

INVESTIGATION OF THE ROLE OF GATA2 IN THE ACTIVATION OF THE
BETA-GLOBIN LOCUS CONTROL REGION DURING EARLY ERYTHROPOIESIS

By

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To my friends and family, with love

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LIST OF ABBREVIATIONS

3C	Chromosome Conformation Capture
ATP	Adenosine-5'-triphosphate
A-USF	Dominant-negative USF
BLAST	Basic Local Alignment Search Tool
BRG1	Brahma-Related Gene 1
ChIP	Chromatin Immunoprecipitation
cDNA	Complementary DNA
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dpc	Days <i>post coitum</i>
EGS	Ethylene glycol bis[succinimidylsuccinate]
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HS	Hypersensitive Site
IgG	Immunoglobulin G
K562 cells	Human Erythroleukemia Cells
LCR	Locus Control Region
mRNA	Messenger RNA
qPCR	Quantitative Polymerase Chain Reaction
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
RNA pol II	RNA Polymerase II
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
shRNA	Small hairpin RNA

siRNA	Small interfering RNA
SWI/SNF	SWItch/Sucrose NonFermentable
TFIID	Transcription factor II D
TG	Transgenic
USF	Upstream Stimulatory Factor
WT	Wild-Type

Abstract of Thesis Presented to the Graduate School
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The beta-globin locus control region (LCR) is a powerful enhancer region located 50 kilobasepairs upstream of the human beta-globin genes that has been shown to be necessary for the high-level expression of these genes. Many events in the activation of the beta-globin gene locus have been shown to first occur at the LCR and subsequently occur at the genes themselves. Thus, understanding the activation of the LCR is crucial to understanding the activation of the beta-globin genes.

BRG1, the central catalytic subunit of the SWI/SNF chromatin remodeling complex, is thought to be the protein responsible for opening the chromatin of the LCR early in erythropoiesis. It is unknown which factor is responsible for the specific targeting and recruitment of BRG1 to the LCR in early erythropoiesis. The experiments presented in this thesis address the hypothesis that GATA2, a transcription factor with a zinc finger DNA-binding domain, is the initial factor that binds to hypersensitive site 2 (HS2) of the LCR and recruits BRG1 to open the LCR.

Chromatin immunoprecipitation experiments were performed to confirm the co-occupancy of GATA2 and BRG1 at HS2 in early erythropoiesis. Co-immunoprecipitation experiments were attempted to detect an interaction between GATA2 and BRG1, but

results from these experiments were inconclusive. Finally, small interfering RNA techniques were utilized to knock down GATA2 to see any indirect effects on the occupancy of BRG1 at HS2 of the LCR. Further work will need to be done to elucidate the potential role of GATA2 in the recruitment of BRG1.

CHAPTER 1 INTRODUCTION

Chromatin Structure and Remodeling

The DNA in the nucleus is organized into chromatin, a complex of DNA and the proteins that help to organize it (1). The building blocks of chromatin are nucleosomes, which consist of DNA wrapped around a complex of highly conserved nucleoproteins called histones (2). In the nucleosome core, two copies of each of the histone proteins H2A, H2B, H3, and H4 assemble to form a disk-shaped octameric structure (3). DNA segments of 145-147 base pairs wrap around these disk-shaped structures in a left-handed superhelix, stabilized primarily by electrostatic interactions and hydrogen bonding between the histone proteins and phosphodiester backbone of the DNA (2). Together, the ~146 base pair DNA segment and the histone octamer form the nucleosome core (3). DNA segments of varying lengths, known as linker DNA, connect one nucleosome core particle to the next in chromatin (3).

Histone Modifications

The amino-terminus of each of the core histone proteins is an unstructured segment of basic amino acid residues that protrudes from the nucleosome core (4). Known as histone tails, these domains do not contribute to the structure or stability of individual nucleosomes (5). Instead, histone tails are essential to the higher-order folding of chromatin (4). The basic histone tails contribute to chromatin condensation through inter-nucleosomal histone-histone interactions, which help the nucleosomes to stack on one another in order to pack DNA (3). Histone tails are so essential to this role that, *in vitro*, their selective proteolytic removal prohibits chromatin from packing beyond the 10nm fiber (6).

The histone tails can be post-translationally modified in a number of ways by a range of enzymes (4). Lysine and arginine residues, which make up around one third of each histone tail sequence, can be methylated or acetylated, and methylation can occur multiple times on a single residue (4,6). Phosphorylation of serines and threonines also occurs, as do ubiquitylation, sumoylation, and ribosylation of other residues (4). Each of these covalent modifications will vary the molecular presentation of a histone tail, allowing it to make different binding contacts to DNA, neighboring histones, and other proteins (7). The ultimate result of the altered molecular presentation of the histone tail via post-translational modification is altered packing of chromatin (4). Some modifications, such as acetylation, are known to be activating modifications which mark open region of chromatin, while others, such as trimethylation of H3K9, correlate with chromatin condensation and transcriptional repression (3,4). Important regulatory regions in DNA, such as promoters, enhancers, and insulators, are often marked by a characteristic pattern of histone modifications that help to define them (4).

Exactly how specific histone modifications alter the chromatin landscape is unknown. It was long believed that alteration in the charge of a histone tail resulting from a modification accounted for the effects of the modification on chromatin packing, as the charge neutralization would affect the electrostatic interactions that help condense chromatin (6). However, further research discouraged this hypothesis, suggesting that the small extent of charge neutralization even on a highly modified histone tail would not be enough to cause the major changes in chromatin structure that result from histone modifications (6). Rather than directly causing changes in chromatin structure, histone modifications are now believed to alter the packing of DNA through a

variety of indirect effects (4). For example, many proteins that bind histones contain small histone binding modules, such as chromodomains to bind methylated lysines or bromodomains to bind acetylated lysines, that enable them to bind to the chromatin fiber via recognition of a specific histone modification (4).

Chromatin Remodeling Complexes

The presence of nucleosomes is generally inhibitory to DNA binding (3). Proteins that regulate gene expression must deal with the repressive nature of nucleosomes in order to bind DNA (3). One mechanism of doing so is to mobilize the nucleosomes, thus relieving repression due to interactions of the core particle with the DNA (3). A number of protein complexes, known as chromatin remodeling complexes, exist to carry out this function (3). These complexes use energy from the hydrolysis of ATP to destabilize nucleosomes by disrupting DNA-histone contacts (3). One such chromatin remodeling complex is SWI/SNF, a complex first identified in yeast that is highly conserved in eukaryotes (8,9). Recruitment of the SWI/SNF complex ultimately leads to removal of nucleosomes from the enhancer and promoter regions to which it is recruited. The resulting nucleosome-free regions are sensitive to digestion of DNase I, and can be referred to as DNase I hypersensitive sites (3).

In mammals, the 2 MDa SWI/SNF complex contains 10-12 subunits, many of which are encoded by multiple genes (9). The complex contains one of two central catalytic ATPase subunits, brahma-related gene 1 (BRG1) or brahma, that share significant sequence homology and have similar activities (10). BRG1 and brahma both contain a DExx catalytic domain that is important in ATP binding and hydrolysis, a HELICc domain that functions in DNA translocation, and a bromodomain that recognizes specific acetylated residues in histone tails (11,12). These features allow the

SWI/SNF complex to disrupt the nuclear architecture at the target site (11). Of the two, BRG1 has been shown to be crucial to many aspects of beta-globin gene regulation, including chromatin remodeling, transcriptional activation, epigenetic modifications, and the looping of the locus (13–15).

The SWI/SNF complex binds with high affinity to DNA and nucleosomes in an ATP-independent manner (8). In addition to the intrinsic DNA-binding abilities of some subunits of the SWI/SNF complex, an initial gene- or cell type-specific factor is thought to be required to recruit the chromatin remodeling complex to a promoter or enhancer region (9). For example, interactions between multiple different nuclear receptors and several SWI/SNF components, including BRG1, have been shown to be involved in the recruitment of SWI/SNF to hormone-responsive promoters (12). Binding of this initial factor is key to the mobilization of nucleosomes that allows initiation of activation of the regulatory DNA region.

Promoter and Enhancer Regions

Several *cis*-regulatory DNA elements act in the regulation of gene expression by RNA polymerase II (RNA pol II) (1). Two such elements are enhancers and promoters. These two DNA elements share several characteristics, but key differences differentiate them as distinct types of functional regions (16).

Promoter Regions

Promoter regions are a type of *cis*-regulatory DNA element located proximal to the target gene (16). The basal promoter contains basal promoter elements, including the TATA box, initiator, and downstream promoter elements, which recruit RNA pol II and general transcription factors, particularly TFIID (16). This region provides a platform for assembly of transcription complexes that are subsequently primed to initiate

transcription of the target gene (16,17). The complete promoter region includes the basal promoter as well as upstream regulatory elements within 1000 base pairs of the transcription start site (16). The upstream regulatory elements provide binding sites for gene-specific transcription factors, which contribute to transcriptional regulation of the target gene (16).

Tissue-specific promoters

Two distinct types of promoters exist. Tissue-specific promoters, which mediate transcriptional initiation on focused, single or several base pair long regions, comprise the promoters for only about 30% of genes (17). These promoters are commonly marked with high H3K4 trimethylation and H3K79 trimethylation (16,18). Tissue-specific promoters contain the previously mentioned basal promoter elements and typically control expression of regulated genes (16,17). Despite making up a minority of the promoters in vertebrates, tissue-specific promoters are heavily studied because of their involvement with significant regulated genes (17).

Housekeeping promoters

The remaining 70% of genes are controlled by housekeeping, or dispersed, promoters (16,17). Typically conveying expression at lower levels than tissue-specific promoters, these contain no basal promoter elements (1,16). Instead, housekeeping promoters are found in CpG islands, granting them a GC-rich nucleotide composition (17). Transcription under the control of a housekeeping promoter initiates at one of many weak start sites scattered over a region of about 50-100 nucleotides (17). Housekeeping promoters are also distinguishable from tissue-specific promoters by their histone modifications (16). Rather than the H3K4 and H3K79 trimethylation seen in

tissue-specific promoters, housekeeping promoters are marked by H3K27 acetylation and H3K20 monomethylation (16).

