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Generation and Characterization of a Knock-In Allele of EKLF: Probing the in vivo Role of the Chromatin Remodeling Domain in Definitive Hematopoietic Cells

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Abstract

The zinc finger-encoding transacting factor EKLF, or erythroid Krüppel-like factor, binds key regulatory elements of many erythroid-specific genes, and is essential for definitive erythropoiesis. Mice lacking this factor die of anemia by E15.5 of gestation, failing to activate β-globin gene transcription, and demonstrating a block in the erythroid differentiation program at the primitive erythroblast stage. In contrast, megakaryocytic progenitors are amplified in EKLF-null embryos, with increased Fli-1 gene expression, a marker of early megakaryocytic differentiation. These observations are consistent with the idea that EKLF modulates the megakaryocytic-erythroid (M-E) differentiation switch.

Our laboratory has previously demonstrated that an amino terminal sequence of EKLF (D221EKLF) is required to induce chromatin remodeling at the β-globin promoter in an EKLF-null erythroid cell line. However, additional amino terminal sequences are required for initiation of β-globin gene transcription. To evaluate the role of this chromatin remodeling domain in erythroid and megakaryocytic differentiation *in vivo*, I have generated a knock-in allele of D221EKLF. Using the recombineering method, a lambda phage-based homolgous recombination method in *E. coli,* cDNA encoding theD221EKLF domain has been inserted into the endogenous initiation site, thus placing the mutant protein under the *cis*-regulatory elements of the endogenous murine EKLF locus. Subsequently, D221EKLF alleles have been generated by gene targeting in ES cells. I have used the mice to probe the *in vivo* role of D221EKLF in definitive hematopoietic cells.

Similar to EKLF-null embryos, mice homozygous for the D221EKLF mutant allele die of anemia by E15.5 of gestation. Molecular analysis ofD221EKLF erythroblasts reveals i) a failure to activate β-globin gene transcription; ii) lack of GATA-1 and NF-E2 recruitment to the β-globin promoter; iii) a block in terminal erythroid differentiation. In contrast to erythroid cells lacking EKLF, D221EKLF erythroid progenitors demonstrate appropriate binding of the D221EKLF encoding domain to all EKLF-regulatory sequences and a chromatin architecture and histone modification pattern at erythroid-specific genes that recapitulate the events observed in wild-type EKLF erythroblasts at a similar stage of erythroid ontogeny.

Examining the role of D221EKLF in megakaryopoiesis, I observed inhibition of megakaryocytic progenitor expansion in D221EKLF fetal hematopoietic cell populations when compared to EKLF-null embryos. Molecular analysis of D221EKLF erythroblasts reveals i) binding of theD221EKLF mutant protein to the Fli-1 promoter with inhibition of gene transcription; ii) hypoacetylation of histone H3 at the Fli-1 promoter; iii) recruitment of a Sin3A-containing corepressor complex to the Fli-1 promoter. Taken together, my results suggest strongly that the unique D221EKLF domain is sufficient to modulate the chromatin-specific roles of EKLF at erythroid- and megakaryocytic-specific loci in definitive hematopoietic cells *in vivo*.

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GENERATION AND CHARACTERIZATION OF A KNOCK-IN ALLELE OF EKLF: PROBING THE *IN VIVO* ROLE OF THE CHROMATIN REMODELING DOMAIN IN DEFINITIVE HEMATOPOIETIC CELLS

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By
Valerie Malyvanh Jansen
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DEDICATION

This dissertation is dedicated in the loving memory of Laura Jeanne Jansen who saw me begin this journey and is watching from above as I finish.

To my husband, Tim, for his unwavering love and support throughout this journey.

To my precious daughter, Hannah, the absolute best result during this journey.

The journey is the reward.
Chinese Proverb

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for leaving and not going to sleep." I am forever grateful and blessed to have my father-in-law, Rick Jansen, who was always ready and willing to help with Hannah. I am thankful to my sister, Phoukhaothong Vichidvongsa, for her encouragement and support as I finished. Finally, I am thankful to my parents for instilling in me the importance of education, for their hard work and struggles to provide me with the best opportunities in life, and for their unconditional love and support.

ABSTRACT

The zinc finger-encoding transacting factor EKLF, or erythroid Krüppel-like factor, binds key regulatory elements of many erythroid-specific genes, and is essential for definitive erythropoiesis. Mice lacking this factor die of anemia by E15.5 of gestation, failing to activate β-globin gene transcription, and demonstrating a block in the erythroid differentiation program at the primitive erythroblast stage. In contrast, megakaryocytic progenitors are amplified in EKLF-null embryos, with increased Fli-1 gene expression, a marker of early megakaryocytic differentiation. These observations are consistent with the idea that EKLF modulates the megakaryocytic-erythroid (M-E) differentiation switch.

