates. Subsequently, pET recombinant colonies were analyzed by double restriction digestion with BamHI-XhoI (New England Biolabs) and successful sub-cloning was confirmed through sequencing at StemCore Laboratories (Ottawa Hospital Research Institute (OHRI)). G9a-Pet 28a vector was amplified and purified by transformed DH5α, GenElute™ HP Plasmid Maxiprep (Sigma-Aldrich).

1 ng of plasmid preparation was used to transform, PLysS (Invitrogen), RIL (Stratagene) and ER 2566 (New England Biolabs) by heat shock, which were used as bacterial host for recombinant protein production. The culture was incubated at 37 °C with shaking (255 rpm) until an OD₆₀₀ of 0.6 was reached and recombinant protein expression was induced at room temperature (25 °C) or 37 °C with 0.4 mM, 0.8 mM or 1mM IPTG (Wiscent Inc) for six hours or overnight. The medium was supplemented with 1% (m/v) L-glucose (Sigma-Aldrich) and/or 30 mM magnesium chloride (Sigma-Aldrich). Following induction, the bacterial culture was pelleted by centrifugation at 10,000 g for 15 minutes at 4 °C. For solubility analysis, the pellet from 10 ml of bacterial culture was mixed into 1ml 25 mM Hepes (pH 7.6) and sonicated on ice 3x 20 %, 2x 25 %, 1x 30 % and 1x 35% with 30-second pulses and 30-second wait-time in between. The soluble fraction was separated from insoluble fraction by centrifuging crude extract at 10,000 g for 30 minutes at 4 °C and analyzed by western blot or Coomassie staining.

For purification purposes, 500 ml of bacterial culture was similarly induced. Bacterial pellet collected was resuspended in 20 ml of equilibration buffer (50 mM NaH₂PO₄, 300mM NaCl, 2 mM Imidazole, 5 % glycerol, 1 mM phenylmethanesulfonylfluoride, and Complete Mini EDTA-free Protease Inhibitor Cocktail tablets (Roche)) at pH 8 or pH 7. Sonication and soluble fraction extraction was similarly performed. The supernatant was incubated with inversion (2 hours at 4 °C) in 1ml beads-bed Talon Metal Affinity Resin (Clontech) equilibrated in sonication buffer. Beads and proteins-bound were centrifuged at 200 g for 5 minutes and subsequently washed in 5X bead volume for 10 minutes at 4 °C in a wash buffer (50 mM NaH₂PO₄, 300mM NaCl , 10 mM Imidazole, 5 % glycerol, 1 mM phenylmethanesulfonylfluoride and Complete Mini EDTA-free Protease Inhibitor Cocktail tablets (Roche)). Using Biorad gravity flow column (Biorad), the proteins were eluted in 4 fractions of 500 µl elution buffer (50 mM NaH₂PO₄, 300mM NaCl, 250 mM Imidazole, 5 % glycerol, 1 mM phenylmethanesulfonylfluoride and Complete Mini EDTA-free Protease Inhibitor Cocktail tablets (Roche)). Total proteins were quantified using Bradford protein assay (Bio-Rad Laboratories) and stored at -80°C until used.

2.9. Blood Sample Processing

Blood was obtained from slaughtered G9a KO and control littermate mice (Fabio Rossi *et al.*) was obtained from E13.5 embryos (kindly provided by Dr. Valerie Wallace and Dr. David Pickett) in 1 M PBS-EDTA (1mg/ml). The red blood cells were precipitated at 1500 RPM, washed twice in PBS, and stored at -80°C until used.

2.10. Hemoglobin quantification

2 µl of blood lysate was suspended in 1ml Drabkin reagent (Sigma-Aldrich) in 1mm cuvette and the absorbance was taken with spectrophotometer at OD₅₄₀ mm to determine the concentration of haemoglobin in the blood. The final haemoglobin concentration (C) was calculated using the following formula as follows:

$$C (\mu\text{g/ml}) = \text{OD}_{540} \div 0.7 \times 50 \div 6$$

2.11. Discontinuous Triton-Acetic acid-Urea (TAU) gel

In order to separate, the different globin chains present in the blood and G9a KD MEL cell samples, blood lysate were run on 12 % poly-acrylamide gel containing 6M urea, 2% triton X-100 and 5 % acetic acid. The separating gel was made by dissolving 18 g of Urea at low heat in 20 mL filtered 30:0.2 (V:V) Acrylamide/bis-acrylamide solution (4°C), 4 ml ddH₂O, 10 ml 10% Triton X-100, and 2.5 mL glacial acetic acid. Prior to gel casting, 0.300 mL Temed and freshly prepared 10% APS were incorporated to the solution. The monomer solution (30 mL) was poured into 1 mm spaced assembled glass plates (PROTEAN® II xi Cell, Bio-Rad Laboratories Inc.) with an overlay of water and left to polymerize at 30°C for 1 hour. Similarly, the stacking gel was prepared by mixing at low heat: 2 mL filtered 30:0.2 (V: V) Acrylamide/ bis-acrylamide solution (4°C), 3.6 g Urea, and 3 ml ddH₂O. Upon urea complete dissolution, 1 ml Triton X-100 10% and 0.55 mL glacial acetic acid were added. 0.1 mL Temed and 0.12 ml 10% freshly prepared APS were incorporated into the solution and poured over the separating gel. The comb was inserted between the glass plates and the gel was left to polymerize at 30°C for 2 hours. The newly formed wells were thoroughly rinsed with distilled water and stored at 4°C until use.

2.12. Triton-Acetic acid-Urea (TAU) western blot

Prior to sample electrophoresis, two pre-runs were performed to remove free radicals that could interfere with the sample migration in 5% glacial acetic acid buffer; first at constant 200V for one hour and then at 150 V for one hour with an overlay of 0.2 mL 1M cysteamine hydrochloride (Sigma-Aldrich). 5 µl of blood pellet was lysed in 20 µl of autoclaved ddH₂O and mixed with 115 µl of sample buffer (3g urea, 2.5 ml ddH₂O, 0.625 mL glacial acetic acid, and 1.25 ml β-mercaptoethanol). Desired haemoglobin amount was loaded onto TAU gel (the wells were fully rinsed to remove traces of Cysteamine) and migrated at 10 mA (constant current) for 16 hours at 4°C with switched electrode leads. Once the run was completed, the proteins were either stained for 1 hour at room temperature with coomassie blue (0.25 % coomassie Blue R-250, 7% glacial acetic acid, 30 % methanol) and destained (5 % glacial acetic acid and 25 % methanol) overnight or transferred to the PVDF membrane. In order to initiate the transfer, the gel was soaked for 15 minutes in the transfer buffer (0.7% glacial acetic acid). Two 15 X 22 cm Immobilon-P PVDF (Polyvinylidene difluoride, Millipore) membranes with pore size of 0.45 µm were activated for 30 second in 100% methanol and subsequently washed for 1 minute with ddH₂O, and soaked for 15 minutes in the transfer buffer (0.7 % glacial acetic acid). The transfer sandwich was assembled as illustrated in Figure 6 and placed in the Trans-blot Cell (Bio-Rad Laboratories Inc.). The transfer was achieved at 4°C for 15 minutes at 400 mA (constant current). The membranes were then stained with ponceau-S (Sigma-Aldrich) for 1 min and destained with water to confirm successful transfer, followed by blocking for 1 hour in 2% skim milk powder in PBS. Primary antibody incubation (1:1000) was performed in 2% skim milk powder in PBS overnight at 4°C. Three PBS washes of five minutes each (at room temperature) were followed by 1 hour

secondary antibody incubation (1:5000) in PBS. Three final washes of five minutes each were performed in PBS supplemented with 0.05% Tween. Pierce® ECL Western Blotting Substrate (Thermo Scientific) was used to reveal and visualize the proteins on Bioflex econo film 8×10in (Clonex corporation). The following primary antibodies were used for blotting: Antibody-Haemoglobin β (37-8) (Santa Cruz biotechnology, sc-21757), and HBE1 Antibody-hemoglobin epsilon (Proteintech Group, 12361-1-AP). The corresponding anti-mouse or anti-rabbit conjugated to horseradish peroxidase (BioRad)

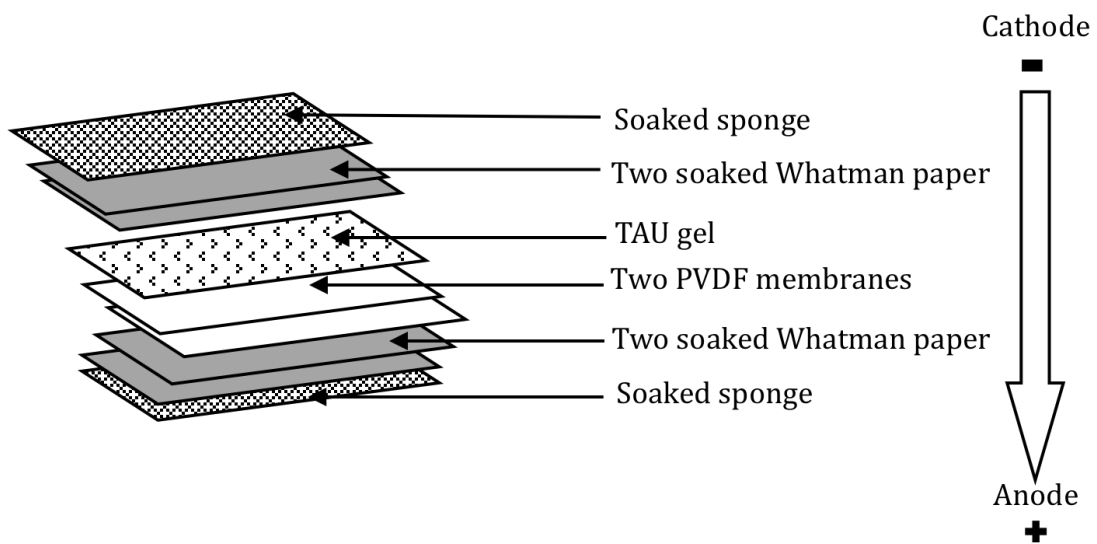


Figure 6: TAU transfer sandwich was assembled accordingly

3. RESULTS

Evidences accumulated so far have shown that the methyltransferase G9a is necessary for the proper regulation of the β -globin genes during erythroid differentiation. Though Makoto Tachibana *et al* in 2005 showed that GLP was necessary for G9a stability and that their heterodimerization was essential for their methyltransferase activity *in vivo* not much is known about GLP's function. Hence we used shRNA-mediated knockdown to characterize GLP role in regulating the globin genes.

3.1. GLP is required for the activation of the adult β -globin genes in MEL cells

To better understand the molecular mechanisms that govern the tissue-specific and temporal expression of the β globin genes, we examined the role of the methyltransferase GLP in their activation and repression within differentiated MEL clones. We hypothesized that GLP doxycycline (dox) inducible knockdown (KD) will impair the adult globin gene activation and relieve the embryonic gene repression. Thereby, we used several MEL clones (40) expressing three different doxycycline inducible shRNA targeting GLP. These clones generated by Dr. Chandra-Prakash Chaturvedi were screened to identify the ones that showed GLP KD both at the protein and mRNA level. The clones were independently grown for five days with dox and with DMSO to induce the MEL erythro-specific differentiation program. Control cells (untreated) were also simultaneously grown. GLP being a nuclear protein, we performed nuclear extraction to enrich the different samples in nuclear proteins and analyzed their content using Western Blot. The blots were then probed with a GLP specific antibody to reveal the changes in expression following dox

treatment. The general transcription factor TFIID antibody was used as an internal control to verify loading between the treated and untreated samples. The western blots were then visually analyzed to identify the clones that showed the greatest GLP reduction (KD). As shown on the illustrative example (Figure 7), 80% of the MEL clones screened such as clone1.2 and clone1.4 did not show changes in GLP expression. While 15 % of the clones displayed a modest decrease (clone 1.3), 5 % had a greater decrease (clone 1.1) in GLP expression upon dox addition when compared to the untreated samples. These clones, which showed reproducible KD were: clone 1 expressing shRNA target sequence 1 (clone 1.1) and clone 2.1 (clone 1 expressing shRNA target sequence 2). Figure 8 illustrate that upon dox treatment clone 1.1 had an overall GLP reduction of 70 % at the protein level (panel A and panel B) and 90 % at mRNA level (panel C). Similarly, clone 2.1 shows 50 % KD at the protein level and consistently a decline of 90 % at the RNA level. To confirm GLP implication in the regulation of globin genes a clone showing a modest decrease in GLP expression, 20% at the protein level and 60% at the transcript level was also retained for downstream analysis. Hence through this screening process we obtained three different MEL clones demonstrating inducible GLP KD where the regulation of the mouse β -globin gene expression by the G9a/GLP complex could be studied. Despite the fact that the KD efficiency varied from clone to clone, perhaps due to the sequences efficiency in down regulating GLP, the number and orientation of the randomly integrated shRNA plasmid into the host genome, we observed a pattern between the clones: all three had the greatest changes in expression at the mRNA level. This could imply that the shRNA sequences were effectively inducing mRNA degradation and that the modest alterations in protein content could result from GLP protein stability.