Enhancer Regions

Enhancers are similar to promoters in their 50-200 base pair length, DNase I hypersensitivity, and inclusion of factor binding elements of 6-10 base pairs (1). However, unlike proximal promoter elements, enhancer regions are located distal from the target gene, often 50 kilobases or more from the target gene and commonly within introns (1,19). Another key distinguishing feature is the enhancer's ability to activate transcription from within a plasmid construct that has been transfected into cells, regardless of the enhancer's location or orientation to the promoter of the target gene (19). The histone modifications that characterize enhancer regions provide an added dissimilarity to promoter regions, as enhancers are marked by high H3K27 acetylation and H3K4 monomethylation and an absence of the significant H3K4 trimethylation found in promoters (16,19). The high H3K27 acetylation could result from the greater extent of histone acetyltransferase p300 binding at enhancers as compared to promoters (19).

Enhancers often act to convey tissue- or developmental-stage-specific expression at the transcriptional level (16). Enhancers can mediate gene expression by opening the chromatin at promoter regions, positioning genes physically close to transcriptionally active regions of the nucleus, directly recruiting transcription complexes, and recruiting elongation factors to affect transcriptional elongation (16). Although some enhancers can directly recruit RNA pol II transcription complexes, enhancers do not contain the basal promoter elements found in promoters (16). Instead, enhancers contain transcription factor binding sites to recruit a variety of ubiquitous or

tissue-specific proteins (16). Many of these proteins also bind promoter regions either directly or through interactions with other factors, creating a complex interplay between enhancer- and promoter-bound proteins (16). Within the past few decades, chromosome conformation capture (3C) experiments have revealed extensive colocalization interactions between enhancers and the promoters of the genes they regulate (19). The “looping” model of enhancer function suggests direct interactions between the enhancer and promoter, with the intervening DNA looped out of the way, are necessary for gene activation by the enhancer (19). These direct interactions could facilitate the transfer of chromatin-modifying proteins, elongation factors, or entire transcription complexes from the enhancer to the promoter to activate expression of the target gene (16).

Organization and Regulation of the Beta-Globin Gene Locus

The beta-globin protein makes up one of two subunits of the protein hemoglobin, the main oxygen carrier in the bloodstream (20). The human beta-globin gene locus is found on chromosome 11 (20). It contains the genes of the beta-globin family, which include epsilon-, gamma-, delta-, and beta-globin (20). These genes are organized and expressed in a developmental stage-specific manner, with epsilon-globin expressed in the embryonic yolk sac, gamma-globin expressed in the fetal liver, and delta-globin and, to a greater extent, beta-globin expressed in the adult bone marrow (20).

The Locus Control Region (LCR)

Upstream of the beta-globin gene locus is a powerful 15 kb enhancer region termed the locus control region (LCR) (20). The LCR contains five DNase I hypersensitive (HS) sites, which contain known binding sites for transcription factors important in the activation of the beta-globin gene locus (20). The LCR has been shown

to be critical for beta-globin gene expression, as absence of an LCR leads to severely reduced beta-globin gene expression (21).

Activation of the Beta-Globin Gene Locus

Erythroid cells differentiate from hematopoietic stem cells through a process known as erythropoiesis, in which they pass through many intermediate stages (20,22). Expression of the developmental stage-appropriate beta-globin gene is not high until late in the process of erythropoiesis (20,23). Activation of the beta-globin gene locus is thought to begin with the opening of the LCR early in erythropoiesis (24). Many events involved in the activation of the beta-globin gene locus have been shown to first occur at the LCR and subsequently occur at the beta-globin promoter regions (24).

Understanding how the LCR is activated is critical for understanding activation of the beta-globin genes as a whole.

Upstream Stimulatory Factor (USF)

Several transcription factors have proven crucial in the activation of the LCR. One of these is a protein known as Upstream Stimulatory Factor (USF) (25). USF is a ubiquitously expressed transcription factor that has a basic helix-loop-helix leucine zipper DNA-binding domain specific for a central E-box motif (26). It has two forms, USF1 and USF2, which share a large amount of sequence homology, and is typically active in the form of the USF1/USF2 heterodimer (26,27). USF enhances the expression of erythropoietic transcription factors and mediates the recruitment of transcription complexes to the beta-globin gene locus (28,29).

A transgenic mouse model has been made which expresses a form of USF in which the basic DNA-binding domain is replaced by acidic residues (25). This protein, known as A-USF, has been shown to inhibit normal activity of both USF1 and USF2 in a

dominant negative manner (25). Erythroid cell-specific expression of A-USF in transgenic mice has been shown to lead to reduced expression of all beta-type globin genes and reduced association of RNA pol II with HS2 and the beta-major globin gene promoter (25). A-USF embryos show an anemic phenotype at 10.5 dpc, and the mutation is embryonic lethal by 12.5 dpc (25).

Co-regulator BRG1

Another critical transcription factor is brahma-related gene 1 (BRG1). As discussed previously in this chapter, BRG1 is the central catalytic ATPase subunit of the SWI/SNF chromatin remodeling complex (12). It uses energy derived from ATP-hydrolysis to disrupt the chromatin architecture at target DNA regions (12). BRG1 is required for beta-globin regulation and erythropoiesis *in vivo* (13–15). The hypomorphic mutant BRG1^{ENU1/-}, which has a mutation in its ATPase domain that uncouples ATPase activity from chromatin remodeling, is recruited to the beta-globin locus, but epigenetic marks, chromatin remodeling, and transcription are all negatively affected (13). BRG1^{ENU1/-} embryos show an anemic phenotype at 12.5 dpc that is similar to the phenotype of A-USF embryos (13,25).

Co-immunoprecipitation experiments show that USF2 and BRG1 appear to be associated in a common protein complex, although the exact nature of their interaction has not yet been determined (30). The pattern of occupancy of BRG1 at HS2 and the beta-major promoter is similar to that of USF (30). Through chromatin immunoprecipitation (ChIP) experiments, BRG1 appears to be associated with the locus early on during differentiation and later dissociates (30). ChIP experiments in 10.5 dpc male embryonic yolk sac cells reveal that BRG1 binding at HS2 appears to be higher in A-USF transgenic cells than in non-transgenic cells (30). Contrastingly, BRG1 binding at

the beta-major promoter appears to be higher in non-transgenic cell than in transgenic cells (30).

GATA2

The SWI/SNF complex, with catalytic subunit BRG1, seems a likely candidate to open the LCR. It remodels nucleosomes, appears to interact with USF2, and is present early in erythropoiesis, later dissociating. As SWI/SNF has no mechanism to direct it specifically to the LCR, another molecule must recruit the complex to the LCR in order for it to play its critical role (9). A likely candidate for this role is GATA2, a transcription factor with a zinc finger DNA-binding domain (32). Other zinc finger proteins have been shown to associate with BRG1 and selectively recruit it to regulatory regions (12). GATA2 is expressed in hematopoietic progenitor cells, and its expression declines through the course of erythropoiesis (32,33). GATA2 has been shown to be essential for the function of hematopoietic progenitor cells (34). GATA2^{-/-} mice exhibit severe defects in hematopoiesis, exhibiting an anemic phenotype that leads to death by 10-11 dpc (35).

Summation

The experiments in this thesis will address the hypothesis that GATA2 is the initial transcription factor that binds to HS2 of the beta-globin LCR and recruits chromatin-remodeling proteins, such as BRG1, to open the LCR.

CHAPTER 2 MATERIALS AND METHODS

Design and Construction of shRNA-Expressing Vectors

Small hairpin RNA (shRNA) oligomers were designed using the Dharmacon siRNA Design Center (<http://www.dharmacon.com/designcenter/designcenterpage.aspx>). Transcript sequences were obtained using the Ensembl Genome Browser (<http://useast.ensembl.org>). Human transcripts used were as follows: Gata2-001, CCDS3049; Gata1-001, CCDS14305; SMARCA4-001 (BRG1), CCDS12253. Two sequences per transcript were chosen from the results of the Dharmacon siRNA Design Center and aligned to the human genome using the Basic Local Alignment Search Tool (BLAST) to check for off-target effects. The selected sequences were then inserted into the RNA Codex Tool (<http://cancan.cshl.edu/cgi-bin/Codex/Tools.cgi>), with AC added before the 19mer sequence and A added after to create a 22mer sequence prior to shRNA optimization. The resulting sense and anti-sense sequences were inserted into an shRNA hairpin sequence, obtained courtesy of the lab of Dr. Suming Huang at the University of Florida. The reverse complement to each sequence was also generated to allow for annealing of the shRNA oligomers and ligation into a double-stranded vector. The IDT DNA Analyzer (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>) was used to analyze the full hairpin sequences to confirm their ability to form hairpins. Complete shRNA sequences are listed in Table 2-1.

Desalted shRNA oligomers were obtained from Sigma, then resuspended and annealed into double-stranded oligomers. The TRIPZ vector (Thermo Scientific) was digested with EcoRI-HF and XhoI (New England Biolabs), and the digested vector was

gel-purified using the QIAGEN Gel Extraction Kit. Double-stranded shRNA oligomers were ligated into the TRIPZ vector. The construct was then transformed into Stbl2 competent cells (Invitrogen). Plasmid DNA was extracted from transformed cells and sequenced to check for mutations in the shRNA sequences.

Cell Culture and Transfections

Human erythroleukemia (K562) cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown at 37°C in an incubator containing 5% CO₂.

shRNA Transfections

K562 cells at a concentration of 5×10^5 cells per milliliter of media were transfected using 10 microliters Lipofectamine 2000 Reagent (Invitrogen) per milliliter of media according to the manufacturer's instructions. The concentration of plasmid DNA for transfections was 2.5 micrograms per milliliter of media. Puromycin (Sigma-Aldrich) at a concentration of 5 micrograms per milliliter of media was added as a selection drug 48 hours post transfection. The concentration of puromycin was lowered to 2 micrograms per milliliter of media 10 days after transfection to maintain selection. Expression of shRNA was induced by adding 1 microgram of doxycycline (Sigma) per milliliter of media. RNA was extracted 72-96 hours after induction with doxycycline.

siRNA Transfections

siGENOME SMARTpool reagents for human GATA2 (M-009024-00), Lamin A/C control (D-001050-01), siGLO Lamin A/C control (D-001620-02), and non-targeting small interfering RNA (siRNA) control (D-001210-02) were obtained from Dharmacon and resuspended in 1x siRNA buffer (60mM KCl, 6mM HEPES-pH 7.5, 0.2mM MgCl₂) according to the manufacturer's instructions. DharmaFECT 1 Transfection Reagent (T-

2001, Dharmacon) was used to transfect K562 cells at a concentration of 2×10^6 cells per milliliter of media according to the manufacturer's instructions. The volume of DharmaFECT 1 Transfection Reagent used per milliliter of media was 2 microliters. The control siRNAs were transfected at 25 micromolar concentrations, and the GATA2 siRNA was transfected at a 50 micromolar concentration. Cells were incubated for 48-72 hours after transfection.