Our laboratory has previously demonstrated that an amino terminal sequence of EKLF (Δ221EKLF) is required to induce chromatin remodeling at the β-globin promoter in an EKLF-null erythroid cell line. However, additional amino terminal sequences are required for initiation of β-globin gene transcription. To evaluate the role of this chromatin remodeling domain in erythroid and megakaryocytic differentiation *in vivo*, I have generated a knock-in allele of Δ221EKLF. Using the recombineering method, a lambda phage-based homolgous recombination method in *E. coli,* cDNA encoding the Δ221EKLF domain has been inserted into the endogenous initiation site, thus placing the mutant protein under the *cis*-regulatory elements of the endogenous murine EKLF locus. Subsequently, Δ221EKLF alleles have been generated by gene targeting in ES cells. I have used the mice to probe the *in vivo* role of Δ221EKLF in definitive hematopoietic cells.

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Examining the role of Δ221EKLF in megakaryopoiesis, I observed inhibition of megakaryocytic progenitor expansion in Δ221EKLF fetal hematopoietic cell populations when compared to EKLF-null embryos. Molecular analysis of Δ221EKLF erythroblasts reveals i) binding of the Δ221EKLF mutant protein to the Fli-1 promoter with inhibition of gene transcription; ii) hypoacetylation of histone H3 at the Fli-1 promoter; iii) recruitment of a Sin3A-containing corepressor complex to the Fli-1 promoter. Taken together, my results suggest strongly that the unique Δ221EKLF domain is sufficient to modulate the chromatin-specific roles of EKLF at erythroid- and megakaryocytic-specific loci in definitive hematopoietic cells *in vivo*.

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LIST OF ABBREVIATIONS

ACH	Active Chromatin Hub
AHSP	Alpha Hemoglobin Stabilizing Protein
ALAD	Aminolevulinate Dehydratase
ALAS2	Aminolevulinic Acid Synthase 2
AMP	Ampicillin
APC	Allophycocyanin
BAC	Bacteria Artificial Chromosome
BFU-E	Burst Forming Unit-Erythroid
BKLF	Basic Krüppel-like Factor
cDNA	Complementary DNA
CFU	Colony Forming Unit
CFU-E	CFU-Erythroid
CFU-GEMM	CFU-Granulocyte-Erythroid-Monocyte-Megakaryocyte
CFU-GM	CFU-Granulocyte-Macrophage
CFU-MK	CFU-Megakaryocyte
ChIP	Chromatin Immunoprecipitation
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
COOH	Carboxyl
CPOX	Coproporphyrinogen Oxidase
Da	Dalton
DAPI	4',6-Diamidino-2-Phenylindole
dsDNA	Double-stranded DNA
DT	Diptheria Toxin
EKLF	Erythroid Krüppel-like Factor
En-1, En-2	Engrailed
EPB4.9	Erythroid Protein Band 4.9
E-RC1	EKLF Coactivator-Remodeling Complex 1
ES	Embryonic Stem
FECH	Ferrochelatase
FITC	Fluorescein Isothiocyanate
FOG1	Friend of GATA-1
GAL4	Galactose 4
H&E	Hematoxylin and Eosin
HA	Hemagglutinin
HCT	Hematocrit
HGB	Hemoglobin
HS	Hypersensitive Site
HSC	Hematopoietic Stem Cell
IP	Immunoprecipitation
IVR	Intervening region
kDa	KiloDalton

KO	Knock-out
LB	Luria Broth
LCR	Locus Control Region
LMO2	LIM Domain Only 2
Lys	Lysine
MEL	Mouse Erythroleukemic
MEP	Megakaryocyte-Erythrocyte Progenitor
mRNA	Messenger RNA
Myf5	Myogenic factor 5
NEO	Neomycin
NF-E2	Nuclear Factor – Erythroid 2
NH2	Amino
NLS	Nuclear Localizing Signal
PAC	P1 Artificial Chromosome
PBGD	Porphobilinogen Deaminase
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PGK	Phosphoglycerate Kinase
PLT	Platelet Count
PMSF	Phenylmethylsulphonyl Fluoride
PolII	Polymerase II
PPOX	Protoporphyrinogen Oxidase
RBC	Red Blood Count
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCL	Stem Cell Leukemia
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error Mean
shRNA	Short Hairpin RNA
TF	Transcription Factor
TK	Thymidine Kinase
UROD	Uroporphyrinogen Decarboxylase
WBC	White Blood Count
WT	Wild Type