Since G9a stability is sensitive to GLP's level in vivo in embryonic stem cells (ES cells), we investigated G9a's overall content in the three MEL GLP KD clones isolated. We examined the nuclear extracts of the MEL clones (Figure 9) by western blot using a G9a specific antibody. The blot shows that during murine erythroid differentiation, overall G9a content is not affected when GLP is knocked down in clone 1.1, clone 2.1 and clone 1.3. Therefore it seems that in MEL cells during differentiation, G9a is less vulnerable to the changes in expression of its interacting partner GLP. Could it be that GLP is not as critical for MEL cells differentiation process? To address this question, we analyzed the bulk levels of H3K9 mono- and di-methyl mark within GLP KD MEL clone. Considering the relative stability of these histone marks, we considered only one clone, clone 1.1 as it manifested 70% reduction in GLP level when treated with dox.

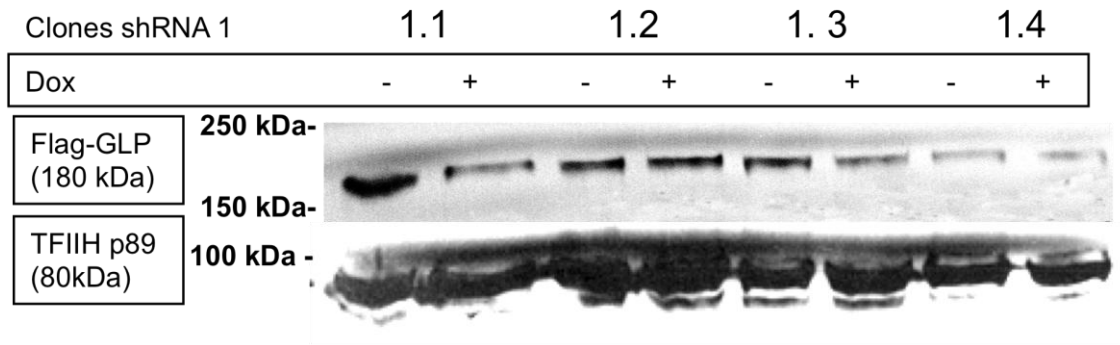


Figure 7: GLP knockdown MEL clones expressing shRNA 1 western blot screen. Nuclear extracts (5 μ g) from four independent MEL clones (5 days culture) were subjected to GLP and TFIIH western blot. TFIIH is used to assess loading between untreated (-dox) and treated (+ dox) samples.

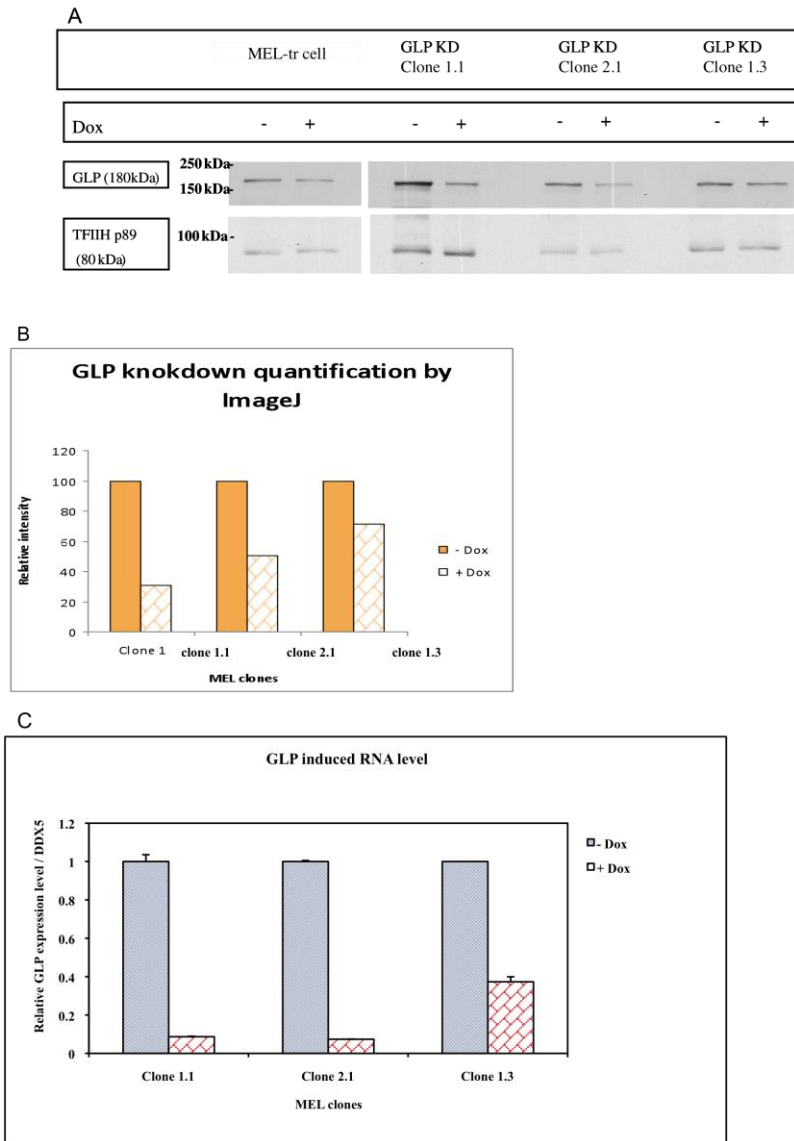


Figure 8: Evaluation of GLP knockdown in MEL clones.

A- Nuclear extracts (5µg) from MEL cells (parent cell line) and three independent MEL clones (5 days culture) were subjected to GLP and TFIIH western blot. TFIIH is used to assess loading between untreated (-dox) and treated (+dox) samples. **B-** Bars represent quantified GLP western blot signals normalized to TFIIH, by Image J. **C-** GLP transcript level assessed by RT-qPCR during differentiation in GLP clones with and without Dox treatment. Transcripts are expressed relative to DDX5 with the highest ratio Set to 1. Average values ± standard deviation represent two PCR replicates.

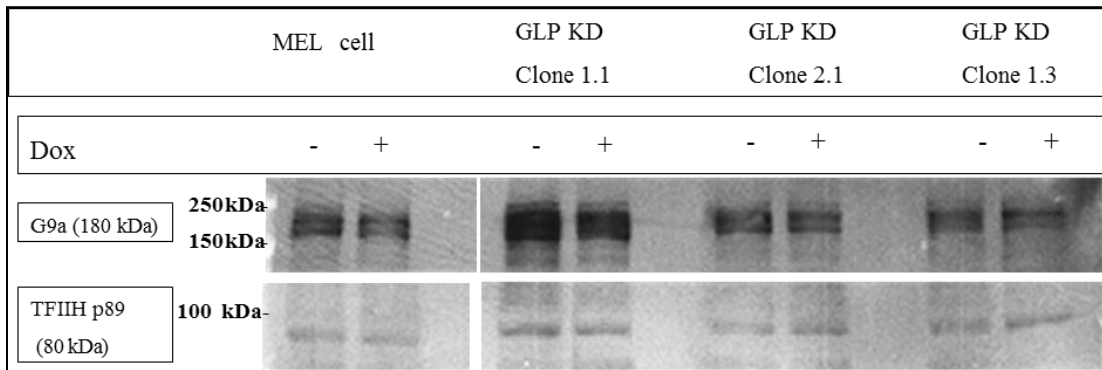
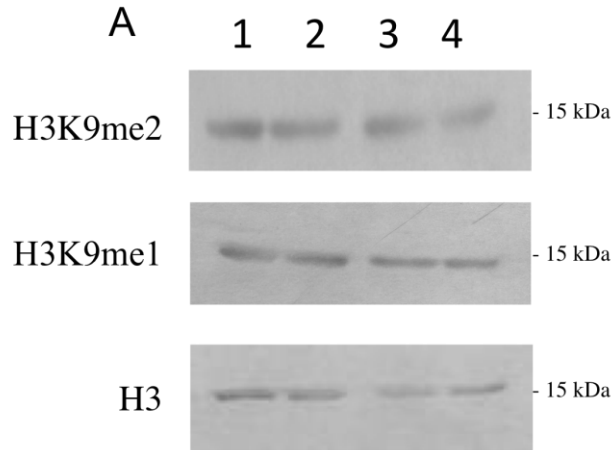


Figure 9: Evaluation of G9a level in GLP knockdown MEL clones.

A- shRNA knockdown of endogenous GLP in MEL cells and three independent MEL clones (5 days) followed by G9a and TFIIH (equal loading control between treated (+dox) and untreated (-dox) samples western blot

As histone 3 (H3) and lysine 9 (K9) methyltransferase, G9a and GLP mediate the formation of H3K9 mono- (me1) and di-(me2) methylation *in vivo* at euchromatin region. Using three MEL clones, we observed that GLP KD did not affect G9a overall content. To assess the biochemical effects of this observation on histone H3 post-transcription modification, we analyzed by western blot the methylation levels within the clone 1.1 (70% KD) using acid-extracted histones. H3, H3K9me1 and H3K9me2 specific antibodies revealed that GLP down regulation leads to a 50% reduction in bulk H3K9me2 but not H3K9me1 (Figure 10). Thus GLP seems to be implicated in the establishment of H3K9me2 specific marks at euchromatin regions during differentiation. Taken altogether, these results point to GLP and G9a being equally important for *in vivo* H3K9 di-methylation in differentiated MEL cells like in ES cells (Makoto Tachibana 2005; Chaturvedi, Hosey et al. 2009). As H3K9me1 level were surprisingly steady upon GLP (Figure 7) and G9a KD, it is possible that other methyltransferases are involved in the maintenance of this mark



1-MEL DMSO
 2-MEL DMSO Dox
 3-GLP KD clone 1.1 DMSO
 4-GLP KD clone 1.1 DMSO Dox

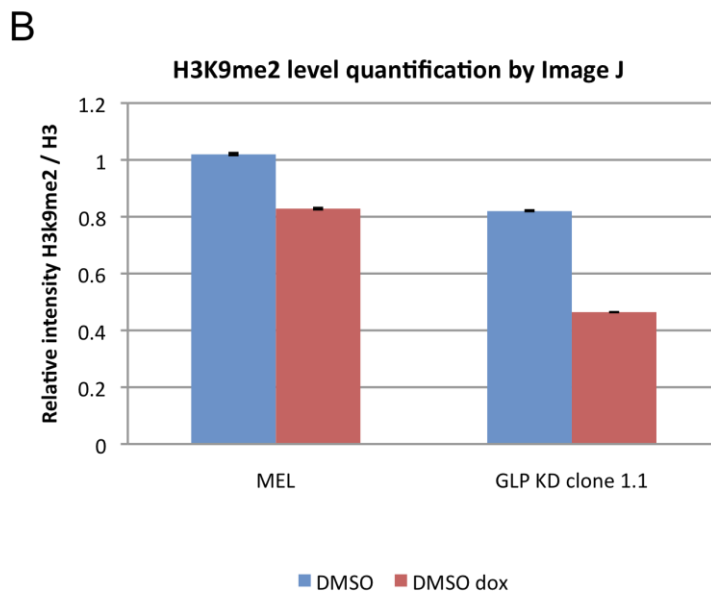


Figure 10: Global H3K9me1, H3K9me2 and H3

A- 1 μ g of acid extracted histones from differentiated MEL cells and GLP KD clone 1.1 were analyzed by Western blot using H3K9me1, H3K9me2 and H3 antibody and B- Bars represent quantified H3K9me2 western blot signals

normalized to H3 by Image J. Average values \pm standard deviation represent two independent experiments

During erythropoiesis, histone posttranscriptional modification participates in the β globin locus remodeling, leading to the activation of the adult globin (β^{maj} & β^{min}) and progressive downregulation of the embryonic genes ($\epsilon\gamma$ and Bh1). Since changes in the level of global H3K9me2 (repression mark) were observed after GLP KD, we further evaluated the effect of these changes on the β -globin genes expression during MEL differentiation. RT-qPCR performed using RNA extracted from three different GLP KD clones (Figure 12, panel A and B) shows that adult globin (β^{maj} & β^{min}) transcript expression is reduced by 50 % in clone 1.1 (70% KD) and 2.1 (50% KD) in presence of dox. As demonstrated in Figure 12 (panel A and B) GLP KD does not lead to an increase in $\epsilon\gamma$ or Bh1 transcript, which contrasts with G9a KD. Therefore seemingly, though G9a and GLP KD lead to a decrease in H3K9me2, they don't affect the β -globin genes in the same manner: GLP is involved in the activation of the β -globin genes while G9a, its interacting partner mediates both activation and repression.