RNA Extraction and Complementary DNA (cDNA) Creation

RNA was extracted from 1×10^6 cells using the RNeasy Mini Kit from QIAGEN. Extractions were performed according to the manufacturer's instructions. The concentration of the RNA was determined using the Implen P300 NanoPhotometer. Complementary DNA (cDNA) was created from 1 microgram of total RNA using the iScript cDNA synthesis kit (Bio-Rad) as described in the manufacturer's protocol, then analyzed via quantitative polymerase chain reaction (qPCR).

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) experiments were performed as described previously (36). The following antibodies were used for ChIP assays: RNA polymerase II (RNA pol II) (CTD4H8; Upstate Biotechnology), rabbit anti-BRG1 (sc-10768, Santa Cruz), rabbit anti-GATA2 (sc-9008x, Santa Cruz), and rat anti-GATA1 (sc-265, Santa Cruz). Normal rabbit IgG (P120-101, Bethyl Labs) was used as a negative control.

Quantitative Polymerase Chain Reaction (qPCR)

Reactions were set up using the SsoAdvanced SYBR Green Supermix (Bio-Rad) and run in the Bio-Rad CFX Connect Real-Time System. Primers used for qPCR experiments are listed in Table 2-2. Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) reactions for RNA analysis were carried out under the

following conditions: 95°C for 5 min, 40 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min (the plate was read after each extension step), 95°C for 10 s, and a melting curve from 60°C to 95°C with a 0.5°C step size. qPCR reactions for ChIP experiments used the following conditions: 95°C for 5 min, 40 cycles of 94°C for 10 s, 59°C for 20 s, and 72°C for 30 s (the plate was read after each extension step), 95°C for 30 s, and a melting curve from 60°C to 95°C with a 0.5°C step size. Analysis was performed using the relative standard curve method, with standard curves generated by 10-fold serial dilutions of wild-type cDNA for RT-qPCR and input DNA of the appropriate cell type for ChIP qPCR. For RT-qPCR, results were reported as expression levels normalized to the messenger RNA (mRNA) of reference gene GAPDH. ChIP qPCR results were normalized as the fraction of input DNA from the appropriate cell type.

Protein Extraction and Western Blotting

At least 1×10^7 cells were used per lane in western blot experiments. Proteins were extracted by resuspending cells in RIPA buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 10 mM EDTA, 0.25% NaDesoxycholate, 1% NP-40, 0.1% SDS) containing protease inhibitors (Roche). Cells were then placed on a rotating wheel at 4°C for 30 minutes. Between 10 micrograms and 60 micrograms of protein extracts were incubated in Laemmli buffer at 95°C for 10 minutes. Denatured proteins were separated by electrophoresis on 4-15% Mini-PROTEAN TGX precast gels (Bio-Rad). The Bio-Rad Mini Trans-Blot apparatus was used for electrotransfer onto a polyvinylidene difluoride membrane, consistent with the manufacturer's instructions. The membrane was blocked with a solution of 5% milk in a TBS solution containing 0.1% Tween-20 (TBST). Antibodies used for probing and detection were incubated with the membrane in a 5% milk solution in TBST. Proteins were detected using the Immobilon Western

Chemiluminescent HRP Substrate (Millipore) and x-ray film (Kodak) according to the manufacturer's protocol. Primary antibodies were the same as those used for ChIP. Mouse anti- β -tubulin (sc-55529, Santa Cruz) was used as an equal loading control for whole cell extracts. Secondary antibodies were goat anti-mouse IgG-HRP (sc-2005, Santa Cruz), goat anti-rabbit IgG-HRP (sc-2301, Santa Cruz), and goat anti-rat IgG-HRP (sc-2006, Santa Cruz).

Co-Immunoprecipitation

Between $1-2 \times 10^7$ cells per antibody were used for co-immunoprecipitation experiments. Cells were washed with PBS and resuspended in lysis buffer (20mM Tris pH 7.5, 100mM NaCl, 0.5% NP-40, 0.5mM EDTA, 0.5mM PMSF) for extraction of protein complexes. After centrifugation of samples and isolation of supernatants, antibodies were added to the extracts and incubated overnight on a rotator at 4°C. Antibodies used in co-immunoprecipitation experiments were the same as those used for ChIP. Next, protein A sepharose beads (GE Healthcare) were added to the immunoprecipitates, which were then incubated on a rotator at 4°C for another 2 hours. After washing the immunoprecipitates three times with lysis buffer, immunoblotting was performed according to the western blot procedure outlined previously.

Table 2-1. List of human shRNA oligomer sequences

Gene	Sequence
GATA2 version 1	Top 5'- tcgagaaggtatattgctgttgacagtgagcgCCCAAAGTGCATGCAGGAGAAAtagtgaa gccacagatgtaTTTCTCCTGCATGCACTTTGGTgcctactgcctcgg-3' Bottom 5'- aattccgaggcagtaggcaCCCAAAGTGCATGCAGGAGAAAatcatctgtggcttcactaT TTCTCCTGCATGCACTTTGGTcgctcactgtcaacagcaatataccttc-3'
GATA2 version 2	Top 5'- tcgagaaggtatattgctgttgacagtgagcgCCGAGAGCATGAAGATGGAAAAtagtgaa gccacagatgtaTTTTCCATCTTCATGCTCTCGTgcctactgcctcgg-3' Bottom 5'- aattccgaggcagtaggcaCCGAGAGCATGAAGATGGAAAAtacatctgtggcttcactaT TTTTCCATCTTCATGCTCTCGTcgctcactgtcaacagcaatataccttc-3'
GATA1 version 1	Top 5'- tcgagaaggtatattgctgttgacagtgagcgCCTGGAAGATCTGGATGGAAAAtagtgaa gccacagatgtaTTTTCCATCCAGATCTTCCAGTgcctactgcctcgg-3' Bottom 5'- aattccgaggcagtaggcaCCTGGAAGATCTGGATGGAAAAtacatctgtggcttcactaT TTTTCCATCCAGATCTTCCAGTcgctcactgtcaacagcaatataccttc-3'
GATA1 version 2	Top 5'- tcgagaaggtatattgctgttgacagtgagcgCCCCGCAAGGCATCTGGAAAAAtagtgaa gccacagatgtaTTTTTCCAGATGCCTTGCGGGTgcctactgcctcgg-3' Bottom 5'- aattccgaggcagtaggcaCCCCGCAAGGCATCTGGAAAAAtacatctgtggcttcactaT TTTTCCAGATGCCTTGCGGGTcgctcactgtcaacagcaatataccttc-3'
BRG1 version 1	Top 5'- tcgagaaggtatattgctgttgacagtgagcgCCGCTCAGAAGAAGAGGAAGAAAtagtgaa agccacagatgtaTTCTTCCTCTTCTTCTGAGCGTgcctactgcctcgg-3' Bottom 5'- aattccgaggcagtaggcaCCGCTCAGAAGAAGAGGAAGAAAtacatctgtggcttcacta TTCTTCCTCTTCTTCTGAGCGTcgctcactgtcaacagcaatataccttc-3'
BRG1 version 2	Top 5'- tcgagaaggtatattgctgttgacagtgagcgCCCCAAGGATTTCAAGGAATAAtagtgaa gccacagatgtaTTATTCCTTGAAATCCTTGGGTgcctactgcctcgg-3' Bottom 5'- aattccgaggcagtaggcaCCCCAAGGATTTCAAGGAATAAtacatctgtggcttcactaT TATTCCTTGAAATCCTTGGGTcgctcactgtcaacagcaatataccttc-3'

In the above sequences, lowercase letters indicate the sequence of the standard shRNA hairpin used. Capital letters signify the specific sense and antisense shRNA sequences. Each version has two strands (top and bottom) that were annealed together to allow ligation into a double-stranded vector.

Table 2-2. List of human primers used for qPCR experiments

Primers	Usage	Sequence
HS2	ChIP qPCR	US 5'-GAGTCATGCTGAGGCTTAGGG-3' DS 5'-GTCACATTCTGTCTCAGGCA-3'
GATA1	RT-qPCR	US 5'-CCACTACCTATGCAACGCCT-3' DS 5'-ACCTGCCCGTTTACTGACAA-3'
GATA2	RT-qPCR	US 5'-CTACAGCAGCGGACTCTTCC-3' DS 5'-CCCACAGTTGACACACTCCC-3'
γ -globin	RT-qPCR	US 5'-TGAATGTGGAAGATGCTGGA-3' DS 5'-CATGATGGCAGAGGCAGAG-3'
GAPDH	RT-qPCR	US 5'-GAAGGTGAAGGTCGGAGTCA-3' DS 5'-GAGGTCAATGAAGGGGTCAT-3'
Lamin A/C	RT-qPCR	US 5'-AGGACCAGGTGGAGCAGTAT-3' DS 5'-CACCAGGTTGCTGTTCTCT-3'

CHAPTER 3 CO-OCCUPANCY OF GATA2 AND BRG1 AT THE LOCUS CONTROL REGION IN EARLY ERYTHROPOIESIS

Introduction

The experiments presented in this thesis aim to evaluate the role of GATA2 in recruiting BRG1, the catalytic subunit of the SWI/SNF chromatin remodeling complex, to hypersensitive site 2 (HS2) of the locus control region (LCR) of the beta-globin gene locus. This recruitment is hypothesized to be necessary to open the LCR in early erythropoiesis, an event important for activation of the beta-globin genes. In order to investigate the possibility of this interaction, it was necessary to first ensure that BRG1 and GATA2 colocalize at HS2 in K562 cells. These cells are a line of human erythroleukemia cells that exist permanently in an early stage of erythropoiesis (37). Previous genome-wide studies have shown that GATA2 and BRG1 can be commonly found to bind to the same regions of DNA (18). Here, chromatin immunoprecipitation (ChIP) experiments were used to investigate the existence of their co-occupancy at HS2 in K562 cells.

Results

ChIP experiments were performed in K562 cells to investigate GATA2 and BRG1 binding at HS2 of the beta-globin LCR. RNA polymerase II (RNA pol II) was used as a positive control, as it is known to bind to HS2 in K562 cells. Rabbit IgG was used as a negative control. The results of the experiments, displayed in Figure 3-1, show BRG1, GATA2, and RNA pol II binding at HS2 in K562 cells.