CHAPTER 1: INTRODUCTION

MOUSE HEMATOPOIESIS

Hematopoiesis is the formation and development of blood cells involving the differentiation of a multipotent progenitor, the hematopoietic stem cell (HSC), and its progeny into all blood cell lineages (Figure 1.1) (Orkin & Zon, 2008). This complex continuous process requires a broad spectrum of lineage-specific transcription factors (TFs), such as SCL, LMO2, c-myb, PU.1, EKLF, GATA-1, NF-E2, and Fli-1 (Shivdasani & Orkin, 1996; Perry & Soreq, 2002; Orkin & Zon, 2008). Genetic studies involving the disruption or over-expression of these genes have facilitated our understanding of the transcriptional regulation of hematopoiesis (Perry & Soreq, 2002). Many of these TFs are beyond the scope of my dissertation and will not be addressed. In the context of this dissertation, I will explore the role of erythroid-Krüppel-like factor, or EKLF, in β-globin gene regulation during erythroid differentiation (Chapter 3). Furthermore, I will demonstrate that the chromatin remodeling encoding domain of EKLF is sufficient to repress expansion of megakaryocytic progenitors observed with complete loss of EKLF (Chapter 4).

Overview of erythropoiesis

Erythropoiesis is the development of mature red blood cells from hematopoietic stem cells (Orkin & Zon, 2008). In mammals, this process occurs in the bone marrow and is characterized by three stages (Palis, 2009). The first stage involves production of lineage –committed progenitors. The earliest recognizable erythroid-specific progenitor is the burst-forming unit erythroid (BFU-E). The BFU-E generates more mature erythroid-committed progenitors termed colony-forming units erythroid (CFU-E) (Socolovsky *et al.*, 1998). BFU-E and CFU-E can be detected *in vitro* using colony-forming assays (Ogawa *et al.*, 2002). The second stage of erythroid differentiation consists of the progression of erythroid precursors from early proerythroblasts into orthochromatic erythroblasts. This stage of erythroid differentiation is characterized by the progressive accumulation of hemoglobin, expansion of erythroblasts, and progressive nuclear pyknosis and ultimately loss of the nucleus. The final stage of erythropoiesis involves maturation of the young red blood cells (reticulocytes) into mature circulating red cells (Palis, 2009).

Erythropoiesis occurs in distinct phases and anatomic sites during murine development (Dzierzak & Medvinsky, 1995; Zon, 1995; Shivdasani & Orkin, 1996; Palis, 2008). The first phase can be identified in the yolk sac at embryonic day 7.5 (E7.5) in mice and is referred to as primitive or embryonic erythropoiesis. Primitive erythrocytes are large cells that retain their nuclei and produce embryonic globin chains (ζ, ε/βh1). The adult β-globin genes are silent. By E11, definitive erythropoiesis is initiated in the fetal liver. At this stage, the adult globin (α, βmaj/βmin) genes are expressed and the embryonic globin genes are silenced. The molecular mechanisms regulating the