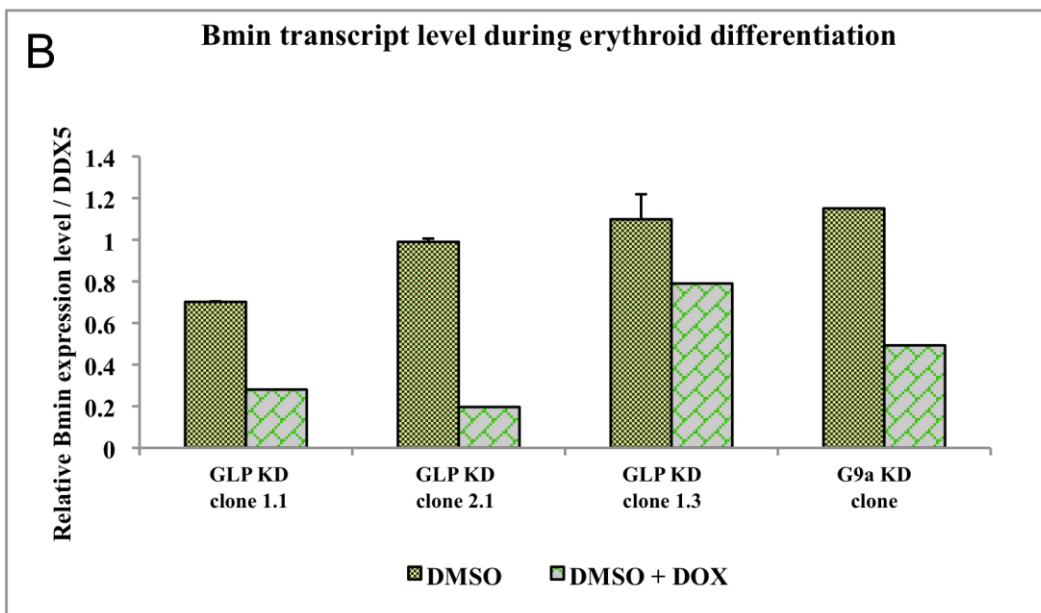
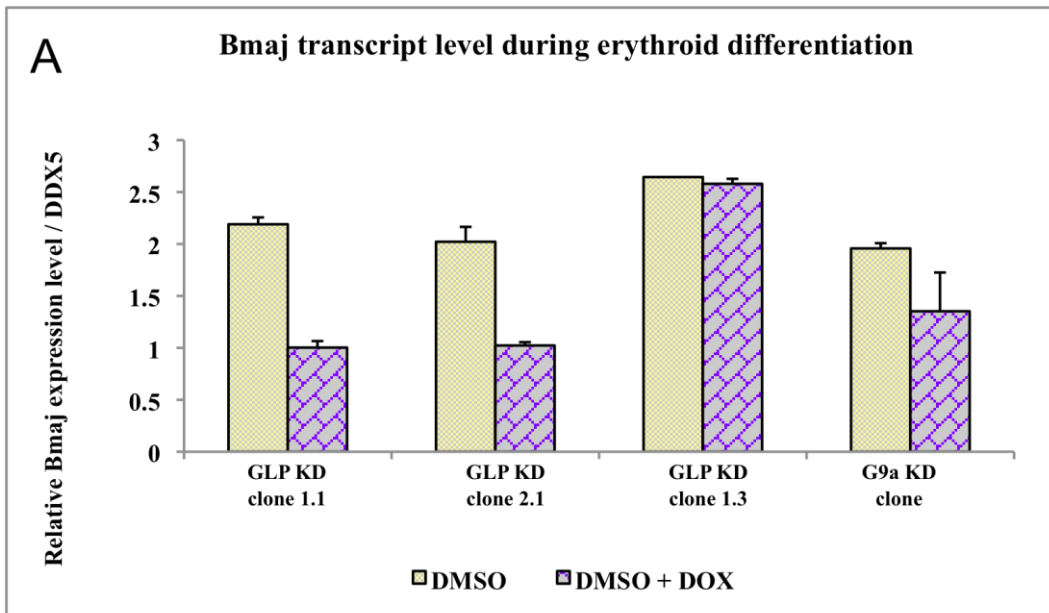


Figure 11: β -globin adult genes transcription.

Level of transcript was analyzed by RT-qPCR in MEL clones treated with or without Dox during differentiation. (A) β maj transcript and β min transcript (B) levels are expressed relative to DDX5. Average values \pm SD represent two PCR experiments.

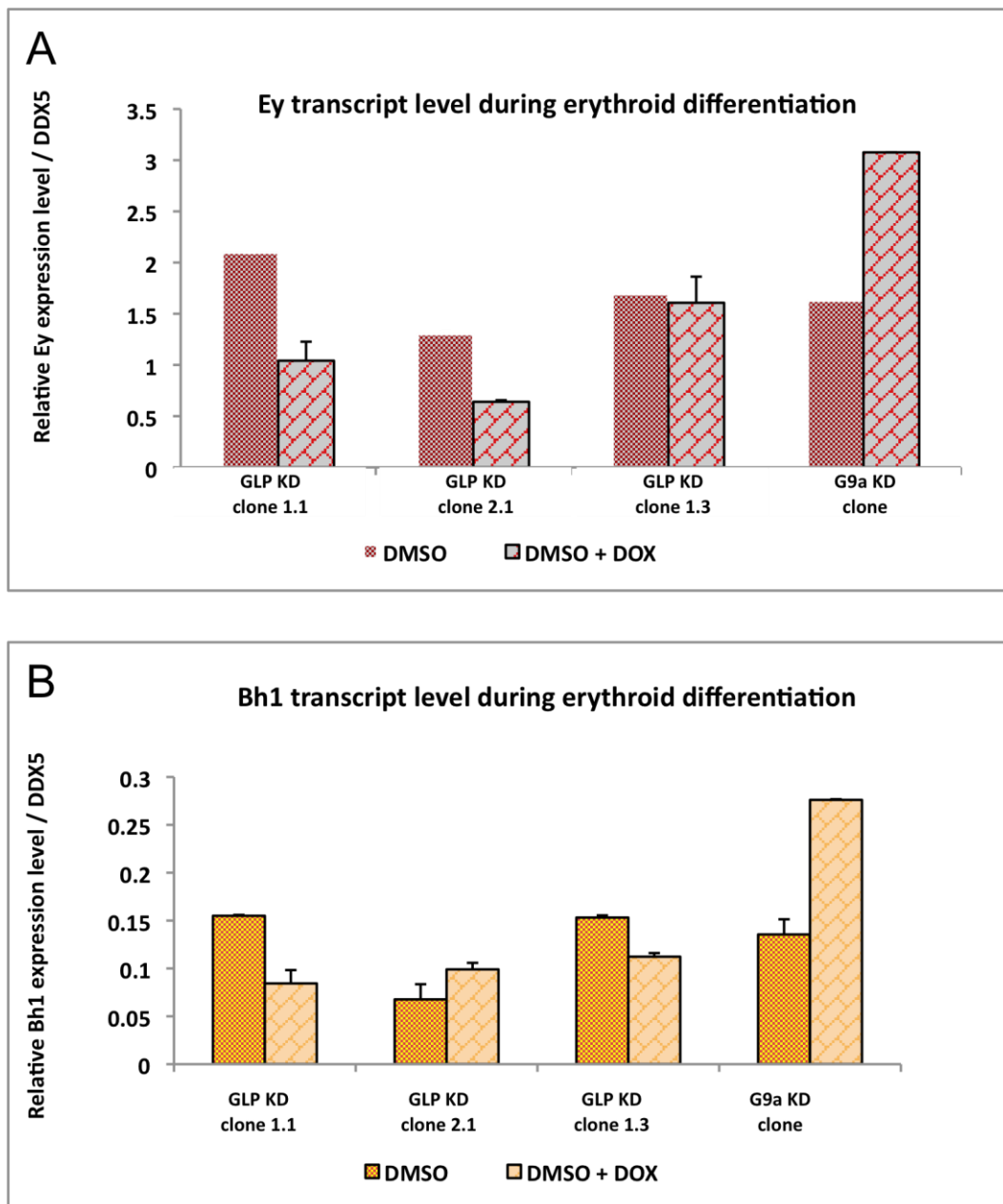


Figure 12: β-globin embryonic genes transcription.

Level of transcript was analyzed by RT-qPCR in MEL clones treated with or without Dox during differentiation. (A) $\epsilon\gamma$ transcript and $\beta h1$ transcript (B) levels are expressed relative to DDX5. Average values \pm SD represent two PCR experiments.

Flag-GLP wild type and mutants activate β -maj

We were able to demonstrate so far that the adult β -globin genes were downregulated upon GLP knockdown in MEL cells. In an attempt to elucidate the mechanism of this regulation, we proposed to investigate the role of GLP's binding through its Ankyrin domain and methyltransferase activity on the expression of the globin genes. We thereby sought to generate MEL clones where doxycycline induced expression of exogenous GLP (wild-type and mutants) tagged with Flag epitope would coincide with endogenous GLP down regulation. We hypothesize that whereas the wild-type construct will rescue the GLP KD phenotype observed at the β -globin locus, GLP Ankyrin and Set domain mutants will not. We thereby generated four GLP constructs: a wild type construct to validate the exogenous expression system, two Ankyrin mutants incapable of binding to H3K9me1/2 mark and one Set domain mutant deficient in methyltransferase activity. Toward this goal, we generated a GLP Flag-tag construct by cloning GLP full-length sequence in a doxycycline inducible Flag-pcDNATM5/TO expression vector. To induce Flag-GLP expression in a GLP-D background we chose to electroporate the construct in one of the GLP KD clone 1.1, for it had the greatest KD (70%). To avoid the degradation the exogenous Flag-GLP by the shRNA sequence 1 expressed within clone 1.1, we modified the Flag-GLP-pcDNATM5/TO vector furthermore by inserting silent point mutations in order to render the construct resistant to the shRNA sequence. Accordingly, six nucleotides were substituted as illustrated in Figure 30 (Appendix I). Based on crystallography results (Collins, Northrop et al. 2008), we generated Ankyrin mutants incapable of binding to the H3K9 methyl mark, abundant at the locus, by performing two non-silent mutations in GLP Ankyrin domain at position tryptophan 791 (W791A) and glutamic acid 817 (E817R). Similarly, deletion of four amino acids (NHHC) in the

SET domain was done to render the Flag-GLP Δ NHHC mutant deficient in its methyltransferase activity (Makoto Tachibana and Shinka 2008).

Exogenous genomic material incorporation in mammalian cell by electroporation presents some challenges when it comes to recombinant gene expression. Hence, to maximize our chances of obtaining high Flag-GLP clones, we used two approaches for MEL electroporation: each construct was either linearized (with restriction enzyme NruI (209)) or used as a super-coiled plasmid (non-linearized). Despite the fact that, super-coiled DNA produces higher transfectant, random integration often leads to a decrease in protein expression when compared to linearized DNA. Indeed, vector linearization promotes proper plasmid integration and hence efficient expression of gene of interest. A total of eight plasmids were used to independently electroporate GLP KD clone 1.1 (four super-coiled and four linearized plasmids) producing a total of eight stable batch cultures. These batch cultures were grown for 5 days with dox then the nuclear proteins were extracted and examined by western blot to detect Flag signals. As shown in Figure 13, we were able to detect consistently of Flag tagged GLP in batch GLP wild type, W791A and E817R with dox treatment. Since Flag GLP Δ NHHC batch (super-coiled and linearized) was not detected, we moved on to generate single clones from batch GLP wild type, W791A, and E817R using 96 well plate dilutions. Each clone was hence grown for five days with DMSO to induce differentiation and dox to induce the expression of exogenous Flag GLP in place of endogenous GLP. The nuclear proteins extracted were analyzed by western blot using Flag antibody to isolate the clones that had the greatest Flag levels with dox addition. GLP blotting was simultaneously performed to ensure that GLP was not over-expressed. Exogenous Flag-GLP wild type was effectively detected in clone 8, 10, 11 and, 12 without an overall increase in GLP signals (Figure

14). In harmony with the batch culture observation, Flag-GLP linearized clones had a much higher Flag signal (Figure 15). Because we are planning to use these clones for Flag-ChIP experiments, we needed to pick the clones that expressed similar levels of Flag expression with minimal or low GLP over expression. GLP (Figure 16) and Flag (Figure 17) expression assessed by western blot show that several clones expressing Flag GLP were obtained. These were FLAG-GLP wild-type clone 6 and 8, FLAG-GLP W791A clone 2 and 5 and FLAG-GLP E817R clone 12 and 15. These clones don't demonstrate GLP over-expression after exogenous expression of Flag-GLP and hence can be used to study the role of GLP Ankyrin domain in the transcriptional regulation of the β -globin genes during MEL erythroid differentiation.

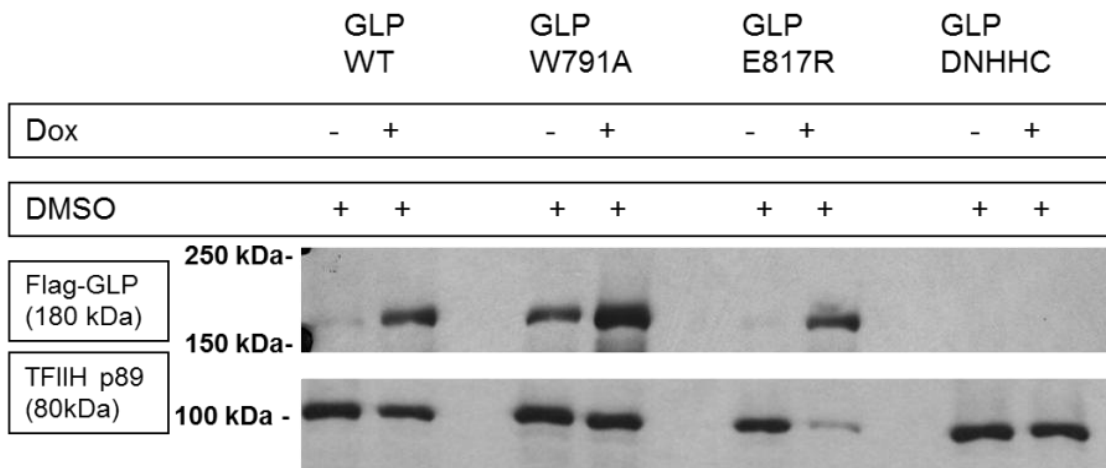


Figure 13: Flag-GLP (linearized) batch culture before and after doxycycline treatment during differentiation. Nuclear extract from 10 ml cell culture was obtained after 5 days culture with 5 μ g/ml Dox + 2% DMSO and migrated on SDS-Page gel. Membranes were blotted with Flag antibody (180 kDa) and transcription factor II H (TF II H, 80 kDa) as a control for equal loading. Batch GLP wild-type (WT), and mutant W791A and E817R have a high level of Flag whereas GLP delta-Set (DNHHC) does not.