Discussion

The presence of colocalization of BRG1 and GATA2 at HS2 was encouraging, as this is the minimum requirement for the hypothesis presented in this thesis to be

plausible. RNA pol II levels were high, as expected at this region in this cell type. BRG1 binding levels were relatively low, possibly because it binds indirectly to DNA. For future experiments, use of an additional crosslinker such as ethylene glycol bis[succinimidylsuccinate] (EGS) could help to capture more of the BRG1 bound.

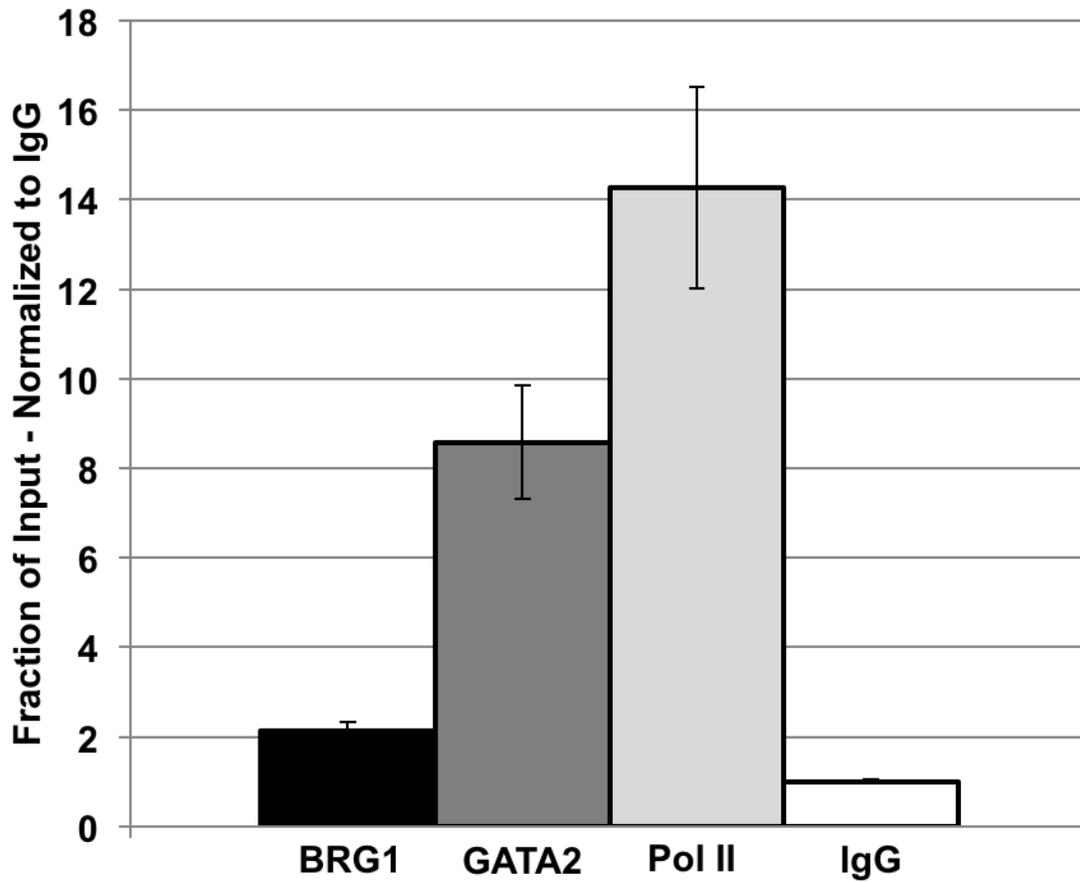


Figure 3-1. ChIP analysis of BRG1, GATA2, and RNA pol II binding to HS2 of the LCR in K562 cells. Antibodies against rabbit IgG were used as a negative control. DNA was analyzed by qPCR using primers specific for HS2. Data were normalized to IgG and are shown as means \pm standard errors of the means of three independent ChIP experiments. qPCRs were performed in triplicate.

CHAPTER 4 THE ASSOCIATION OF GATA2 AND BRG1

Introduction

In order for GATA2 to recruit BRG1, protein-protein interactions must exist between the two factors. If they exist, these interactions should be detectable by co-immunoprecipitation experiments. Previous co-immunoprecipitation studies have shown an interaction between BRG1 and GATA1, a transcription factor in the same family as GATA2 that shares many redundant functions (30, 38). Attempts were made to determine the existence of an interaction between GATA2 and BRG1.

Results

Prior to beginning co-immunoprecipitation experiments, antibodies used for immunoblotting were tested by western blot. Figure 4-1 shows the results of a western blot performed using K562 whole cell protein extracts and optimized antibody dilutions. Bands for BRG1 and GATA2 can be clearly seen at 205 kDa and 50 kDa, respectively. This shows that the blotting technique and antibodies used in these experiments were effective.

The results of the co-immunoprecipitation experiments showed severely overexposed pull-down lanes, with the highest concentration of overexposure being centered around 50 kDa. The input lanes were generally unaffected by the overexposure, and continued to demonstrate the presence of the target protein in the protein extracts through successful immunoblotting. However, the amount of overexposure in the lanes with the immunoprecipitated protein complexes prohibited visualization of any possible interaction between GATA2 and BRG1.

Discussion

Co-immunoprecipitation experiments presented several problems. As seen in Figure 4-2, severe overexposure was present in all lanes containing antibody pull-downs. This overexposure was absent from the input lane, indicating that the likely cause of the overexposure was something found only in the lanes containing immunoprecipitated protein extracts. The most probable cause of the overexposure was the presence of antibodies used for pull-down in the gel. These antibodies, even when denatured, would be bound by the secondary antibodies used to probe the membrane. Heavy chain immunoglobulins have a molecular weight of 50 kDa, which would explain the centering of the overexposure at this weight.

Unfortunately, the protein GATA2 has a molecular weight of 50 kDa, placing it at the center of the overexposure. This prevents the visualization of even a positive control immunoprecipitation using GATA2, where a GATA2 antibody would be used for both the pull-down and probing. BRG1, with a molecular weight of 205 kDa, could theoretically still be detected. However, the level of overexposure was so high that it would often obscure past this molecular weight. In Figure 4-2, BRG1 can be detected in the positive control lane, where the BRG1 pull-down is probed using an anti-BRG1 antibody. Unfortunately, BRG1 can also be detected in the negative control IgG pull-down, which calls into question the significance of the positive control. BRG1 is notably absent from the GATA2 pull-down lane. With the lack of a negative control or any confirmation that the GATA2 pull-down was effective, this absence contributes nothing to the body of knowledge surrounding a possible interaction between BRG1 and GATA2.

Several methods can be used to overcome the problems caused by the presence of pull-down antibodies in the gel. Use of cross-species antibodies for pull-down and

probing was attempted, but cross-species antibodies were still recognized, leading to the same issue with overexposure. As an alternative to secondary antibody usage, the Clean-Blot IP Detection Reagent (Thermo Scientific) was utilized in several attempts. However, the blots resulting from these experiments were extremely questionable, and detection of proteins even in a non-immunoprecipitated western blot was not effective. Binding the pull-down antibodies to protein A sepharose beads was also unsuccessfully attempted.

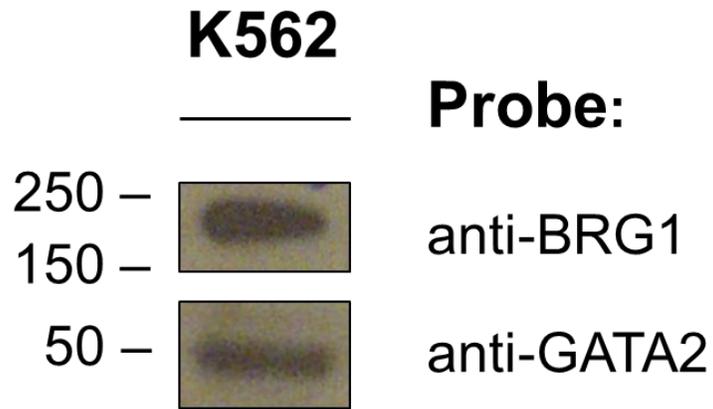


Figure 4-1. Immunoblot analysis of protein extracts from K562 cells. 15 micrograms of protein extracts were loaded per lane.

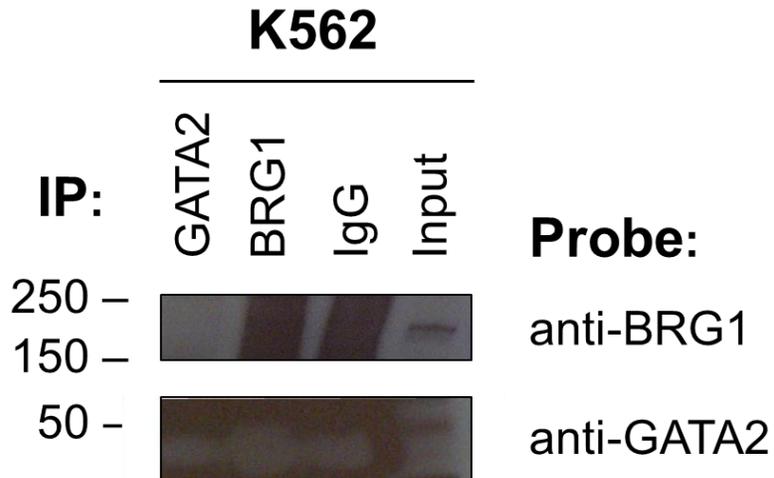


Figure 4-2. Immunoblot analysis of co-immunoprecipitation on whole cell protein extracts from K562 cells. Antibodies against rabbit BRG1, rabbit GATA2, and rabbit IgG were used to pull down protein complexes. Membrane was probed using antibodies against either rabbit BRG1 or rabbit GATA2.

CHAPTER 5 STABLE KNOCKDOWN OF GATA2 USING SMALL HAIRPIN RNA

Introduction

If the hypothesis that GATA2 recruits BRG1 to hypersensitive site 2 (HS2) during early erythropoiesis is correct, it should follow that knocking down GATA2 in K562 cells would reduce BRG1 occupancy at HS2. Thus, small hairpin RNA (shRNA) was used to knock down GATA2 in K562 cells in order to elucidate any direct effect on BRG1 occupancy at HS2. This particular method can be used to create a stable line of cells in which expression of an shRNA from a vector could be induced to knock down a gene. The TRIPZ vector (Thermo Scientific) was chosen for its many features, including a Tet-On system to allow for expression of the shRNA only in the presence of doxycycline, puromycin and ampicillin resistance genes, and the coexpression of red fluorescent protein (TurboRFP) in conjunction with the shRNA so that successfully transfected cells could be visualized using fluorescence microscopy.