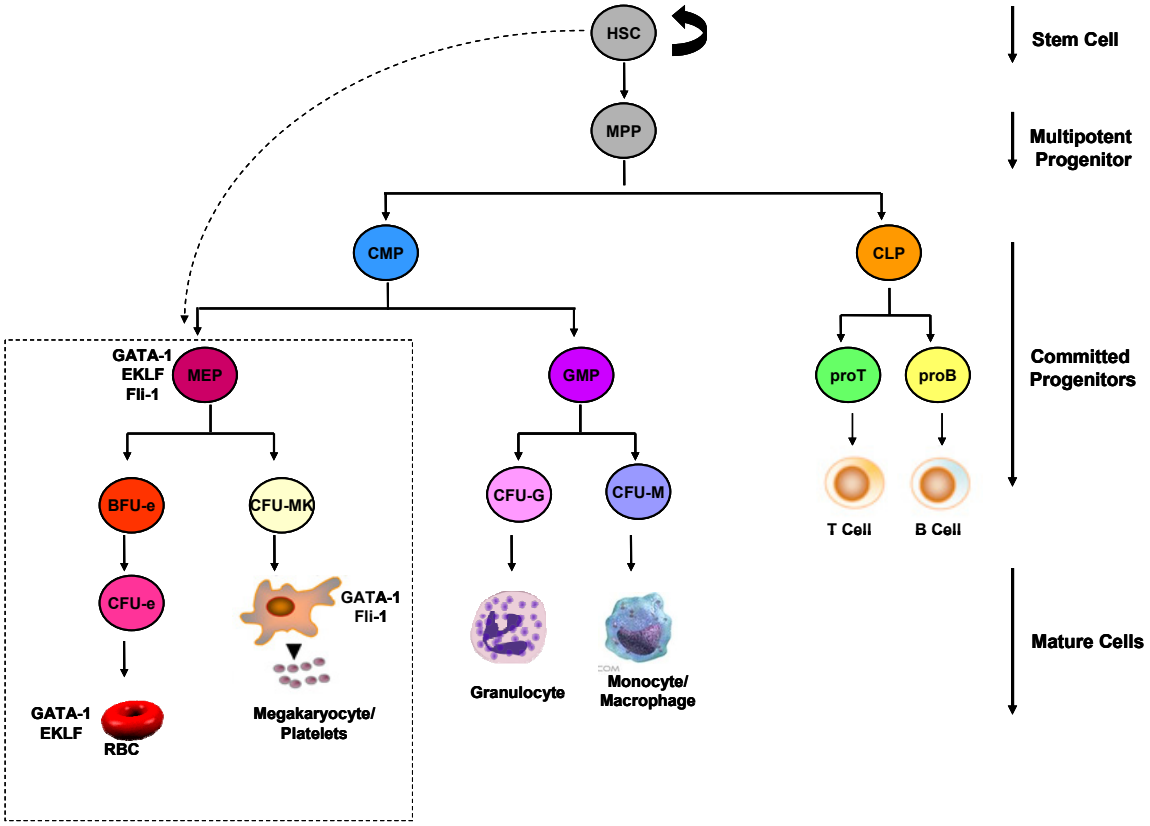


Figure 1.1. Overview of murine hematopoiesis. All blood cell types arise from the self-renewing hematopoietic stem cell (HSC) that differentiates into committed progenitor cells to produce mature blood cells. The erythroid and megakaryocytic lineage is thought to have come from a common bipotent progenitor (megakaryocytic-erythroid progenitor (MEP) (dotted box). The MEP population is thought to be formed from common myeloid progenitor (CMP); recent evidence proposes a direct pathway from HSCs to MEP (dotted line). Key transcription factors regulating this process are listed by the cell type in which they are expressed. Abbreviations: MPP, multipotent progenitor; CLP, common lymphoid progenitor; GMP, granulocyte and macrophage progenitor; CFU-G, colony-forming unit granulocyte; CFU-M, colony-forming unit monocyte/macrophage; BFU-E, blast-forming unit erythrocyte; CFU-E, colony-forming unit erythrocyte; MK-P, megakaryocyte progenitor.

switching of globin gene expression has been the intense focus of many laboratories. They have provided a critical foundation for our understanding of the molecular mechanism underpinning erythropoiesis.

Overview of megakaryopoiesis

The erythroid and megakaryocyte lineages are thought to be derived from a common precursor, the bipotent megakaryocyte-erythroid progenitor (MEP) (Debili *et al.*, 1996). In the most established pathway, HSCs generate common myeloid progenitor (CMP) cells from which the MEP is formed (Akashi *et al.*, 2000). However, it has been recently proposed that HSCs can give rise to MEP population without an intermediate progenitor (Adolfsson, 2005).

Megakaryopoiesis is the process by which HSCs differentiate into mature megakaryocytes through a series of differentiated progenitors. Megakaryocytic progenitors are detected in the yolk sac and fetal liver at approximately E7.5-10.5 and E11.5, respectively (Tober *et al.*, 2007). The earliest committed MK progenitor is the burst-forming unit megakaryocyte (BFU-MK) that gives rise to the more mature colony-forming unit megakaryocyte (CFU-MK) (Briddellet al., 1989). The CFU-MK then gives rise to megakaryoblasts that in turn differentiate into mature megakaryocytes.

Transcription factors in erythropoiesis and megakaryopoiesis

The precursor cells express many common hematopoietic transcription factors that are essential to both erythropoiesis and megakaryopoiesis, including GATA-1 (Pevny *et al.*, 1995; Shivdasani *et al.*, 1997), FOG1 (Tsang *et al.*, 1997), and SCL (Hall *et al.*, 2003). The MEP lineage differentiation is regulated in part by the differential expression and combinatorial action of these transcription factors.

GATA-1

GATA-1, the founding member of the GATA family of zinc finger proteins, is an erythroid transcription factor that binds to the DNA sequences WGATAR found at the *cis*-regulatory sequences of nearly all erythroid genes, including the β-globin gene (Martin & Orkin, 1990; Weiss & Orkin, 1995). GATA-1 is also expressed and has defined functional activities in megakaryocytes, eosinophils, and mast cells (Zon *et al.*, 1993). Hemizygous deletion of GATA-1, located on the X-chromosome, leads to loss of erythropoiesis and embryonic lethality by E11.5 (Fujiwara *et al.*, 1996). GATA-1 null mice also display a block in megakaryocyte development. However, GATA-1 null ES cells can develop into other hematopoietic lineages (Kitajima *et al.*, 2006). Similarly, forced expression of GATA-1 in an early myeloid cell line promotes megakaryocytic differentiation, suggesting that GATA-1 has a key role in lineage determination (Shivdasani & Orkin, 1996; Shivdasani *et al.*, 1997). Studies to identify proteins that

bind to GATA-1 led to the discovery of Friend of GATA (FOG-1), a nuclear zinc finger protein that binds the amino zinc finger of GATA-1 (Tsang *et al.*, 1997). Expression of FOG-1 is similar to that of GATA-1. Like mice lacking GATA-1, FOG-1 null mice do not form megakaryocytic progenitors and are embryonic lethal at E11.5 (Tsang *et al.*, 1998).