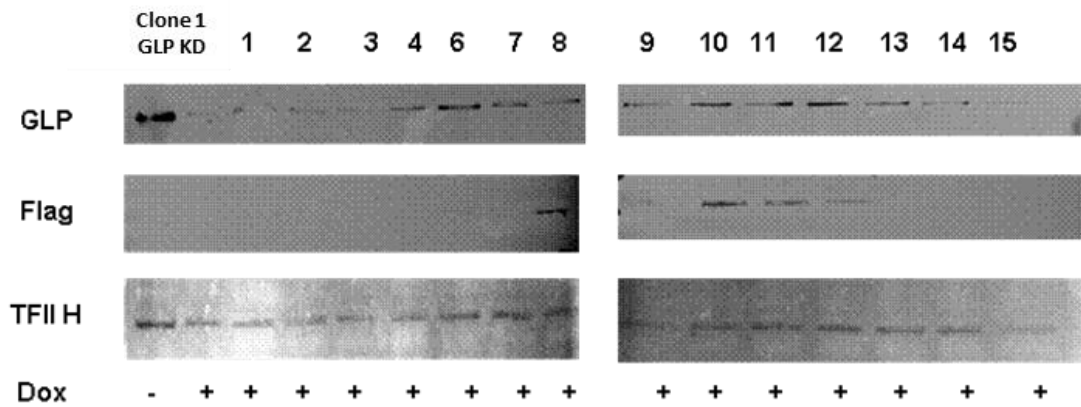


Figure 14: Flag-GLP (supercoiled) batch culture before and after doxycycline treatment during differentiation. Nuclear extract from 10 ml cell culture was obtained after 5 days culture with 5 µg/ml Dox + 2% DMSO and migrated on SDS-Page gel. Membranes were blotted with Flag antibody (180 kDa) and transcription factor II H (TF II H, 80 kDa) as a control for equal loading.

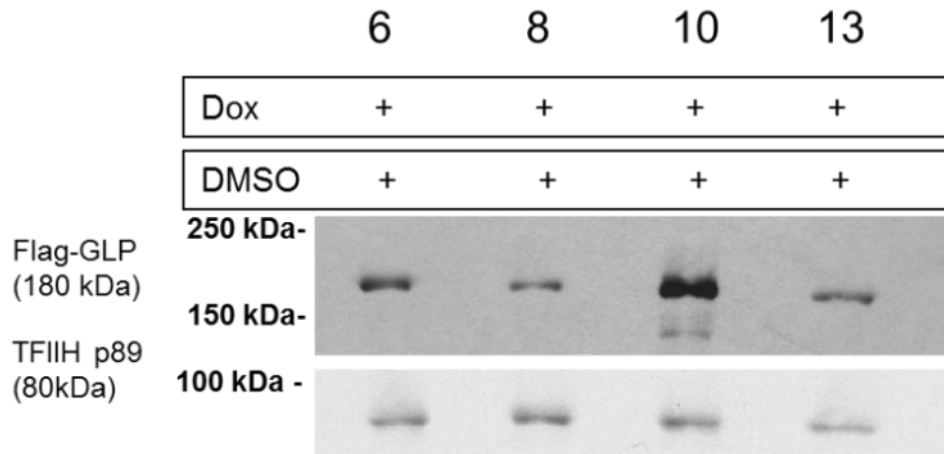


Figure 15: Selected Positive Flag-GLP (linearized) wild type clones after doxycycline treatment. Nuclear extract from 10 ml cell culture was obtained after 5 day culture with 5 μ g/ml Dox + 2% DMSO and migrated on SDS-Page gel. Membranes were blotted with GLP antibody (180 kDa) Flag and transcription factor II H (TF II H, 80 kDa) as a control for equal loading.

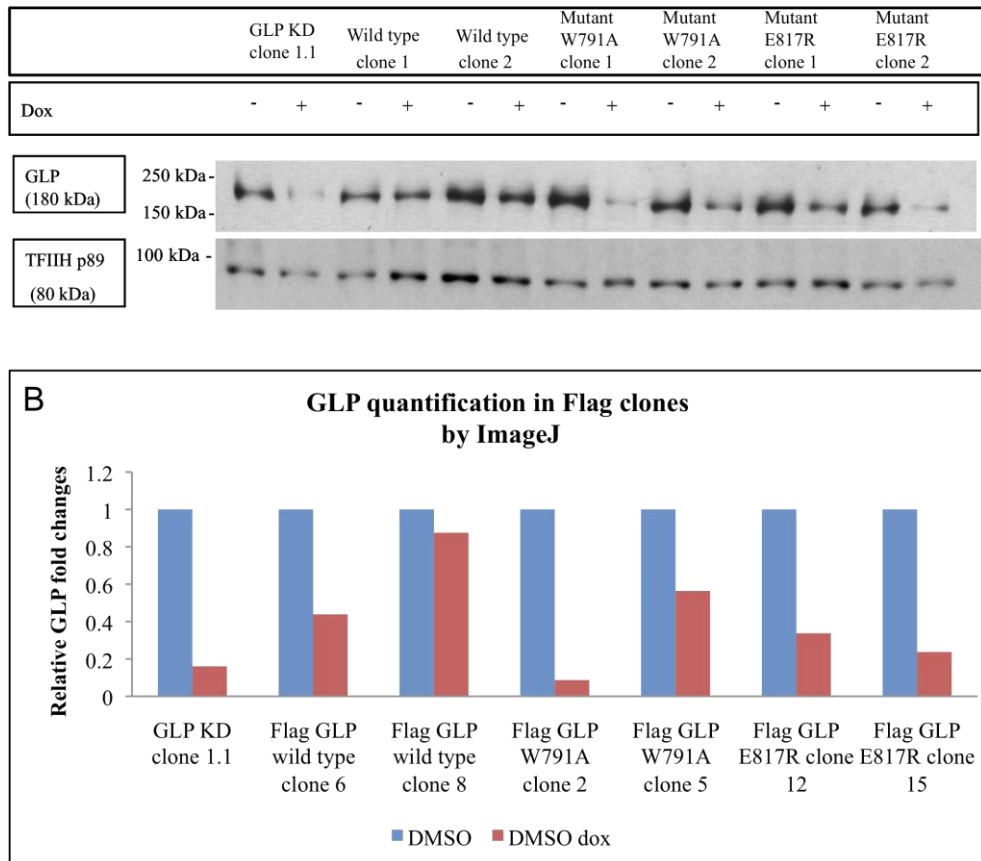


Figure 16: Evaluation of GLP signal in Flag-GLP MEL clones

Nuclear extract from 10 ml cell culture was obtained after 5 days culture with 5 $\mu\text{g/ml}$ Dox + 2% DMSO and migrated on SDS-Page gel. Membranes were blotted with GLP antibody (180 kDa). B- Bars represent quantified GLP western blot signals normalized to TFIIH, by Image J.

A

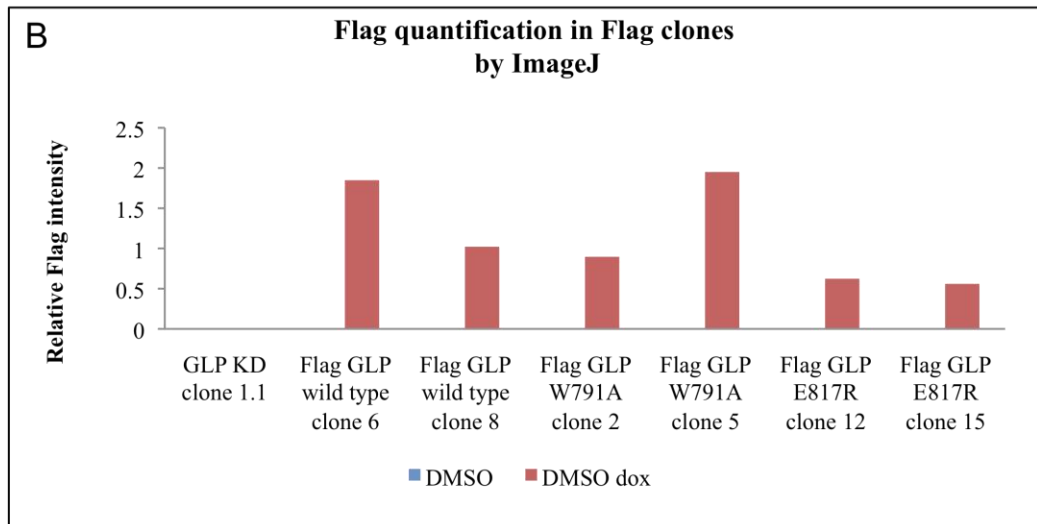
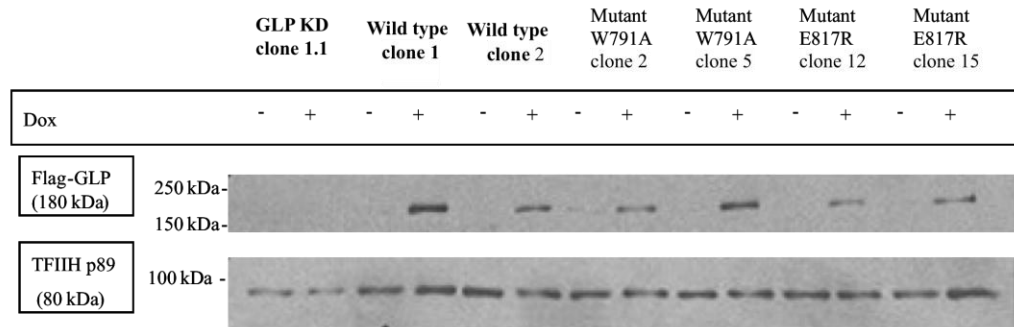


Figure 17: Evaluation of Flag signal in Flag-GLP MEL clones

A- Nuclear extract from 10 ml cell culture was obtained after 5 days culture with 5 µg/ml Dox + 2% DMSO and migrated on SDS-Page gel. Membranes were blotted with Flag antibody(180 kDa). Endogenous level of GLP and Flag was similar across Flag-GLP clones. B- Bars represent quantified GLP western blot signals normalized to TFIIH, by Image J.

Western blot analysis showed that Flag-GLP wild type and Ankyrin mutants can be effectively induced by dox treatment without GLP overexpression. To assess the physiological effect of GLP Ankyrin mutations on the activation of the globin genes, we examined β^{maj} gene expression in these clones during MEL differentiation. By RT-qPCR we assay the mRNA transcript derived from the Flag-GLP clones isolated previously: FLAG-GLP wild-type clone 6 and 8, FLAG-GLP W791A clone 2 and 5 and FLAG-GLP E817R clone 12 and 15. Using the delta CT methods we generated the relative transcript levels normalized to GAPDH expression. Figure 18 display the results plotted in a graph form. Consistent with the previous findings, β^{maj} activation is impaired in GLP KD clone 1.1 (decreased in transcript) and steadily rescued within Flag-GLP wild type (clone 6 and 8) as endeavored by an increase in transcript with dox treatment. Noticeably, β^{maj} activation is also greatly enhanced in Flag-GLP W791A clone 2 and E817R clone 15 when dox is added and to a lesser extent, in Flag-GLP W791A clone 5 and E817R clone 12. Collectively these results reinforce GLP activation function at the β -globin locus by promoting the transcription of the adult gene β^{maj} .

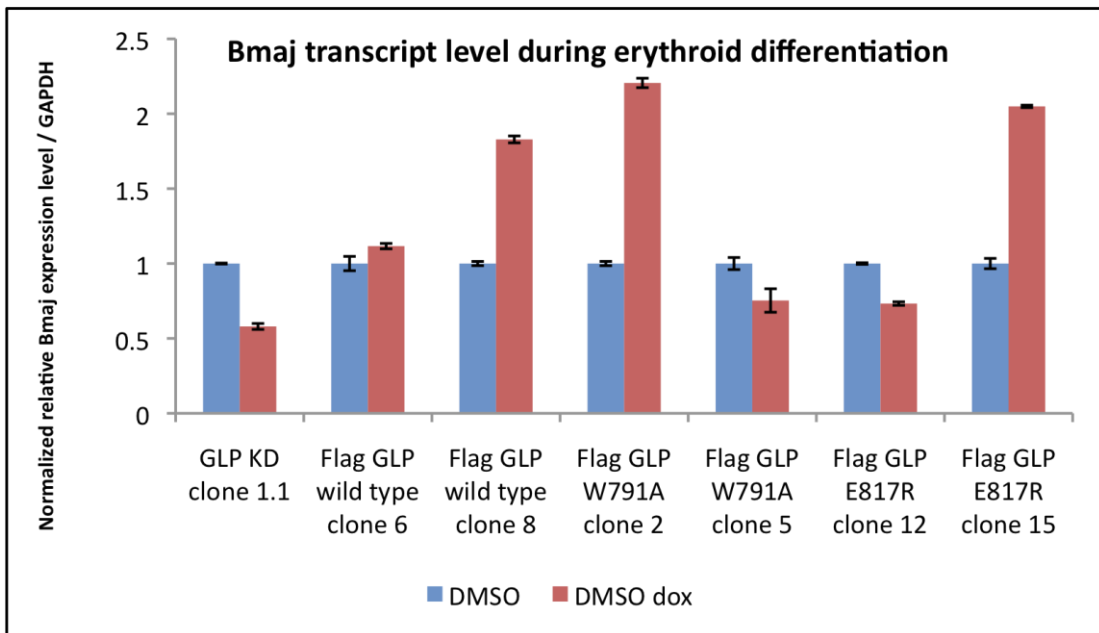


Figure 18: β -globin genes transcription.