Results

Six shRNAs were acquired, two versions each for BRG1, GATA2, and GATA1. Version one of both BRG1 and GATA2 failed to anneal, despite many attempts, leaving experiments to be carried out with only one version of the shRNAs for these two genes. The other four remaining shRNAs that did successfully anneal were ligated into the TRIPZ vector, transformed into bacteria, and then sequenced to ensure that the 110 base pair shRNA sequences contained no mutations. Figure 5-1 shows the outcome of a Basic Local Alignment Search Tool (BLAST) alignment of the 110 base pair shRNA sequences with their corresponding sequencing results.

Plasmid DNA of each of the four mutation-free shRNA constructs was extracted from bacteria. Independent populations of cells were transfected with one of these four constructs. After a month of selection with puromycin, BRG1 version 2 shRNA-transfected cells were frozen down to maintain them for future experiments. Following a three-day induction with doxycycline to induce shRNA expression, analysis was performed on the three remaining transfected cell populations to analyze the expression levels of the genes targeted for knockdown. The results of these experiments are shown in Figure 5-2. A Student's *t*-test revealed that GATA1 version 1 and GATA2 version 2 shRNAs produced significant results, successfully knocking down their target genes upon induction of expression of the shRNA with doxycycline.

After this check for successful knockdown on the population level, single-cell clones were generated from the three shRNA-transfected populations. Surviving clones were induced with doxycycline and checked with an inverted fluorescence microscope for RFP expression, which would indicate expression of the shRNA. RNA extractions were performed on those clones that expressed RFP. Expression levels of target genes were analyzed using RT-qPCR (data not shown). Knockdown levels varied with each clone, and were inconsistent between two separate extractions and analyses.

Before further analysis, including chromatin immunoprecipitation (ChIP) experiments, could be performed, all clones died from contamination. Frozen cell populations were thawed and expression analysis was performed to check for knockdown of target genes upon induction with doxycycline. Results are shown in Figure 5-3. No difference in the levels of GATA2, the shRNA target gene, was seen

between untreated shRNA-transfected control cells and doxycycline-induced shRNA-transfected cells.

Discussion

Use of shRNA to knock down genes seemed moderately effective on the population level, as successful knockdowns of GATA2 and GATA1 were achieved. However, single-cell clones originating from these populations did not fare well. Knockdown levels varied significantly and were inconsistent between two extractions. Additionally, many of the clones failed to grow. One possible explanation for this phenomenon is leaky expression of the shRNA from the vector, potentially through trace amounts of doxycycline contained in the fetal bovine serum (FBS) used in the media. GATA2 and GATA1 are important proteins involved in many different cellular processes. Altering the levels of these two proteins on a long-term basis could negatively affect their growth and survival. An attempt to surmount this problem by using tetracycline-free FBS (Clontech) ultimately led to the demise of all of the cells, as the laboratory stock of this reagent was contaminated with bacteria.

The decision to create single-cell clones was made in an attempt to see higher knockdown levels than those observed in the transfected populations. However, knockdown levels in single-cell clones, if present, were equal to or less than the levels seen in the populations and were inconsistent. Future attempts at knocking down GATA1 and GATA2 with this specific shRNA expression system should focus experimentation first on the population level, since minimal problems were encountered at this stage. If desired, generation of single-cell clones concurrent with experimentation on the population level would be an efficient strategy, as generation of single-cell clones takes an extensive amount of time.

```

A      Score = 199 bits (220), Expect = 9e-56
        Identities = 110/110 (100%), Gaps = 0/110 (0%)
        Strand=Plus/Plus
Query 1  TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCTGGAAGATCTGGATGGAAAAATAGTGA 60
        |||
Sbjct 46  TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCTGGAAGATCTGGATGGAAAAATAGTGA 105

Query 61  AGCCACAGATGTATTTTCCATCCAGATCTTCCAGTTGCCTACTGCCTCGG 110
        |||
Sbjct 106  AGCCACAGATGTATTTTCCATCCAGATCTTCCAGTTGCCTACTGCCTCGG 155

B      Score = 199 bits (220), Expect = 9e-56
        Identities = 110/110 (100%), Gaps = 0/110 (0%)
        Strand=Plus/Minus
Query 1  TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCCGCAAGGCATCTGGAAAAATAGTGA 60
        |||
Sbjct 151  TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCCGCAAGGCATCTGGAAAAATAGTGA 92

Query 61  AGCCACAGATGTATTTTCCAGATGCCTTGCGGGTTGCCTACTGCCTCGG 110
        |||
Sbjct 91  AGCCACAGATGTATTTTCCAGATGCCTTGCGGGTTGCCTACTGCCTCGG 42

C      Score = 199 bits (220), Expect = 9e-56
        Identities = 110/110 (100%), Gaps = 0/110 (0%)
        Strand=Plus/Minus
Query 1  TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCGAGAGCATGAAGATGGAAAAATAGTGA 60
        |||
Sbjct 153  TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCGAGAGCATGAAGATGGAAAAATAGTGA 94

Query 61  AGCCACAGATGTATTTTCCATCTTCATGCTCTCGTTGCCTACTGCCTCGG 110
        |||
Sbjct 93  AGCCACAGATGTATTTTCCATCTTCATGCTCTCGTTGCCTACTGCCTCGG 44

D      Score = 199 bits (220), Expect = 9e-56
        Identities = 110/110 (100%), Gaps = 0/110 (0%)
        Strand=Plus/Minus
Query 1  TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCCAAGGATTTCAAGGAATAATAGTGA 60
        |||
Sbjct 151  TCGAGAAGGTATATTGCTGTTGACAGTGAGCGCCCAAGGATTTCAAGGAATAATAGTGA 92

Query 61  AGCCACAGATGTATTTATTCCTTGAAATCCTTGGGTTGCCTACTGCCTCGG 110
        |||
Sbjct 91  AGCCACAGATGTATTTATTCCTTGAAATCCTTGGGTTGCCTACTGCCTCGG 42

```

Figure 5-1. BLAST alignment of the sequenced bacterial plasmids with the corresponding 110 base pair shRNA sequence. 100% identity was found between the correct sequence and the observed sequence for each. A) GATA1 version 1 shRNA. B) GATA1 version 2 shRNA. C) GATA2 version 2 shRNA. D) BRG1 version 2 shRNA.

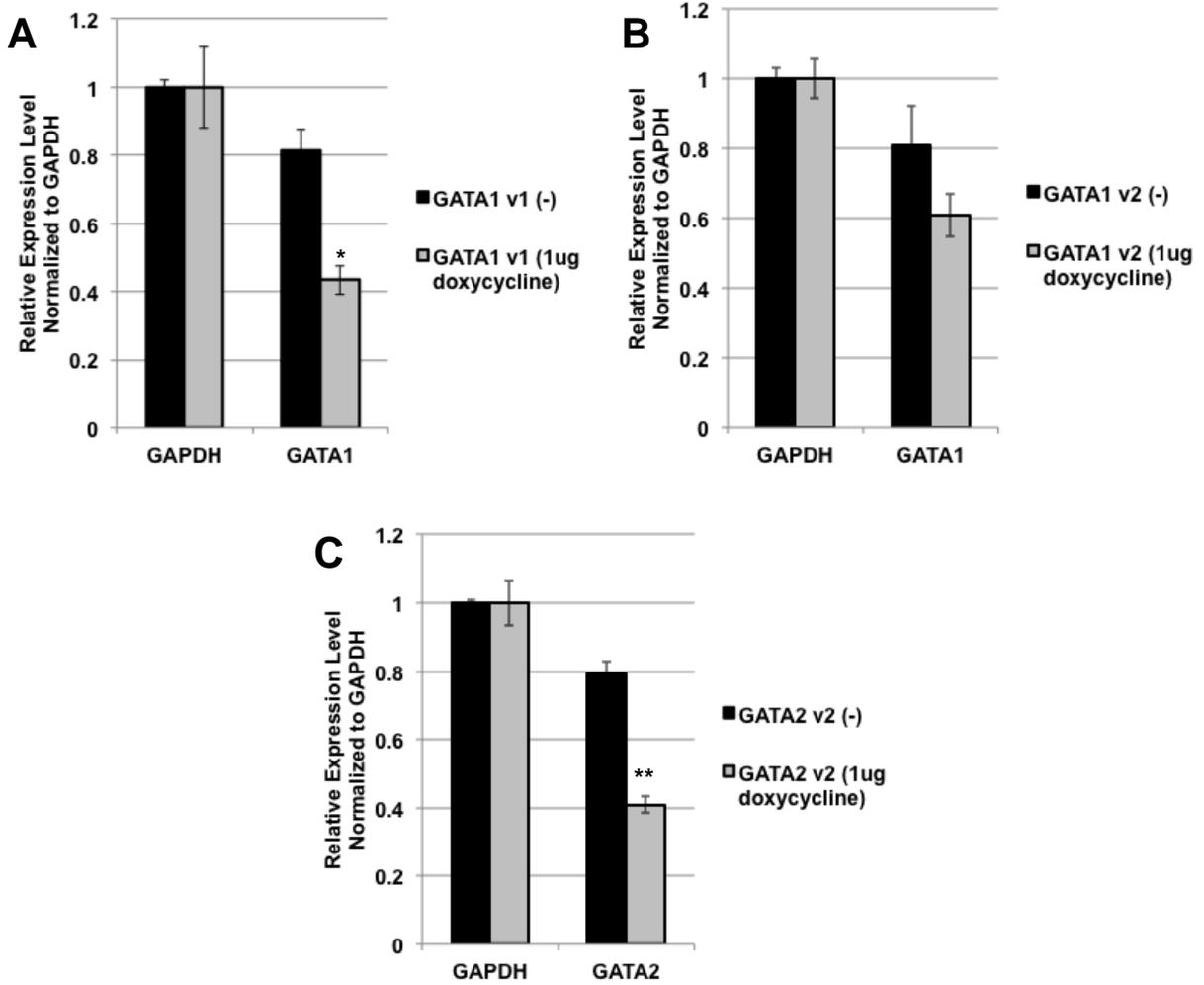


Figure 5-2. RT-qPCR expression analysis results for populations of shRNA-transfected K562 cells. Results are depicted as means \pm standard deviations for one experiment and are normalized to GAPDH levels. The black bars represent expression levels in transfected cells untreated with doxycycline, which should not express the shRNA. The gray bars indicate expression levels in transfected cells induced to express the shRNA through treatment with 1 microgram doxycycline for three days. Significance was analyzed using a two-tailed Student's *t*-test. A) GATA1 version 1 shRNA (* p <0.001). B) GATA1 version 2 shRNA. C) GATA2 version 2 shRNA (** p <0.0001).