NF-E2

NF-E2 is a hematopoietic transcription factor belonging to the basic-leucine zipper family of dimeric proteins consisting of a ubiquitously expressed 18 kDa subunit and a tissue-specific 45 kDa subunit (Chan *et al.*, 1993). Initial studies in cell lines provided evidence that NF-E2 is essential for β-globin gene expression (Lu *et al.*, 1994). Surprisingly, mice lacking the 45 kD hematopoietic-restricted subunit develop only a mild erythroid phenotype, but exhibit severe thrombocytopenia with bone marrow showing excessive immature and dysplastic megakaryocytes (Shivdasani & Orkin, 1995). The subtle effects on erythroid maturation are presumably due to functional redundancy between NF-E2 and other basic leucine zipper family proteins (Sawado *et al.*, 2001). By contrast, the molecular basis for the severe defects in megakaryocyte differentiation and platelet release remains to be elucidated.

Fli-1

Fli-1 is a member of the Ets family of transcription factors (Watson *et al.*, 1992) containing a conserved winged helix-loop-helix DNA binding (ETS) domain which has critical functions in development and oncogenesis (Jackers *et al.*, 2004). Fli-1 is preferentially expressed in cells of the hematopoietic lineages and vascular endothelium. Fli-1 has been shown to transcriptionally activate many genes, including those involved in megakaryopoiesis. In undifferentiated hematopoietic cell lines, overexpression of Fli-1 can induce megakaryocytic features and inhibit erythroid differentiation (Pereira *et al.*, 1999; Athanasiou *et al.*, 2000). Moreover, Fli-1 knock-out mice either have abnormal megakaryocytes with associated thrombocytopenia (Hart *et al.*, 2000) or fail to develop recognizable megakaryocytes (Kawada *et al.*, 2001). These observations are consistent with the idea that Fli-1 is a key factor in the lineage fate decision leading to the production of megakaryocytes.

Interestingly, these factors are interconnected with my gene of interest, EKLF (reviewed in a separate section below). EKLF, GATA-1, and Fli-1 are all expressed in the MEP (Orkin & Zon, 2008). GATA-1 transcriptionally activates EKLF (Crossley *et al.*, 1994), while Fli-1 and EKLF interactions have been noted (Starck *et al.*, 2003). Moreover, recent data including that from our laboratory suggest that EKLF represses Fli-1 expression (J.M.C unpublished data; Frontelo *et al.*, 2007). Similarly, both EKLF and NF-E2 are essential for high level β-globin gene transcription (Asano & Stamatoyannopoulos, 1998; Sawado *et al.*, 2001). However, the relationship between EKLF and NF-E2 in megakaryopoiesis is relatively unknown. Although associations

between these transcription factors and many others are recognized in erythroid and megakaryocytic differentiation, the mechanisms underlying these relationships have yet to be resolved.

ERYTHROID KRÜPPEL-LIKE FACTOR

Discovery of EKLF in MEL cell line

The murine erythroleukemia (MEL) cell line has proven a popular and highly important murine model system to study erythroid-specific gene expression. These erythroid progenitor cells, immortalized by infection with Friend virus (Friend, 1957; Antoniou, 1991), are arrested at the proerythroblast stage of development. This cell can be maintained in tissue culture indefinitely (Friend, 1957; Antoniou, 1991). However, upon treatment with various chemical agents, MEL cells can be induced to undergo erythroid differentiation with the induction of globin and other erythroid genes involved in the terminal erythroid differentiation program (Marks & Rifkind, 1988; Radhika *et al.*, 1995). These cells have also proven to be useful to study megakaryocytic differentiation (Bouilloux *et al.*, 2008).

This transformed cell line is an ideal model to identify novel genes which may play a role in erythropoiesis. Using subtractive hybridization and enriching for genes expressed in a MEL cell line, but not in a murine monocyte-macrophage cell line, a novel erythroid cell-specific zinc finger protein was isolated (Miller & Bieker, 1993). Close inspection of the zinc finger region of this factor revealed that it is similar to the *Drosophila* pattern-determining gap gene *Krüppel*. Therefore, the protein was named EKLF/KLF1, for erythroid Krüppel-like factor (Miller & Bieker, 1993).