Level of transcript was analyzed by RT-qPCR in MEL clones treated with or without Dox during differentiation. Gene specific transcript levels are expressed relative to GAPDH and normalized, Average values \pm SD represent two PCR experiments.

3.2. Bacterial systems are not suitable for G9a full-length recombinant protein production

G9a and GLP have the unique ability to bind the products of their own enzymatic activity (H3K9me1/2) through their Ankyrin domain. Though G9a was found to spread on the β globin locus (Brand *et al*, 2009), the contribution of each domain (Set and ankyrin) in the process remained to be elucidated. Hence to understand GLP Ankyrin domain role in the complex spreading, we aimed to study both G9a/GLP wild type and G9a/GLP ankyrin mutant complexes spreading at the β -globin locus using G9a-ChIP experiments. As there is no good G9a ChIP-grade antibody commercially available we attempted to produce recombinant G9a protein using bacterial systems.

For our experiment, pET28a was used as G9a expression vector. This translation vector contains the highly efficient ribosome-binding site from the phage T7 major capsid protein necessary for the production of His-tag recombinant protein in *Escherichia coli* (*E. coli*) by the addition of Isopropyl β -D-1-thiogalactopyranoside (IPTG) to the bacterial culture (Studier and Moffatt 1986). This system will enable not only the production of G9a recombinant protein tagged with a histidine tag (His-tag) but also its purification using either Nickel or cobalt column. Hence we cloned G9a full length (180kDa) into the Pet 28a between the Bam HI, and XhoI sites of the multiple cloning sites (MCS). G9a-pET28a sequence was validated by sequencing and further tested for recombinant expression in different bacteria host.

Different bacterial host strains have been extensively used in our laboratory for protein expression: PLYS, RIL, and ER 2566. The bacterial strain PLYS (Invitrogen) is a DE3 bacterium or a λ prophage (carries a chromosomal copy

of T7 RNA polymerase gene under the control of lacUV5); it contains T7 lysozyme coding sequence that suppresses the basal expression from the T7 promoter. The bacterial strain RIL (Stratagene) is also DE3 but its specificity resides in the extra copies of tRNA genes that are normally rare in *E. coli*, improving the production of some heterologous proteins. ER 2566 (New England Biolabs) on the other hand, is an *E. coli* strain deficient in both lon and ompT proteases, which minimizes protein degradation during induction. All these strains were tested to identify the conditions that would yield highest recombinant protein.

Initially, several induction conditions were tested using RIL. The bacteria were transformed by heat shock with G9a-pET 28a plasmid, then different clones were screened by restriction enzyme digestion different clones to confirm sub-cloning and then further tested the expression conditions. Six conditions were used to test the effect of IPTG () concentration (0.4 mM or 0.8 mM) and induction temperature (37°C or room temperature-RT) on the G9a recombinant expression. Bacterial pellet obtained from 50mL LB medium culture overnight were subjected to whole cell lysis in sodium dodecyl sulfate (SDS) loading buffer and loaded on to an 8 % polyacrylamide gel for western blot analysis. As illustrated on Figure 19, in the absence of IPTG (lane 1 and 2), recombinant G9a is not expressed. By adding either 0.4 (lane 3 and 4) or 0.8 mM (lane 5 and lane 6) IPTG we were able to induce G9a expression. However, we observed the presence of smear in all 4 lanes suggesting that the protein relative stability was being compromised though it seems that 0.8 mM IPTG expression at room temperature promoted the accumulation of stable G9a. Indeed, when expression at RT (lane 5) is compared to expression at 37°C (lane 6) in the presence of 0.8 mM IPTG we noticed that expression at RT was beneficial for G9a full length stability. Hence, all the subsequent induction was performed at RT.

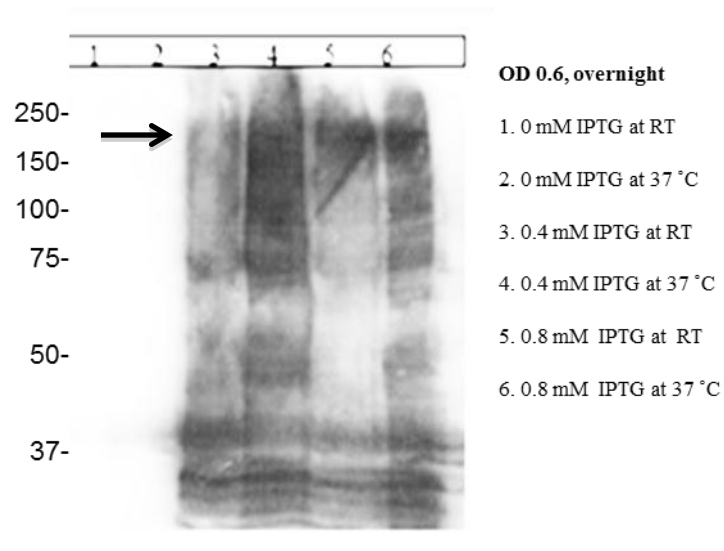


Figure 19: RIL IPTG induction G9a western blot. Whole bacterial extracted from 50 ml LB culture was migrated onto SDS-Page gel. Membrane was blotted using G9a antibody (180kDa). RT- room temperature. The arrow indicates G9a full length theoretical.

Similarly, we sought to determine the conditions that would enhance G9a solubility using the bacteria strains ER2566 and PlysS. The bacteria were independently grown to stationary phase and pelleted by centrifugation and then lysed in SDS-PAGE loading buffer. The boiled soluble fractions were separated from the insoluble ones by centrifugation and were loaded on a 10% SDS page gel and transferred onto a nitrocellulose membrane. The membranes were then blotted using a G9a specific antibody. Figure 20 show that in the absence of IPTG (0mM IPTG), recombinant G9a (lane 1, 2 and 5-6). Addition of 1mM IPTG is sufficient to induce the production of G9a as early as 6h after addition of IPTG (lane 3, 4) though G9a expression is maximal in bacterial cultured grown overnight (lane 7, 8) . A close examination at the results shows that while G9a expression in ER2566 overnight seems to decrease G9a solubility (lane 7), in PLYS G9a full length (180kDa) solubility is not affected (lane 8). Seemingly, G9a-pET basal expression is not tightly regulated when PLYS E. coli are grown to stationary phase (overnight culture) because leaky expression was observed in the absence of IPTG (lane 5 and 6).

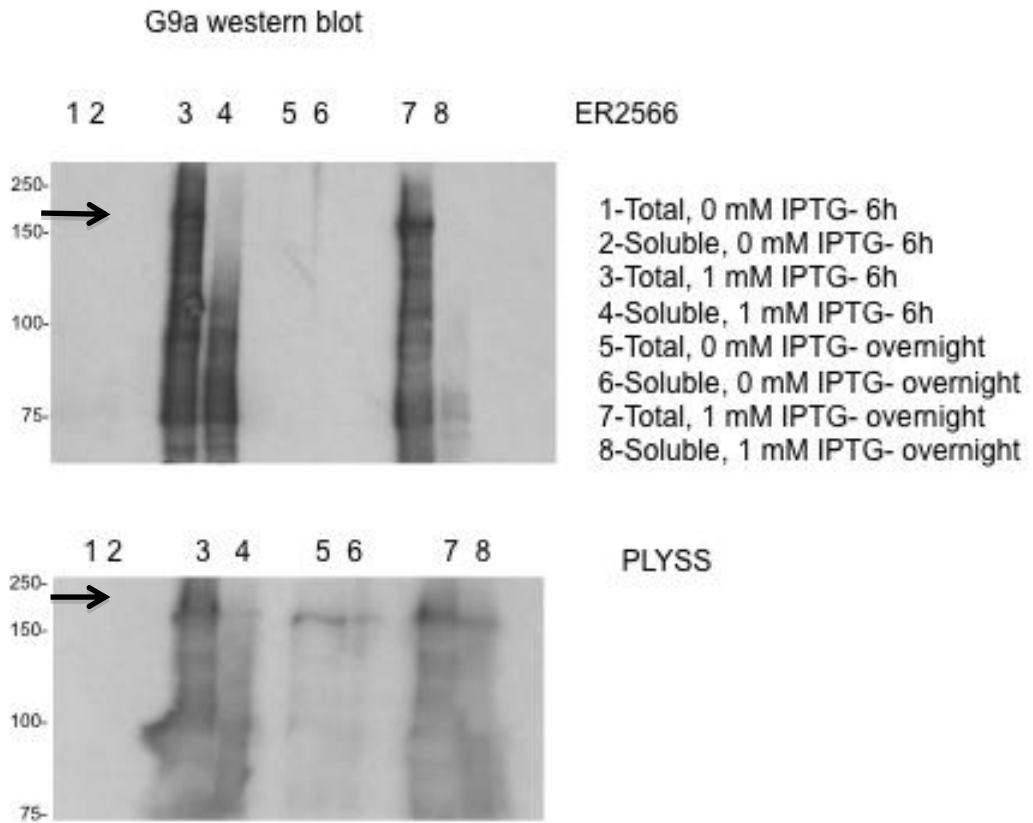


Figure 20: ER2566 and PLYSS IPTG induction G9a western blots. Whole and soluble bacterial fraction extracted from 50 ml LB culture was migrated onto SDS-Page gel. Membrane was blotted using G9a antibody (180kDa). 6h-harvest 6 hours after induction; overnight-harvest after overnight culture. The arrow indicates G9a full-length position.

Though G9a recombinant expression in Plysis was leaky, it gave the best result in term of protein stability. Thereby we proceeded to produce recombinant G9a in pLysS induction using 1 mM IPTG overnight at room temperature.

In addition, alternative sources of sugar and divalent cation have been shown in the past to stabilize recombinant protein expressed in *E. coli* when cultures are left to reach stationary phase (overnight) (Thorens, Sarkar et al. 1988; Marshall and Piddock 1994). Hence we test tested the expression of recombinant G9a in LB media supplemented with 1% glucose and 30 mM magnesium chloride. Since recombinant G9a is flanked by 6 N-histidines, a cobalt resin was used to purify the produced recombinant protein. Cobalt resins having less affinity for host proteins providing a great selectivity compared to nickel resin and hence allowing milder elution conditions (less imidazole). As the manufacturer protocol (Talon) suggested, G9a full-length purification was attempted at pH=7. G9a western blot (Figure 21) shows that His-tag G9a was detected in the wash fraction, indicating that G9a does not bind to the talon resin at pH 7. Consequently, purification was attempted at pH=8. Coomassie staining (Figure 22) shows that G9a full length, expressed in the bacteria, was effectively isolated however many unknown contaminants and/or degradation products were also detected.

As our ultimate goal was to obtain sufficient recombinant protein (about 300mg) to produce an antibody; the amount generated was insufficient. Indeed, 30ug being the lowest amount of protein that is detectable by coomassie staining, the tested expression and purification conditions generated approximately 100 ug of protein from a 500mL culture, which average to a yield of 0.2 mg/mL which was too low. For

future application, the baculovirus system could be used an alternative as it is a much more efficient method to produce high levels of recombinant large proteins.

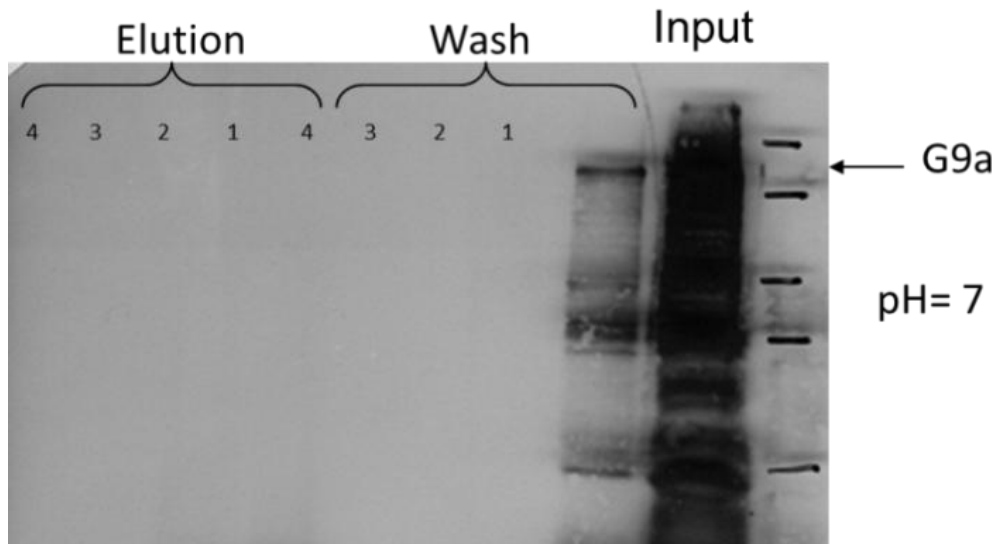


Figure 21 : Purification of G9a full length upon induction of PLysS at pH=7. 500 ml LB culture was grown overnight with 1mM IPTG, 30 mM Mgcl2 and 1% glucose. Solubles fractions obtained after sonication were incubated 2h in with 1 ml cobalt beads. Washes were performed using 50mM sodium phosphate, 300mM Nacl and 20 mM imidazole. Elution buffer contains 50mM sodium phosphate, 300mM Nacl and 150 mM imidazole. The different fractions were migrated onto SDS-Page gel. Membrane was blotted using G9a antibody (180kDa). The arrow indicates G9a full-length position.