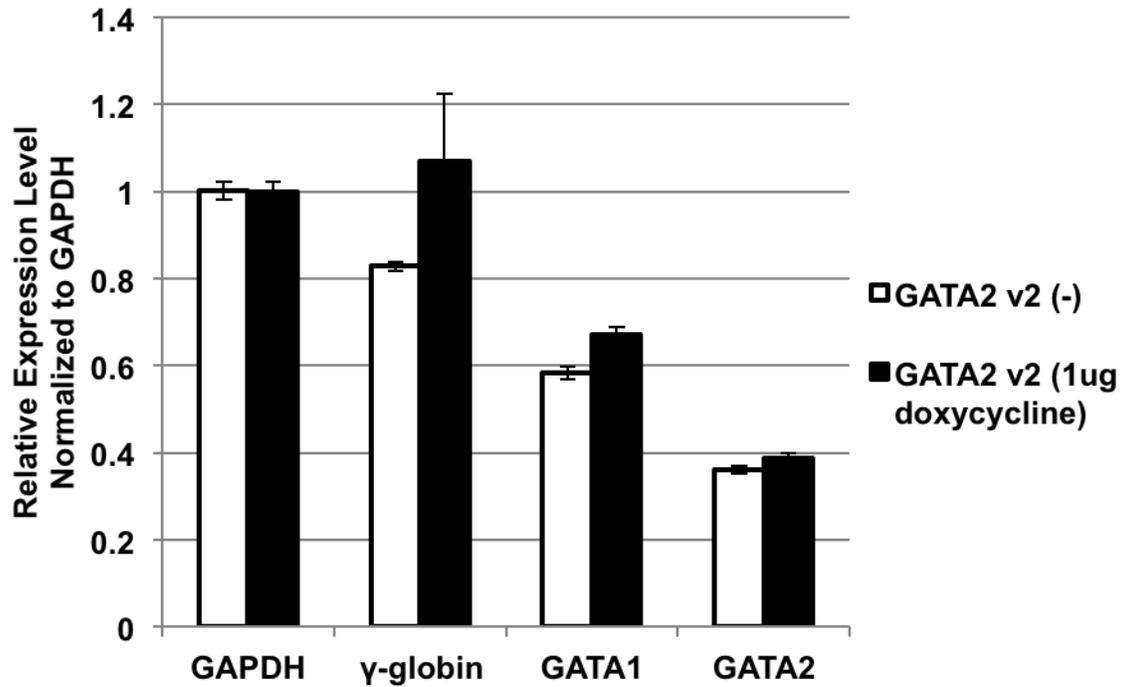


Figure 5-3. RT-qPCR analysis of expression levels of gamma-globin, GATA1, and GATA2 in GATA2 version 2 shRNA-transfected cells after recovery from thawing. Shown here are the means \pm standard deviations of one experiment. Results were normalized to the levels of GAPDH. The black bars represent expression levels in transfected cells untreated with doxycycline, which should not express the shRNA. The gray bars indicate expression levels in transfected cells induced to express the shRNA through treatment with 1 microgram doxycycline for three days.

CHAPTER 6 TRANSIENT KNOCKDOWN OF GATA2 THROUGH SMALL INTERFERING RNA

Introduction

In order to circumvent the problems encountered with stable transfections of small hairpin RNA (shRNA)-expressing vectors, transfections were performed using small interfering RNA (siRNA). This technique would allow analysis of knockdown levels as soon as 24 hours after transfections, minimizing the potential adverse, unintentional effects of knocking down important transcription factors. The goal of these experiments was to determine the effect of reducing the levels of GATA2 on the occupancy of BRG1 at hypersensitive site 2 (HS2) of the locus control region (LCR). If GATA2 does recruit BRG1 to this region, knocking down GATA2 through siRNA should reduce the levels of BRG1 binding at HS2.

Results

Transfections were performed using siRNAs acquired from Dharmacon. An siRNA against lamin A/C was used as a positive control to evaluate the effectiveness of the transfection technique. A non-targeting siRNA was used as a negative control to discern any effects on gene expression caused solely by the transfection procedure. A pool of GATA2 siRNAs was used as the experimental condition.

First, transfection conditions were optimized using the positive control siRNA. RT-qPCR analysis was performed 48 hours after transfection with lamin siRNA at a 25 micromolar concentration. The conditions evaluated included two concentrations of cells (2×10^5 and 4×10^5 cells per milliliter of media) and three concentrations of DharmaFECT 1 reagent (1, 2, or 3 microliters of DharmaFECT per milliliter of media). Figure 6-1 shows that optimal conditions for transfection were found at a cell density of 2×10^5 cells

per milliliter of media and a DharmaFECT concentration of 2 microliters DharmaFECT per milliliter of media.

Once transfection conditions were optimized, GATA2 siRNA was used to transfect K562 cells. RNA was extracted 72 hours after transfection and GATA2 expression levels were analyzed using RT-qPCR. Results are displayed in Figure 6-2. The results of three independent experiments indicated a statistically significant knockdown in the GATA2 siRNA-transfected cells when GATA2 expression levels were compared to those in non-targeting siRNA-transfected cells or to untreated cells.

Discussion

Use of siRNA to knock down GATA2 appears promising. Preliminary optimization experiments to knock down lamin using a positive control siRNA were successful, and lamin knockdown levels appeared consistent between experiments. Encouragingly, small-scale transfections of GATA2 siRNA into K562 cells resulted in a statistically significant reduction of GATA2 messenger RNA (mRNA) levels. It has yet to be determined if the ~40% reduction in the levels of GATA2 mRNA in these siRNA-transfected cells will translate to any changes in GATA2 or BRG1 binding at HS2 in chromatin immunoprecipitation (ChIP) experiments. More work remains to be done before GATA2 can be knocked down on the scale required for ChIP experiments.

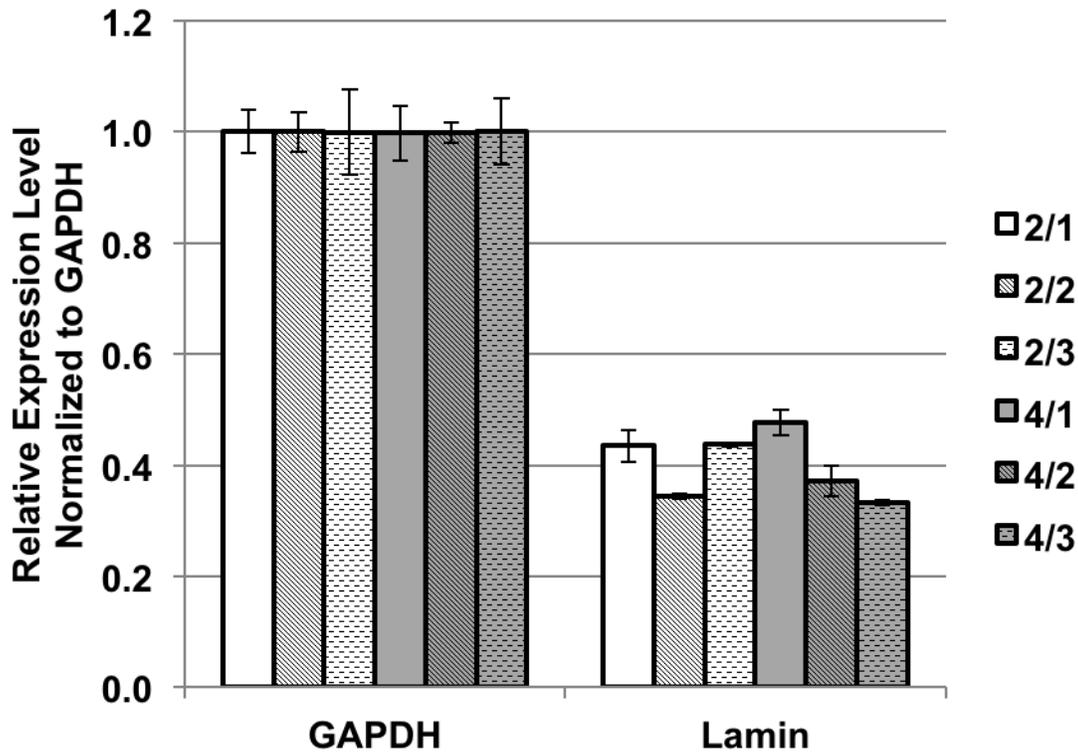


Figure 6-1. RT-qPCR analysis of K562 cells 48 hours after transfection with siRNA against lamin a/c. Expression levels shown are the means \pm standard deviations of one experiment, with the qPCR performed in triplicate. Expression levels were normalized against levels of GAPDH. In the figure legend, the first number refers to the concentration of cells used. The number 2 indicates a concentration of 2×10^5 cells per milliliter of media, while the number 4 indicates a concentration of 4×10^5 cells per milliliter of media. The second number, located after the forward slash, shows the amount of DharmaFECT 1 reagent used in each transfection. The number 1 indicates that 1 microliter of DharmaFECT was used per milliliter of media. Similarly, the number 2 indicates that 2 microliters DharmaFECT was used, and the number 3 indicates that 3 microliters DharmaFECT was used. The optimal conditions were found when the concentration of cells was 2×10^5 cells per milliliter of media and the concentration of DharmaFECT 1 reagent was 2 microliters DharmaFECT per milliliter of media.