Molecular properties of EKLF

EKLF maps to a region on mouse chromosome 8 (Jenkins *et al.*, 1998) and human chromosome 19 (Bieker, 1996; van Ree *et al.*, 1997). The EKLF gene spans ~6.5 kb and contains 3 exons. There are two major transcripts produced, the results of alternative transcriptional start sites at nucleotides 41 and 55. This is not uncommon as multiple transcription start sites have been observed for other tissue-specific genes, namely the heterogeneous 5' ends of both *c-myb* and GATA-1 (Bender & Kuehl, 1986; Tsai *et al.*, 1989). Sequence analysis reveals open reading frames beginning at the in-frame methionines 1 and 19. Because methionine 1 does not match the Kozak consensus sequence, translation of the major product starts from methionine 19, encoding an EKLF protein of 358 amino acids (37,755 Da) (Miller & Bieker, 1993). The protein has a carboxy-terminal DNA-binding domain consisting of three C2H2 zinc fingers and a proline-rich amino domain that has structural similarities to other transactivation domains (Figure 1.2).

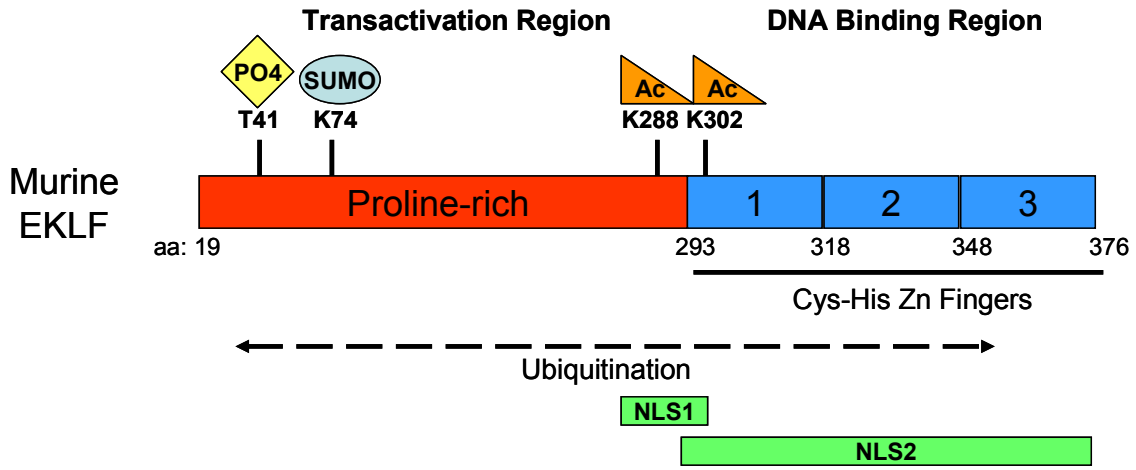


Figure 1.2. Domain mapping of murine EKLF. This schematic diagram summarizes the EKLF protein showing its transactivation (amino terminal) and DNA binding (carboxyl terminal) regions. EKLF encodes a protein of 376 amino acids characterized by a proline-rich transactivation domain. The DNA binding domain contains three C2H2 zinc fingers. Also noted in the diagram are the locations of important phosphorylation (T41), sumoylation (K74), acetylation (K288 and K302), and ubiquitination (throughout protein) sites, and two NLS discussed in the text.

EKLF/KLF1, the founding member of a 15 gene clade, interacts with the DNA consensus sequence CCNCNCCCN found at many promoters (Miller & Bieker, 1993). EKLF has two nuclear localization signals (NLSs) corresponding to a region adjacent to the zinc finger DNA binding domain within a stretch of highly basic amino acids 275-296 (Quadrini & Bieker, 2002) and another more efficient signal within the zinc finger domain itself encompassing amino acids 293-376 (Pandya & Townes, 2002; Quadrini & Bieker, 2002). Interestingly, each zinc finger is important for the overall function of the NLSs, and a complete zinc finger structure is necessary for efficient nuclear localization.

Expression of EKLF

Both human and murine EKLF have been cloned and show a high degree of homology with >90% similarity in the zinc fingers and approximately 70% within the proline-rich amino domain (Bieker, 1996; van Ree *et al.*, 1997). Expression of both factors is restricted to the erythroid lineage with high levels in murine and human definitive hematopoietic progenitors (Miller & Bieker, 1993; Bieker, 1996; van Ree *et al.,* 1997). During *in vitro* hematopoietic cell differentiation of murine ES cells, EKLF is expressed in the CMP and MEP population (Frontelo *et al.*, 2007). EKLF expression is absent in lymphoid cell lines (Miller & Bieker, 1993) and the CLP and their progeny (Frontelo *et al.*, 2007).