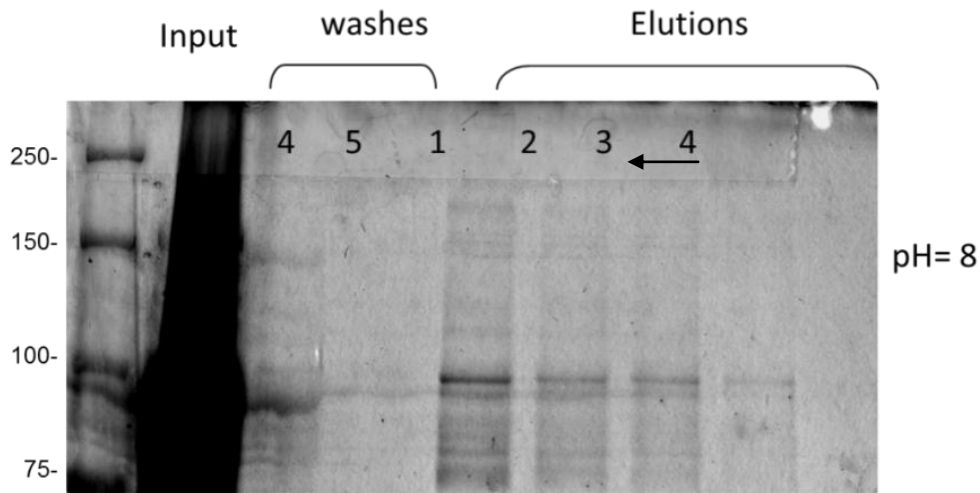


Figure 22: Purification of G9a full length upon induction of PLysS at pH=8. 500 ml LB culture was grown overnight with 1mM IPTG, 30 mM Mgcl2 and 1% glucose. Soluble fraction obtained after sonication was incubated 2h in with 1 ml cobalt beads. Washes were performed using 50mM sodium phosphate, 300mM Nacl and 20 mM imidazole. Elution buffer contains 50mM sodium phosphate, 300mM Nacl and 150 mM imidazole. The different fractions were migrated onto SDS-Page gel and stained using coomassie stain. The arrow indicates G9a full-length position (180kDa).

3.3. $\epsilon\gamma$ reactivation can be detected at the protein level using Triton-Acetic Acid-Urea (tau) western blot

In 2009, Brand *et al* observed that the embryonic β globin gene $\epsilon\gamma$ repression was relieved when the methyltransferase G9a was knocked down by RT-qPCR (Chaturvedi, Hosey et al. 2009). However, it was not possible to assess whether the 10-fold increase in mRNA could be visible at the protein level due to the challenge in separating the globin chains by SDS-Page electrophoresis, due to their similarity in size. One technique that has been used to separate very similar proteins based on differences in size and effective charge is: Triton-urea-acetic acid gel (TAU). Since this technique requires low amount of material, it is efficient to separate proteins based on their charge, mass, and hydrophobic properties (Hossainy, Zweidler et al. 1973; Zweidler 1978). Therefore, we hypothesized that this technique will enable us to visualize $\epsilon\gamma$ reactivation at the protein level after G9a KD. We planned to analyze whole cell lysate from differentiated MEL GLP and G9a (a clone used in the past by Brand *et al* to demonstrate $\epsilon\gamma$ reactivation at the protein level) KD clones. To validate the technique, we started by analyzing the different globin chains contained in different blood samples: one sample was from an adult mouse, samples from two different human adult donors and one sample from a mouse E13.5 embryo (Embryonic day 13.5). The globin chains contained in these samples were efficiently separated using the TAU gel followed by coomassie staining (Figure 23). As expected, mouse peripheral blood contains exclusively adult globin chains (α , β_{maj} , β_{min}). Likewise mature human hemoglobin is comprised of adult globin polypeptides (α , β , δ). At E13.5, in the mouse embryonic hemoglobin only the α -globin genes have switched from the embryonic ζ to the α chain (E 8.5). Therefore we can still detect the presence of embryonic β -globin genes $\epsilon\gamma$, β_{h1} . As we noted, terminally differentiated

MEL cells do not exclusively contain hemoglobin (Figure 23) because these cells do not lose their nucleus like erythrocyte; their lysate contains a mixture of hemoglobin and other proteins. In an attempted to reduce these proteins, that could have originated from the media, the cells were either washed twice or thrice with PBS before lysis and analysis on TAU gel. As demonstrated in Figure 24, increasing the number of washes does not decrease number bands (Figure 24). Hence these protein signals can certainly be attributed the MEL cells. Therefore, MEL cells pose significant challenges as a model to investigate $\epsilon\gamma$ re-activation when G9a is KD.

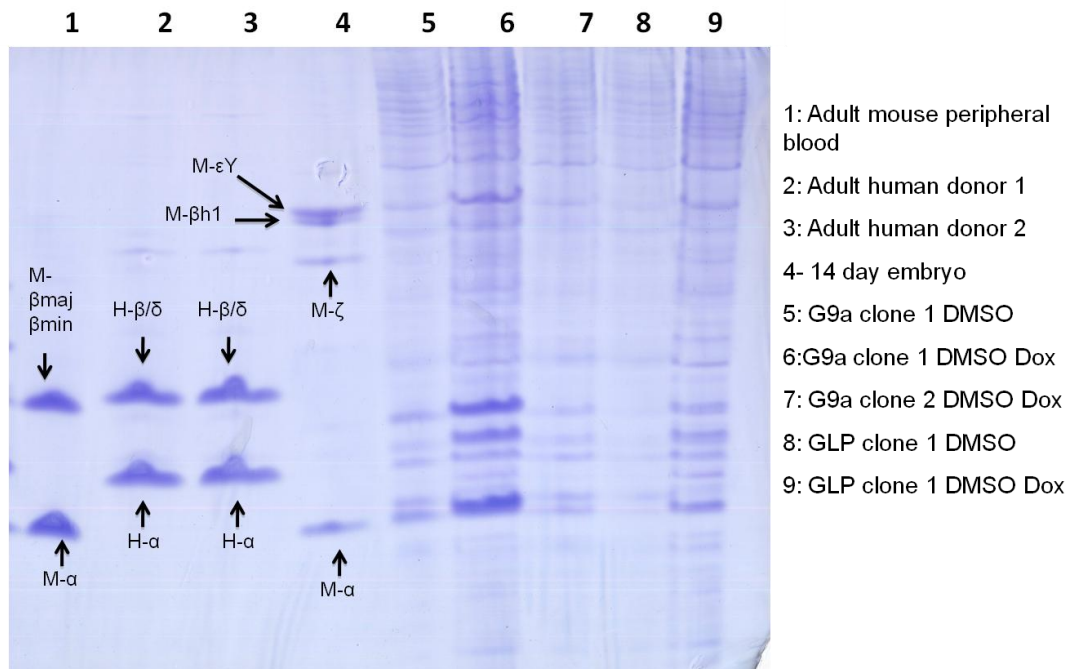


Figure 23: Separation of mouse globin chains by electrophoresis using a coomassie stained Triton- Acetic-Urea gel. The mouse adult β -globin chains (M- β_{maj} / β_{min}), the mouse embryonic β -globin chains (M- $\epsilon\gamma$ and M- $\beta h1$), the mouse adult α -globin chain (M- α) and the mouse embryonic α -globin chain (M- ζ) are shown along with the human adult β -globin chains (M- β/δ) and the human adult α -globin chain (α) Adult globin chain (β_{maj} / B min), Adult α globin chain (α) are shown.

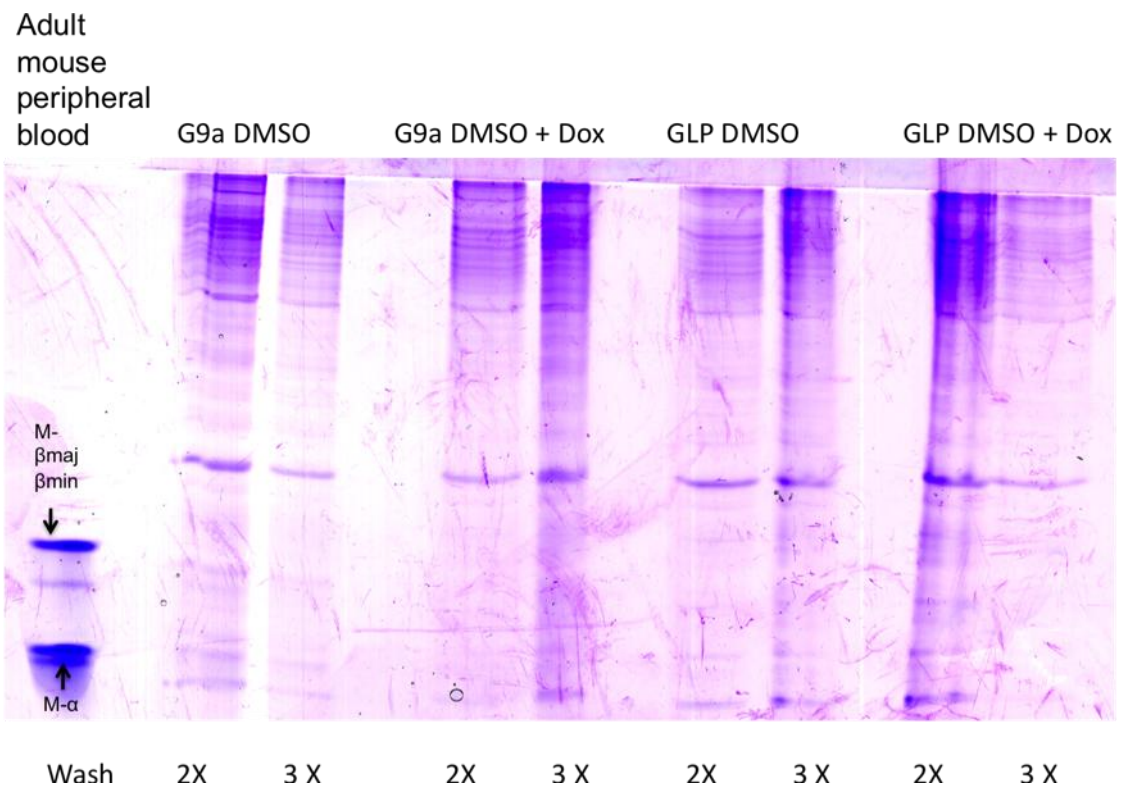


Figure 24: Separation of mouse globin chains by electrophoresis using a coomassie stained Triton- Acetic-Urea gel. The mouse adult β -globin chains (M- β maj / β min) and the mouse adult α -globin chain (M- α) are shown. The cell pellets were washed twice and thrice with 1X PBS (Phosphate buffered saline)

During the course of this study, one of our collaborators Dr. Fabio Rossi developed conditional knockout G9a mice (Lehnertz, Northrop et al. 2010). Rossi and colleagues were able to induce G9a knockout in the blood cells of these mice using a Cre-mediated excision of G9a. Using the TAU technique followed by coomassie staining, we analyzed twenty different peripheral blood samples from G9a knockout mice. The different globin chains present in the blood samples were efficiently separated as Figure 25 and Figure 26 illustrate. As expected, the adult globin chains alpha and beta were present in all the specimens. However we could not detect embryonic $\epsilon\gamma$ protein in the peripheral blood of the G9a KO mice due to the multiplicity of bands. This result suggests that perhaps $\epsilon\gamma$ protein, reactivated by G9a KO might be too low to be detected by coomassie staining.