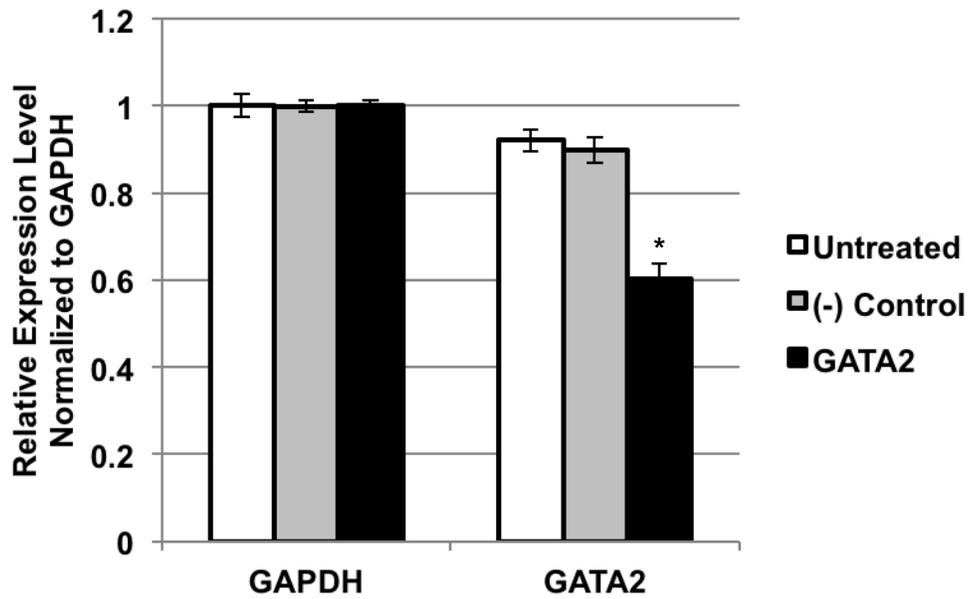


Figure 6-2. RT-qPCR analysis of K562 cells 72 hours after transfection with non-targeting (negative control) or GATA2 siRNA. Results are shown as means \pm standard errors of the means of three independent experiments and are normalized to the levels of GAPDH. The asterisk indicates a statistically significant difference between the expression levels of GATA2 in GATA2 siRNA-transfected cells (black) and GATA2 expression levels in both negative control siRNA transfected cells (gray) and untreated cells (white) ($p < 0.0001$).

CHAPTER 7 CONCLUSIONS AND FUTURE DIRECTIONS

The overall hypothesis presented in this thesis is that the transcription factor GATA2 recruits BRG1, the catalytic subunit of the SWI/SNF chromatin remodeling complex, to hypersensitive site 2 (HS2) of the beta-globin locus control region (LCR) during early erythropoiesis. Preliminary chromatin immunoprecipitation (ChIP) experiments in K562 cells reveal that BRG1 and GATA2 can be found to co-occupy HS2, although BRG1 levels were relatively low. However, low levels of BRG1 binding should be expected due to the indirect nature of its binding interactions with HS2. Future ChIP experiments should include the use of a supplemental crosslinker, such as EGS, to pull down a greater proportion of the BRG1 bound to HS2. Additionally, the use of a negative control DNA region, such as the linker DNA between HS2 and HS3 in the LCR, would be advisable. This type of negative control would provide additional evidence of the validity of the binding of BRG1, GATA2, and RNA polymerase II (RNA pol II) observed at HS2. Still, these ChIP experiments provide the minimum support necessary to merit further exploration of the hypothesis.

Co-immunoprecipitation experiments have been inconclusive so far. The detection of pull-down antibodies in the blot has proven to be a truly challenging problem to circumvent. Many different approaches to fix the problem, including crosslinking the antibodies to protein A sepharose beads, using special detection reagents, or pulling down protein complexes with a cross-species antibody, failed to yield successful results. Recently, attempts at co-immunoprecipitation experiments using a protocol obtained from the lab of Dr. John Strouboulis have showed promising

results. Future efforts on co-immunoprecipitation experiments will involve optimization of this protocol to attempt to detect an interaction between GATA2 and BRG1, if present.

Knockdown of GATA2 through expression of small hairpin RNA (shRNA) in K562 cells after transfection of an shRNA-expressing vector produced encouraging results at the population level. However, the reproducibility of these results is questionable, since all cells were used in the process of generating single-cell clones before replicate experiments were performed. The extreme variations in knockdown levels after doxycycline induction in the single-cells clones suggests that the reproducibility on the population level may also have been questionable. Still, future researchers would be wise to at least attempt to perform experiments on the population level, as cells appeared to be much more stable and healthy at this point and knockdown levels were still around 50%. It is possible that the variegated expression between cells on the population level would diminish the possibility of viewing a significant difference in factor binding at HS2 through ChIP. This effect might even be large enough to obscure any differences in binding when considering GATA2, which should have its binding directly affected by its own knockdown. Only experimentation will be able to put these questions to rest. Given the lack of success seen in experiments performed on the single-cell clone level and the tremendous amount of time required to get to this stage, it would certainly be worth the time of a future researcher to attempt the ChIP experiment at the population level.

Regardless of what stage the transfected cells are in, care should be taken to use only media containing tetracycline-free fetal bovine serum (FBS). Leaky expression of the shRNA through trace amounts of doxycycline contained in the media, although

not confirmed, can only diminish the results of any experiments performed and damage the life of the transfected cells. Experiments should also be done as quickly as possible once cells are through the selection phase. Delaying experiments prolongs the amount of time cells must be maintained in culture and increases the risk of cell death due to contamination or old age.

Transient transfections using small interfering RNA (siRNA) were far faster and simpler than stable transfections using shRNA. Preliminary experiments knocking down lamin using a positive control siRNA were extremely successful and consistent, as were transfections of a GATA2 siRNA to knock down GATA2 in small-scale experiments. Future experiments will include analysis of the expression of gamma-globin (the beta-globin family gene expressed in K562 cells) and GATA1 in GATA2-knockdown cells. Transfected cells will also be used for ChIP experiments to examine binding of GATA2 and BRG1 to HS2. The results of these experiments, in conjunction with the potential results from co-immunoprecipitation experiments, should finally put to rest the question posed in the hypothesis of this thesis.

APPENDIX A
PROTOCOL FOR SHRNA DESIGN AND TRANSFECTIONS

shRNA Design

- **Purpose:** Design shRNAs against Gata2, Brg1, and Gata1.
1. Using ensembl.org, I searched Human for Gata2, Brg1, and Gata1.
 2. I then clicked Transcript>Human, followed by the first transcript result that popped up.
 3. Where it said, “This transcript is a product of gene _____,” I clicked the ID number to view all transcripts.
 - a. **Gata2:** six transcripts; I chose CCDS3049, Gata2-001 (1443 nt)
 - b. **Gata1:** three transcripts; I chose CCDS14305, Gata1-001 (1242 nt)
 - c. **Brg1 (SMARCA4):** nine transcripts; I chose CCDS12253, SMARCA4-001 (4944 nt)
 4. I copied these transcripts into a word document for storage. I then inserted each sequence into the Dharmacon siRNA design center (<http://www.dharmacon.com/designcenter/designcenterpage.aspx>). I entered the sequences in FASTA format. In all cases, I chose the first and second results generated so that I would have two shRNAs to work with.
 5. To generate a 22mer sequence, I used the RNA codex tool (<http://cancan.cshl.edu/cgi-bin/Codex/Tools.cgi>). I inserted AC before the 19mer sequence and A after to create the 22mer sequence. After pressing submit, I grabbed the two 22mer sequences highlighted in red. These should be optimized for shRNA. I inserted both into the hairpin sequences from Jared/the Huang lab. In both the forward and reverse cases, I inserted the sense sequence first and the antisense second. Since they are reverse complements, this allows them to pair with each other.
 6. After generating the full hairpin sequences, I used the IDT DNA analyzer (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>) to check their ability to form hairpins.
 7. I also blasted each sequence to its respective genome to make sure that the only perfect match was in the target gene.

Anneal oligos

- **Purpose:** My oligos are all single-stranded, so I have to anneal each to its complement in order to ligate them into a double-stranded vector.
1. I received the technical datasheet from my oligos. It gives a column that reads “uL for 100uM.” Halve that number of uL to find out the volume of nuclease-free water necessary to resuspend them at 200uM.
 2. Use a 30uL reaction to anneal the oligos. Most reagents are found in the enzyme box.
 - 8uL Nuclease-free H₂O
 - 7.5uL Oligo (use both top and bottom oligo, so 15uL total, each at 50uM)
 - 3uL 10x PNK Buffer
 - 2uL 10mM ATP
 - 2uL T4 Polynucleotide Kinase
 3. Incubate at **37°C** for *1.5 hours*.
 4. Add 4uL of 0.5M NaCl and incubate the solution at **95°C** for *5 minutes* in a water bath.
 5. Turn the water bath off and let the solution cool slowly to room temperature, or until a minimum of 30C.
 6. Store at -20C.

Check integrity of double-stranded oligos

1. Prepare a 500nM solution of each single-stranded oligo for a control.
 - a. This is a 1:400 dilution from the 200mM stock.
2. Prepare a 500nM solution of each double-stranded oligo from the annealing reaction.
 - a. This is a 1:100 dilution from the annealing reaction
3. Add 2uL 6x dye and 5uL of each prepared solution. Run these samples on a 4% agarose gel.
 - a. The double-stranded oligo should run slower than the single-stranded oligo. There should also be no contamination of single-stranded oligo in the double-stranded oligo lane.

Digest vector

- **Purpose:** Perform a double digestion of the TripZ vector to prepare it for shRNA ligation.
1. Set up a 40uL reaction:
 - a. 2uL TripZ vector (concentration=1.7ug/uL, so 3.4ug total)
 - b. 2uL EcoRI
 - c. 2uL XhoI
 - d. 4uL Buffer 4
 - e. 4uL 10x BSA
 - f. 26uL Nuclease-free water
 2. Incubate at **37°C** for *2 hours*.
 - g. EcoRI has star activity, meaning that it can cleave the 4bp core of its 6bp cleavage sequence. Incubations longer than 2 hours aren't good for this enzyme.
 3. Add 1uL calf intestine phosphatase, or CIP. Incubate for *30 minutes* at **37°C**.
 - h. CIP removes phosphate groups from the ends of your vector so that it cannot self-ligate.
 4. Heat-inactivate CIP by incubating at **65°C** for 30 minutes.
 5. Run digest on a gel. Excise the correct band and purify with the Qiagen gel extraction kit to remove the small digested fragment.

Ligate oligos to vector

- **Purpose:** Ligate double-stranded shRNA into vector.

1. Make ligation mastermix:

Total	Single	Item
81uL	5.5uL	Nuclease-free water
13.5uL	1.5uL	Ligase buffer
9uL	1uL	T4 Ligase
18uL	2uL	Digested vector (want 50-100ng vector)

2. Aliquot 10uL of master mix into 8 tubes. Label tubes appropriately.
3. Add 5uL of each double-stranded oligo into the appropriate tube.
4. Incubate the ligation reaction *overnight* at **16°C**.

Transform bacteria

1. Thaw competent cells for *5-10 minutes* on ice. Use a 50uL aliquot for each tube.
2. Take ligation mixture out of incubator, flash spin down, then pipet up and down to mix.
3. Add 2uL of vector (from the ligation reaction) to each tube of 50uL competent cells. Pipet just on the surface, then flick carefully a few times.
 - a. Positive control: add 1uL of standard TripZ vector
4. Incubate on ice for *30 minutes*.
5. Heat shock cells at **42°C** for *30 seconds*.
6. Incubate on ice for *3-5 minutes* to allow bacteria to relax.
7. Add 450uL SOC or LB (use flame) and incubate for *90 minutes* at **30°C** while shaking at 225rpm.
8. Pellet cells at 13,200rpm for 1min. Remove most of the supernatant, leaving 30-40uL of media. Resuspend with pipet.
9. Plate all 50uL of cells on LP AMP plates.
 - a. If colonies grow, then that means transformation was successful.