During murine ontogeny, EKLF is expressed early and at different anatomical sites (Southwood *et al.*, 1996). EKLF mRNA is detected first at the neural plate stage (at E7.5) within the blood islands in the yolk sac. EKLF is then expressed within the hepatic tissue beginning with the earliest stage of hepatic formation at E9 and continuing until E14.5 when the liver becomes the only source of EKLF. Concomitantly with EKLF mRNA, EKLF protein is also expressed in primitive cells and in the fetal liver. In the adult animal, EKLF expression is strictly localized to the red pulp of the spleen.

Regulation of EKLF

EKLF is a stage-and lineage-specific transcription factor, its expression requiring tight regulation. EKLF expression is induced by Bmp4/Smad signaling and GATA-1 (Adelmann *et al*., 2002; Lohmann & Bieker, 2008). However, regulation of EKLF activity is achieved in part by post-translational modifications of the EKLF protein (see Figure 1.2). First, EKLF is a phosphoprotein whose transcriptional activity is dependent on the phosphorylation status at threonine 41 (T41) (Ouyang *et al.*, 1998). On the other hand, sumoylation of EKLF at lysine 74 (K74) promotes transcriptional repression of megakaryopoiesis (Siatecka *et al.*, 2007). Similarly, EKLF is acetylated by CBP/p300 (Zhang & Bieker, 1998; Zhang *et al.,* 2001). This modification increases EKLF's affinity for the SWI/SNF components of chromatin remodeling complexes which has been postulated to maintain chromatin in an open configuration (Armstrong *et al.*, 1998; Kadam *et al.*, 2000; Zhang *et al.*, 2001). On the other hand, EKLF acetylation can also result in interaction with co-repressors Sin3A and recruitment of histone deacetylases

(HDACs) to promoters to inhibit gene transcription (Chen & Bieker, 1996; Chen & Bieker, 2004). Finally, EKLF can be ubiquitinated and degraded through the ubiquitin-mediated proteosome pathway (Quadrini & Bieker, 2006).

The role of EKLF at the β-globin locus

Disruption of the EKLF gene by homologous recombination has demonstrated its non-redundant role in erythropoiesis. EKLF-null embryos die of a lethal anemia by embryonic day 16 (E16), as definitive erythroid cells fail to produce β-globin transcripts *in vivo* (Nuez *et al.*, 1995; Perkins *et al.*, 1995), consistent with the idea that EKLF is essential for β-globin gene transcription. However, it is now recognized that EKLF also regulates expression of multiple erythroid-specific genes, including cytoskeletal proteins (Nilson *et al.*, 2006) and alpha hemoglobin stabilizing protein (AHSP) (Pilon *et al.*, 2006). Moreover, analysis of EKLF-null embryos that are transgenic for the human β-globin locus confirmed the necessity of EKLF for human β-globin gene transcription (Perkins et al., 1996; Wijgerde *et al.*, 1996). Studies in EKLF-null animals have delineated three mechanisms of action for EKLF in regulating β-globin gene transcription: i) chromatin remodeling, ii) modulation of transactivation, and iii) stabilization of the locus control region (LCR)/β-globin promoter interaction.

EKLF alters chromatin structure at the β-globin promoter

Local chromatin structure plays a critical role in regulating gene expression. Transcriptionally active genes are typically found in regions of open chromatin structure characterized by DNase I-sensitivity and histone hyperacetylation whereas inactive genes are packaged in a highly condensed chromatin configuration that is typically DNase I-insensitive and under-acetylated (Harju *et al.*, 2002). Chromatin structure may be altered by changing the organization of the nucleosome(s) at the gene promoter or by modifying the histones (Harju *et al.*, 2002). Thus, chromatin remodeling is an essential event for the initiation of gene transcription *in vivo*.

EKLF is a key player in activating β-globin gene transcription. Not only has it been postulated to be necessary for transactivation but it was the first factor implicated in erythroid-specific promoter remodeling of the β-globin promoter. Analysis of EKLF-null embryos revealed a specific loss of a developmentally specific DNase I hypersensitive site in the proximal β-globin promoter (Wijgerde *et al.*, 1996). Since the degree of DNase I hypersensitivity of a given locus correlates with nucleosomal remodeling (Steger & Workman, 1996; Pazin *et al.*, 1997), these findings strongly suggest that EKLF is required for chromatin reorganization at the β-globin promoter in definitive erythroid cells. A loss of DNase I hypersensitivity was also observed in hypersensitive site 3 (HS3) of the LCR, but to a lesser extent. Furthermore, utilizing chromatin immunoprecipitation (ChIP) analysis, we observed increased acetylation of histone H3 at the β-globin promoter after induction of EKLF in an EKLF-inducible erythroid cell system. This change correlates with activation of β-globin gene transcription (J.M.C

unpublished data). These results confirmed the role of EKLF as a chromatin modulator and transcriptional activator of the β-globin gene. However, the specific domains that fulfill this role *in vivo* and the molecular mechanisms responsible for chromatin modification remain to be elucidated.