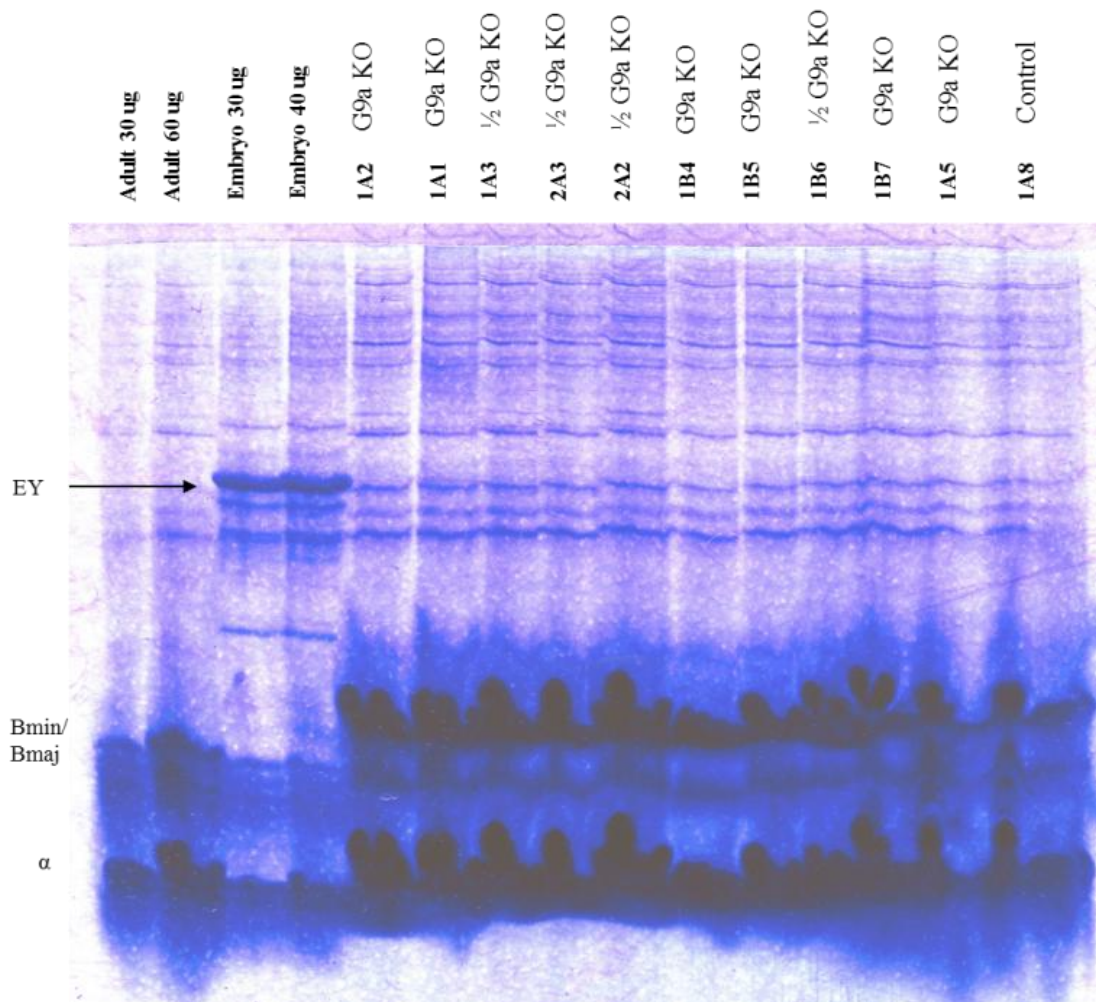


Figure 25: Separation of mouse globin chains by electrophoresis using a coomassie stained Triton- Acetic-Urea gel. Embryonic globin chain (Ey), Adult globin chain (β maj / B min), Adult α globin chain (α), G9a KO (G9a homozygous knockout), $\frac{1}{2}$ G9a KO (G9a heterozygous knockout), control (G9a KO littermate) are shown.

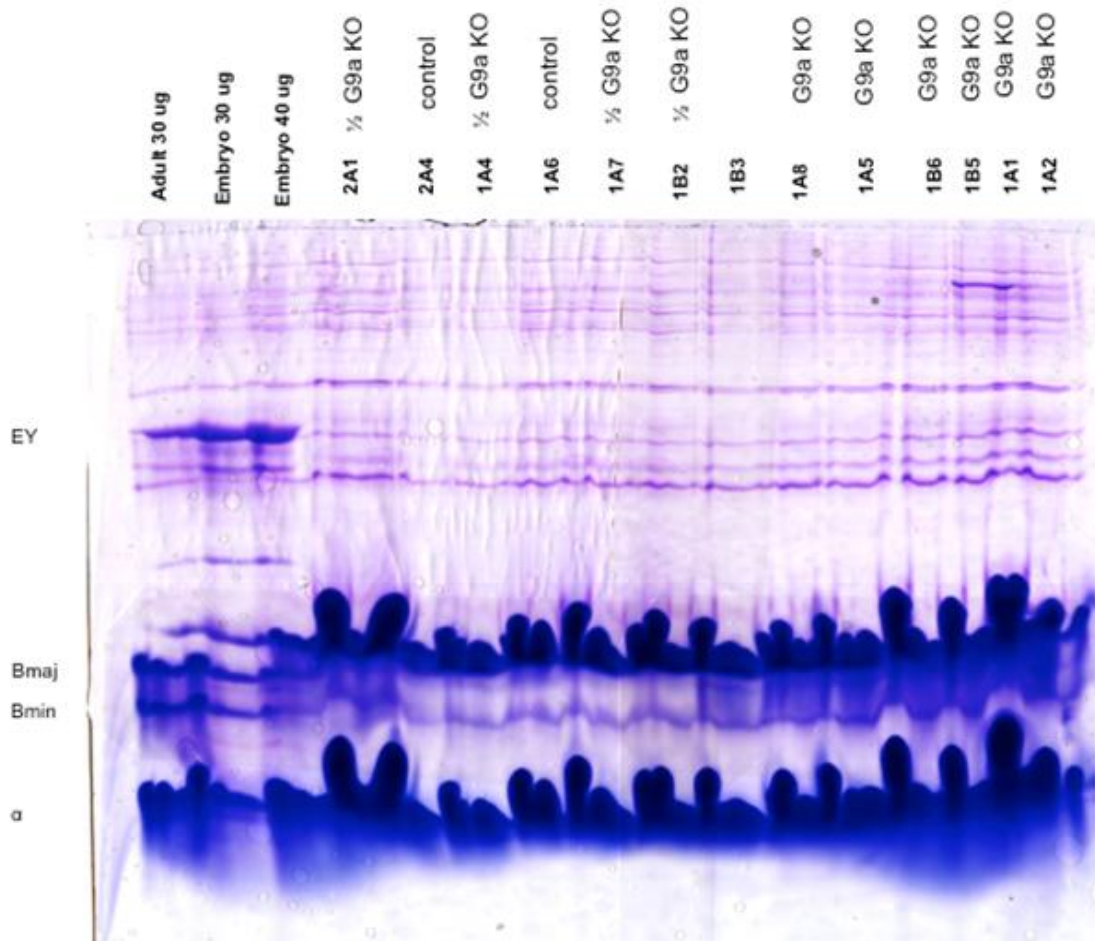


Figure 26: Separation of mouse globin chains by electrophoresis using a coomassie stained Triton- Acetic-Urea gel. Embryonic globin chain (Ey), Adult globin chain (β maj / B min), Adult α globin chain (α), G9a KO (G9a homozygous knockout), $\frac{1}{2}$ G9a KO (G9a heterozygous knockout), control (G9a KO littermate) are shown

Due to the low sensitivity of the coomassie staining technique we were not able to detect $\epsilon\gamma$ protein in the knockout mice thus, we performed TAU Western blot using the same blood samples to further our investigation. As previously described, the samples were loaded on a TAU gel, separated and stained by coomassie; the proteins were transferred onto a PVDF membrane and blotted with hemoglobin specific antibodies to detect $\epsilon\gamma$.

Since no commercial mouse embryonic $\epsilon\gamma$ -specific antibody is available, mouse monoclonal antibody raised against human hemoglobin was used for the blotting. Increasing concentrations of hemoglobin from peripheral human and mouse blood, human cord blood and one G9a KO and its littermate blood were loaded on two TAU gel. While one was stained with coomassie, the other was transferred onto a PVDF membrane and probed with human hemoglobin antibody (Santa Cruz biotechnology, sc-21757). As illustrated (Figure 27), this antibody did not permit the detection of the embryonic mouse globin chains $\epsilon\gamma$.

Similarly, we used the embryonic human hemoglobin epsilon specific antibody (HBE1 Proteintech Group, 12361-1-AP) to analyze blood samples from G9a KO mice, their control littermate, one E13.5 embryo and one adult mouse. Figure 28 show that this antibody allows the visualization of $\epsilon\gamma$ protein. Though the amounts detected were low, it is evident that $\epsilon\gamma$ is present consistently across five G9a KO mice

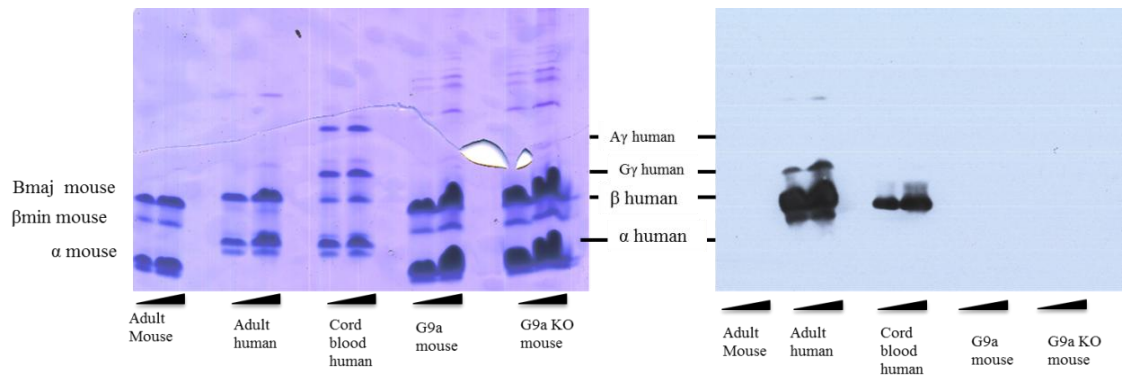


Figure 27: Separation of mouse globin chains by electrophoresis using a coomassie stained Triton- Acetic-Urea gel (TAU) and TAU western blot. The membrane was blotted using human hemoglobin antibody (Santa Cruz biotechnology, sc-21757). Mouse adult globin chain (β_{maj} / β_{min}), human and mouse adult α globin chain (α), human gamma-A ($A\gamma$), human gamma-G ($G\gamma$) and human β are shown.

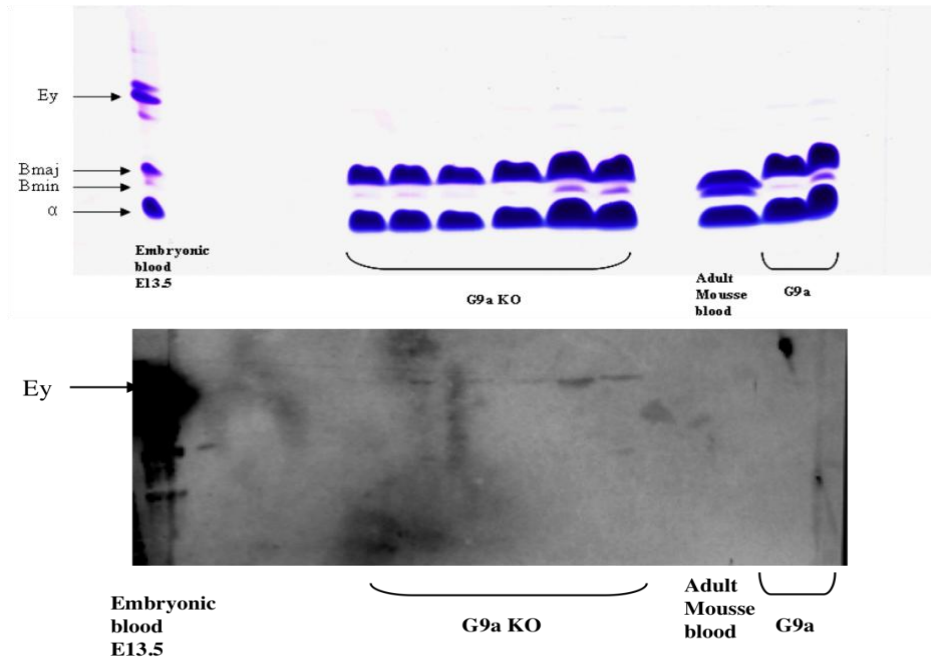


Figure 28: Separation of mouse globin chains by electrophoresis using a coomassie stained Triton- Acetic-Urea gel (TAU) and TAU western blot. The membrane was blotted using human hemoglobin beta antibody (Proteintech Group, 12361-1-AP)). Embryonic globin chain (Ey), mouse adult globin chain (β maj / B min), mouse adult α globin chain (α) are shown.

4. DISCUSSION

4.1. GLP participates in the Activation of the Adult β -Globin Genes by Stabilising the General Transcription Machinery in MEL cells

Studies on the globin genes regulation have provided great insights on eukaryote genes expression during development. The β globin-locus for decades has served as a model to understand the elements involved in developmental specific gene transcription regulation. The mouse β globin-locus has the characteristic to encompass four genes ($\epsilon\gamma$, $\beta h1$, β^{maj} , β^{min}), expressed in a developmental and tissue specific manner, suggesting the involvement of complex regulatory elements. Evidences gather thus far have implicated histone modifications to play a significant role in the spatial and temporal regulation of the β globin genes: Histone 3 Lysine 4 (H3K4) methylation and H3 acetylation together mark extensive active chromatin, $\epsilon\gamma$, $\beta h1$ in the foetus and β^{maj} , β^{min} in the adult. Histone 3 Lysine 9 (H3K9) methylation, strongly associated with silent heterochromatin, has been found recently to be enriched both at active genes coding regions along with Heterochromatin Protein 1 γ (HP1 γ) and at repressed genes promoter (Vakoc, Mandat et al. 2005). In accordance with this observation, G9a a euchromatin-associated histone 3 Lysine 9 methyltransferase was found to have a dual function at the β globin-locus. Through a spreading mechanism, G9a establishes an H3K9 di-methyl environment over the entire locus, represses the embryonic genes ($\epsilon\gamma$, $\beta h1$) in a methyltransferase dependent manner and participates in the adult β globin genes (β^{maj} , β^{min}) activation by stabilizing the pre-initiation complex (PIC) encompassing RNA pol II during erythroid differentiation¹⁹. On a genome wide scale, G9a was also found to be responsible for the formation of differentiation specific large organized chromatin K9-modifications (LOCKs)

covering up to 4.9 mega bases (Wen, Wu et al. 2009). Does G9a-Like-Protein (GLP), G9a's interacting partner participate in the establishment of these repressive domains? To what extent does GLP participate in the complex spreading? All these questions are elements that prompted the present study. To address the hypothesis that GLP regulates the β globin genes in a similar manner as G9a, its interacting partner, we generated MEL shRNA mediated GLP knockdown clones. In a doxycycline inducible manner, we studied the globin genes expression level during erythropoiesis before and after GLP KD. In agreement with the results observed for G9a, we observed by RT-QPCR a decrease in adult globin genes (β^{maj} and β^{min}) transcript (Figure 12) pointing at a diminution in RNA Pol II transcriptional activity. Indeed previous work done in our lab showed that G9a participates in the activation of the globin genes by stabilizing the pre-initiation complex (PIC) through interaction with RNA Pol II and other general transcription factors at β^{maj} promoter thereby enabling the full activation of the globin genes (Jorgensen and Fisher 2009). Hence, GLP KD would also alter the integrity of the PIC complex thus leading to significant reduction in transcriptional activity at this location. Interestingly we detected a reduction in global H3K9me2 which seems to correlate with a decrease in transcription as well (Vakoc, Mandat et al. 2005). Evidently, during MEL differentiation, β^{maj} activation is linked to transcription elongation marked by an increase in H3K9 di-methyl (me2) and tri-methyl (me3) at the β^{maj} coding region (Vakoc, Mandat et al. 2005). Therefore, a decrease in phosphorylated RNA Polymerase II (elongating RNA Pol II) activity would be marked with a decrease in H3K9me2/me3 suggesting that the GLP/G9a complex is recruited to actively transcribed genes along with the general transcription machinery at the β^{maj} promoter.