Miniprep Protocol

1. **Grow bacteria:** Pick colonies. Incubate *overnight* at **30°C** while shaking in 3ml LB with ampicillin (100mg/ml stock, use 1ul amp/ml LB).
2. **Pellet bacteria:** Pour culture into two microcentrifuge tubes (1.5ml into each tube). Pellet bacteria by centrifuging for 2 minutes at 8000rcf.
3. **Lyse bacteria:** Remove supernatant. Using your pipet, resuspend pellet in 100ul cold GTE buffer with lysozyme (0.25mg in 5ml). Incubate on ice *5 minutes*.
4. **Denature proteins and chromosomal DNA:** Add 200ul buffer P2 (make fresh). Mix by inversion five times. Incubate on ice for *5 minutes*.
 - a. For steps 4 and 5, try not to extend incubation time past 5 minutes!

To make 1ml Buffer P2:

930ul H₂O
20ul 10M NaOH
50ul 20% SDS

5. **Precipitate proteins and chromosomal DNA:** Add 150ul 5M potassium acetate. Invert and tap to mix. Incubate on ice for *5 minutes*.
6. **Pellet proteins and chromosomal DNA:** Centrifuge *5 minutes* at max speed.
7. **Precipitate plasmid DNA and RNA:** Transfer 400ul of supernatant to a new tube. Add 1000ul 100% ethanol (or 2.5x volume). Incubate at RT for *5 minutes*, or in freezer *overnight*.
 - a. A longer incubation will allow for increased precipitation of your plasmid DNA.
 - b. If any of the white stuff gets taken in while pipetting, do a phenol-chloroform-isoamyl alcohol precipitation to improve purity.
8. **Pellet plasmid DNA and RNA:** Centrifuge for *15 minutes* at max speed at **4°C**.
9. **Wash:** Remove supernatant and wash with 300ul 70% ethanol. Do not resuspend. Centrifuge for *10 minutes* at max speed at **4°C**.
10. **Eliminate remaining ethanol:** Remove supernatant by tilting tube sideways and aspirating ethanol as it flows down. Try to dry out as much ethanol as possible without aspirating your pellet. Let stand around *10 minutes*.
11. **Degrade RNA:** Resuspend in 30ul TE pH 8.0 plus 0.5ul RNAse per sample. Incubate at **37°C** for *30 minutes*.
12. Quantitate, and store at -20°C.

LIST OF REFERENCES

1. *Molecular cell biology*. (W.H. Freeman and Co, 2013).
2. Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F. & Richmond, T. J. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389**, 251–260 (1997).
3. Kornberg, R. D. & Lorch, Y. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98**, 285–294 (1999).
4. Peterson, C. L. & Laniel, M.-A. Histones and histone modifications. *Current Biology* **14**, R546–R551 (2004).
5. Ausio, J., Dong, F. & Van Holde, K. E. Use of selectively trypsinized nucleosome core particles to analyze the role of the histone ‘tails’ in the stabilization of the nucleosome. *J. Mol. Biol.* **206**, 451–463 (1989).
6. Hansen, J. C. Conformational Dynamics of the Chromatin Fiber in Solution: Determinants, Mechanisms, and Functions. *Annual Review of Biophysics and Biomolecular Structure* **31**, 361–392 (2002).
7. Jenuwein, T. & Allis, C. D. Translating the histone code. *Science* **293**, 1074–1080 (2001).
8. Peterson, C. L. & Workman, J. L. Promoter targeting and chromatin remodeling by the SWI/SNF complex. *Curr. Opin. Genet. Dev.* **10**, 187–192 (2000).
9. Euskirchen, G., Auerbach, R. K. & Snyder, M. SWI/SNF chromatin-remodeling factors: multiscale analyses and diverse functions. *J. Biol. Chem.* **287**, 30897–30905 (2012).
10. Tang, L., Nogales, E. & Ciferri, C. Structure and function of SWI/SNF chromatin remodeling complexes and mechanistic implications for transcription. *Prog. Biophys. Mol. Biol.* **102**, 122–128 (2010).
11. Liu, N., Balliano, A. & Hayes, J. J. Mechanism(s) of SWI/SNF-induced nucleosome mobilization. *Chembiochem* **12**, 196–204 (2011).
12. Trotter, K. W. & Archer, T. K. The BRG1 transcriptional coregulator. *Nucl Recept Signal* **6**, e004 (2008).
13. Bultman, S. J., Gebuhr, T. C. & Magnuson, T. A Brg1 mutation that uncouples ATPase activity from chromatin remodeling reveals an essential role for SWI/SNF-related complexes in beta-globin expression and erythroid development. *Genes Dev.* **19**, 2849–2861 (2005).

14. Griffin, C. T., Brennan, J. & Magnuson, T. The chromatin-remodeling enzyme BRG1 plays an essential role in primitive erythropoiesis and vascular development. *Development* **135**, 493–500 (2008).
15. Kim, S.-I., Bultman, S. J., Kiefer, C. M., Dean, A. & Bresnick, E. H. BRG1 requirement for long-range interaction of a locus control region with a downstream promoter. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 2259–2264 (2009).
16. Stees, J., Varn, F., Huang, S., Strouboulis, J. & Bungert, J. Recruitment of Transcription Complexes to Enhancers and the Role of Enhancer Transcription. *Biology* **1**, 778–793 (2012).
17. Juven-Gershon, T. & Kadonaga, J. T. Regulation of gene expression via the core promoter and the basal transcriptional machinery. *Developmental Biology* **339**, 225–229 (2010).
18. Dunham, I. *et al.* An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57–74 (2012).
19. Bulger, M. & Groudine, M. Functional and Mechanistic Diversity of Distal Transcription Enhancers. *Cell* **144**, 327–339 (2011).
20. *The Molecular basis of blood diseases.* (W.B. Saunders, 1994).
21. Higgs, D. R. Do LCRs open chromatin domains? *Cell* **95**, 299–302 (1998).
22. Vernimmen, D., De Gobbi, M., Sloane-Stanley, J. A., Wood, W. G. & Higgs, D. R. Long-range chromosomal interactions regulate the timing of the transition between poised and active gene expression. *EMBO J.* **26**, 2041–2051 (2007).
23. Tsiftoglou, A. S., Vizirianakis, I. S. & Strouboulis, J. Erythropoiesis: Model systems, molecular regulators, and developmental programs. *IUBMB Life* **61**, 800–830 (2009).
24. Levings, P. P. & Bungert, J. The human beta-globin locus control region. *Eur. J. Biochem.* **269**, 1589–1599 (2002).
25. Liang, S. Y. *et al.* Defective erythropoiesis in transgenic mice expressing dominant-negative upstream stimulatory factor. *Mol. Cell. Biol.* **29**, 5900–5910 (2009).
26. Corre, S. & Galibert, M.-D. Upstream stimulating factors: highly versatile stress-responsive transcription factors. *Pigment Cell Res.* **18**, 337–348 (2005).
27. Sirito, M. *et al.* Members of the USF family of helix-loop-helix proteins bind DNA as homo- as well as heterodimers. *Gene Expr.* **2**, 231–240 (1992).

28. Crusselle-Davis, V. J. *et al.* Recruitment of coregulator complexes to the beta-globin gene locus by TFII-I and upstream stimulatory factor. *FEBS J.* **274**, 6065–6073 (2007).
29. Crusselle-Davis, V. J., Vieira, K. F., Zhou, Z., Anantharaman, A. & Bungert, J. Antagonistic regulation of beta-globin gene expression by helix-loop-helix proteins USF and TFII-I. *Mol. Cell. Biol.* **26**, 6832–6843 (2006).
30. Liang, S. Y. An investigation into the role of USF in beta-globin regulation. (2010).at
<<http://search.proquest.com/docview/822234848/abstract/13C7D79D5CAF266992/1?accountid=10920>>
32. Bresnick, E. H., Katsumura, K. R., Lee, H.-Y., Johnson, K. D. & Perkins, A. S. Master regulatory GATA transcription factors: mechanistic principles and emerging links to hematologic malignancies. *Nucleic Acids Res.* **40**, 5819–5831 (2012).
33. Merryweather-Clarke, A. T. *et al.* Global gene expression analysis of human erythroid progenitors. *Blood* **117**, e96–108 (2011).
34. Tsai, F. Y. & Orkin, S. H. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood* **89**, 3636–3643 (1997).
35. Tsai, F. Y. *et al.* An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221–226 (1994).
36. Leach, K. M. *et al.* Characterization of the human beta-globin downstream promoter region. *Nucleic Acids Res.* **31**, 1292–1301 (2003).
37. Yoo, J., Herman, L. E., Li, C., Krantz, S. B. & Tuan, D. Dynamic changes in the locus control region of erythroid progenitor cells demonstrated by polymerase chain reaction. *Blood* **87**, 2558–2567 (1996).
38. Grass, J. A. *et al.* Distinct Functions of Dispersed GATA Factor Complexes at an Endogenous Gene Locus. *Molecular and Cellular Biology* **26**, 7056–7067 (2006).

BIOGRAPHICAL SKETCH

Stephanie Noel Morton began her studies at the United States Military Academy (USMA) majoring in life science with the intent to become a physician. After completing her second year, Stephanie transferred to the University of Florida (UF), where she majored in biology. In the summer of 2011, she started a supplemental instruction (SI) tutoring program for the undergraduate biochemistry course coordinated by Dr. Phillip Laipis. The success of the SI program prompted Dr. Laipis to request that Stephanie remain at UF as a master's student in the Department of Biochemistry and Molecular Biology to grow and stabilize the new program. In August 2011, Stephanie graduated *cum laude* from UF with her bachelor's degree in biology and immediately transitioned into the master's program. During her time as a master's student, Stephanie established a multifaceted tutoring program that includes group study sessions, private tutoring, walk-in tutoring, and supplemental videos. In the six semesters during which she ran the program, over 1,300 students utilized the group study sessions alone. She oversaw the development of 48 group leaders and 57 other tutors. Upon graduating, Stephanie will enter medical school at the Geisel School of Medicine at Dartmouth. She would like to pursue a career in academia, splitting her time between clinical practice and medical education.