The resolution of G9a and GLP crystal structures followed by in vitro binding experiments have identified several amino-acids (within the ankyrin domain) to be key in mediating G9a and GLP specific binding to H3K9me1 and H3K9me2, the product of their own enzymatic activity (Collins, Northrop et al. 2008). Accordingly, through ChIP experiments, Brand et al showed that G9a is recruited to the β -globin LCR in a NF-E2/p45 dependent manner and spreads across the locus thereby maintaining $\epsilon\gamma$ in a repressed state while mediating β^{maj} activation (Chaturvedi, Hosey et al. 2009). With G9a binding preferentially to H3K9me2 and GLP to H3K9me1 we proposed that the participation of both G9a and GLP Ankyrin and Set domain is necessary for the complex spreading. This could be achieved by G9a/ GLP complex recruitment at the LCR followed by nearby H3K9 residues methylation. Then, by GLP recognition and binding to H3K9me 1 or G9a to H3K9me2 the complex would be able to propagate itself through a rolling mechanism. Hence their physical interaction with the histones marks at the β -globin locus could be key to their function. Therefore to test the possibility that GLP binding to H3K9me 1/2 is linked to its activating function, we tested GLP Ankyrin domain mutants (incapable to bind H3K9me1/2) ability to activate β^{maj} in MEL differentiated cells. Noticeably, the defect in β^{maj} activation is rescued by expression of exogenous both Flag tagged wild type and Ankyrin mutated GLP (Figure 18). As Ankyrin mutants are unable to bind to H3k9me1/2 mark but activate of β^{maj} , it seems that GLP binding is not required for its function suggesting that GLP could act through an indirect effect on the transcriptional machinery: Hence once recruited at the gene promoter, the basal transcription machinery (RNA polIII complex and PIC) activity could be enhanced by the recruitment of G9a/GLP/WIZ complex promoting adult genes full activation (Figure 29).

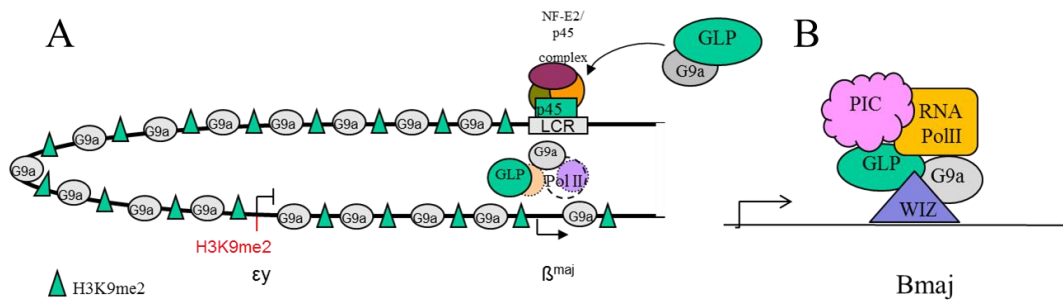


Figure 29: Model of GLP and G9a spreading on the β -globin locus during terminal erythroid differentiation. Figures not drawn to scale The circles illustrate the different transcription factors and cofactors, the green triangle-histone 3 lysine 9 dimethylation mark, grey rectangle-LCR, the yellow box-RNA pol II complex. A- G9a/GLP complex is recruited in an NF-E2/P45 dependent manner at the LCR and spreads on the globin locus creating an H3K9me2 repressive environment and activating the β ^{maj}. B- GLP is necessary for the assembly of the general transcription machinery complex (RNA pol II, the pre-initiation complex (PIC)) along with G9a and the Widely-interspaced zinc finger-containing protein (WIZ) at the β -maj promoter.

Though GLP involvement in activating the adult globin genes (β_{maj} and β_{min}), many questions remain: How GLP and G9a stabilize the general transcription machinery? Does GLP methyltransferase activity necessary for the complex spreading or the β -globin genes expression? Do other G9a and GLP dependent developmental specific genes regulated in the same manner as the β -globin locus?

4.2. Magnesium chloride and glucose improve pET expression systems for recombinant proteins

As observed, the production of G9a recombinant protein in PlysS was low and leaky (Figure 22), highlighting one of the drawbacks of the pET system expressed in the DE3 hosts. Indeed an article from Robert Novy and Barbara Morris in 2001 reported the same observation. In the pET system, the bacteriophage T7 promoter controls the transcription of the target gene. Expression is induced by providing a source of T7 RNA polymerase in the host cell through transcription of the T7 gene 1 under the control of a lac promoter derivative (L8-UV5). In the absence of IPTG, a lac repressor (lacI gene product present in the E. coli genome) binds to the lac operator preventing T7 RNA polymerase mediated transcription. Once provided, an inducer like IPTG binds to the repressor and decreases its affinity for the lac operator, enabling transcription. For efficient transcription, cyclic AMP (cAMP) has to bind to its receptor cyclic AMP protein (CAP) upstream of the lac promoter and directly stimulate RNA polymerase transcription. Hence, induction of transcription depends on cAMP level in the host. However, cAMP level is a metabolic product; its level is low when glucose is used as the primary carbon source and high when alternative source are used. Accordingly when cAMP levels are sufficiently high, the CAP/cAMP complex is formed and binds immediately upstream from the promoter to fully stimulate transcription even in the absence an inducer(Grossman, Kawasaki et

al. 1998). Furthermore, expression hosts containing the pLysS plasmid express T7 lysozyme, which binds to and inhibits T7 RNA polymerase to reduce basal expression. Hence, as the source of glucose is reduced in stationary phase (overnight), the level of cAMP becomes elevated in the host cells increasing overall transcription: T7 RNA polymerase but also T7 Lysozyme. Consequently, high level of T7 lysozyme would sequester the RNA polymerase and inhibit transcription of the target protein, G9a in our case. Therefore, addition of glucose to the media reduces T7 Lysozyme production and could promote G9a production as pET vectors contain mutations that reduce their sensitivity to cAMP level. In 2004, Yongde Luon *et al* showed that recombinant production of fibroblast growth factor 7 (FGF7) could be enhanced by the addition of 30 mM magnesium chloride. Robert Novy and Barbara Morris in 2001 reported that addition of glucose to bacteria culture media reduces pET vectors sensitivity to cAMP level accumulated when bacteria is cultured overnight and hence reduce basal expression in the absence of an inducer. In fact, magnesium chloride is known to improve bacterial growth and recombinant plasmid stability when culture is grown to stationary phase by reducing glucose fermentation (Wang and Germaine 1993; Luo, Cho et al. 2004; Wu, Tian et al. 2009). Though this approach was undertaken production of substantial quantities of G9a recombinant proteins was not achieved.

4.3. $\epsilon\gamma$ Reactivation Detection by Triton-Acetic Acid-Urea (TAU) Western Blot

Brand *et al* in 2009 demonstrated that during erythropoiesis, the maintenance of the β -globin genes expression involved the mammalian methyltransferase G9a. Particularly, G9a repressive function was found to be critical for the maintenance of embryonic β -globin gene $\epsilon\gamma$ in a repressed state through its methyltransferase activity. Indeed evidence of $\epsilon\gamma$ at the mRNA level (Chaturvedi, Hosey et al. 2009) in MEL cells and at the protein level (Figure 28) in mouse peripheral blood upon G9a down regulation implies that G9a alone is sufficient to repress the embryonic gene. For β -thalassemia patients, this opens the avenue for new treatments. Indeed, it was found that patients with β -thalassemia, who had aberrant expression of high level of hemoglobin HbF ($\alpha_2\gamma_2$), hereditary persistence of fetal hemoglobin, had less severe anemia because the γ chain could compensate the absence of β globin subunit. As we have shown new insights into the mechanisms of globin genes regulation, new and more rational treatments can thus be developed for patients with β -thalassemia were G9a could be used as a potential therapeutic target for gene therapy alone or in combination with other treatments.

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APPENDIX I

ShRNA 1 target sequence:

GATCAAACCTGCTCGGAAATTCAAGAGATTTCCGAGCAGGTTGATCtt
tttggaaa

ShRNA 2 target sequence:

GGCGGAGAAGTAGAAAGAAATCAAGAGATTCTTTCTACTTCTCCGCCtt
tttggaaa

ShRNA 3 target sequence:

GCTACATGGCCACCACAAATCAAGAGATTTGTGGTGGCCATGTAGCtt
tttggaa

ShRNA 1 target sequence 1295-1314: G ATC AAA CCT GCT CGG AAA

19 nucleotides

Predicated translated protein sequence: I K P A R K

ShRNA resistance mutations: G ATA AAG CCA GCG AGA AAG

6 nucleotides

Predicated translated protein sequence: I K P A R K

after mutagenesis



Figure 30: Silent Mutations introduced into GLP Coding Sequence

1.

GLP-W791A (1210)a.a



From W (TGG) to A (GCG)

2.

GLP-E817R (1210)a.a



From E (GAG) to R (AGG)

GLP-1112 Δ NHHC 1115 (1207)a.a



Delta NHHC (delta AACCACCACTGC)

Figure 31: Non-silent Mutations introduced into GLP Coding Sequence

Table 1: Primers used for site directed mutagenesis using QuikChange®

Site-Directed Mutagenesis Kit

ShRNA 1 resistance mutagenesis primer
Forward: GACAGCCCCTGG <u>GATAAAGCCAGCGAGAAAGAGGAGGCGGAGAAG</u>
Reverse: CTTCTCCGCCTCCT <u>CTTTCTCGCTGGCTTTATCCAGGGGCTGTC</u>
Tm: 84.51 % GC: 59 Length: 44
Point Mutations Primers
GLP-W791A primer (ANK 1)
Forward: CGGTGGATGGACACCTATGATCGCGGCCACTGAGTACAAGCACG
Reverse: CGTGCTTGTA CT CAGTGGCCGCGATCATAGGTGTCCATCCACCG
Tm: 84.51 % GC: 59 Length: 44

GLP-E817R primer (ANK 2)

Forward:

CAACATCCGGGACAACGAGGAGAACATTTGTCTGCACTGGGCAG

Reverse:

CTGCCCAGTGCAGACAAATGTTCTCCTCGTTGTCCC GGATGTTG

Tm: 85.43 % GC: 55 Length: 44

GLP -1112DNHHC1115 primer (SET)

Forward:

TCTATGGGAATGTCAGCCGGTTCATAGAACCCAACCTTGTGCC

Reverse:

GGCACAAGGTTGGGTTCTATGAACCGGCTGACATTCCCATAGA

Tm: 82.11 % GC: 51 Length: 43

Table 2: Primers used for Real-Time RTqPCR

$\epsilon\gamma$ exon 2
Forward: GCAAGAAGGTGCTGACTGCTT
Reverse: GTAGCTTGTCACAGTGCAGTTCACT
TaqMan probe FAM: TGGAGAGTCCATTAAGAACCTAGACAACCTCAAGTC-MGBNFQ
β^{maj} exon 3
Forward: TCTACAGTTATGTTGATGGTTCTTCCA
Reverse: CAGGACAATCACGATCATATTGC
TaqMan probe FAM: TCCCACAGCTCCTG-MGBNFQ
β^{min} exon 3
Forward: GCAATGCGATCGTGATTGTG
Reverse: CAGCCACCACCTTCTGGAA
TaqMan probe FAM: CCCCTGCTGCACAGG-MGBNFQ

Garite, Bibi

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Title of Publication: The role of GLP domains in spreading of the G9a/GLP complex and regulation of -globin gene expression
Authors/Editors: Camilia thieba / University of Ottawa Date of Publication: april 2012
Publisher: University of Ottawa
Title of CSHP Journal/Book: Genes & Development Title of Article/Chapter: H3K9 methyltransferase G9a and the related molecule GLP CSHL Authors/Editors: Yoichi Shinkai and Makoto Tachibana Page Numbers: 781-788 Figure Numbers: Figure 1 Figure Page Numbers: 782 Copyright Date: 2011
Language: English
Territory:
Format: print

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By Lauren Cornell, Ph.D. 2/1/12
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