EKLF-mediated transactivation

EKLF is an erythroid-specific transcription factor containing a carboxy-terminal zinc finger DNA-binding domain and a proline rich amino terminal domain. Initial structure-function studies, utilizing chimeric proteins consisting of the DNA-binding domain of the yeast factor GAL4 fused in frame to various EKLF sequences, demonstrate that the amino terminal region could be divided into two domains with opposing functions (Chen & Bieker, 1996). In these assays, the first 104 amino acids activate GAL4-dependent transcription, whereas an internal domain (aa 196-291) mediated transcriptional repression. In contrast to these studies that utilize heterologous promoters, our laboratory has shown that an internal domain of EKLF is sufficient for activation of the endogenous β-globin promoter (Brown *et al.*, 2002). However, it remains unclear what functions of EKLF are required for β-globin gene transactivation *in vivo*.

The role of EKLF in LCR/β-globin promoter interaction

Regulation of the β-globin locus is believed to occur in part by competition of each globin gene promoter for direct interaction with the LCR with the intervening region looping out. Direct support for EKLF being involved in stabilizing the LCR/β-globin promoter interaction was provided by utilizing chromosome conformation capture (3C) technology (Dekker *et al.*, 2002) to study the spatial organization of the β-globin locus. In erythroid cells, the hypersensitive sites of the LCR are in close physical proximity to the active globin genes with the intervening DNA sequence containing the inactive globin genes looped out forming the Active Chromatin Hub (ACH), a nuclear compartment dedicated to RNA polymerase II mediated transcription (Palstra *et al.*, 2003). Subsequent studies in EKLF-null fetal livers demonstrated that EKLF is required for ACH formation and active β-globin gene transcription (Drissen *et al.*, 2004). Despite the significant insights into the binding sites, and the effects of wild-type EKLF action, it remains unclear whether differing domains of EKLF are required for alteration of the β-promoter and LCR architecture *in vivo*.

Other EKLF target genes

Early observations alluded to a broader role for EKLF in the regulation of genes other than β-globin. First, the EKLF-null phenotype is more severe than that observed in a murine model of β-thalassemia in which the adult β-globin genes are deleted by homologous recombination (Ciavatta *et al.*, 1995). Furthermore, enforced expression of an EKLF-independent globin transgene fails to rescue the lethal phenotype (Perkins *et al.*,

2000). Gene profiling studies by our laboratory and many others provide a list of potential target genes (see Appendix Table A.1). Subsequent studies utilizing chromatin immunoprecipitation (ChIP) analysis have confirmed dematin (Hodge *et al.*, 2006), AHSP (Keys *et al.*, 2007; Pilon *et al.*, 2006), ankyrin, Band 3 (Nilson *et al.*, 2006), and BKLF (Funnell *et al.*, 2007) as direct EKLF target genes. Together these observations support a broader role for EKLF in the regulation of other erythroid-specific genes and provide additional evidence that defects in addition to β-globin deficiency contributes to the lethal phenotype in EKLF-null mice.

EKLF possesses distinct and separable chromatin remodeling and transactivation domains

To explore the determinants of EKLF-dependent β-globin gene activation, our laboratory utilized an erythroblast cell line lacking endogenous EKLF expression, J2eΔ*eklf* (Coghill *et al.*, 2001). Briefly, this line was prepared by immortalization of fetal liver erythroblasts derived from E14.5 EKLF-null embryo by transduction with a raf/myc retrovirus. Subsequently, the cells were transduced with retroviral constructs containing EKLF cDNA fused in frame with the influenza hemagglutinin (HA) epitope at the amino terminus and the tamoxifen-binding domain of the estrogen receptor (ER^{TM}) at the carboxyl terminus. Constructs containing full length human EKLF and a series of amino terminal mutants were studied (Figure 1.3A) (Coghill *et al.*, 2001; Brown *et al.*, 2002). Subsequently, these cell lines were utilized to study the distinct domains of EKLF in β-globin gene activation.

To determine the effects of the amino terminal deletions on formation of hypersensitive sites at the β-globin promoter, nuclei of induced cells for each mutant were incubated with increasing amounts of DNase I (Figure 1.3B). Contrary to an *in vitro* study that demonstrated the DNA binding domain of EKLF alone could induce a specific DNase I hypersensitive site of chromatinized β-globin template (Kadam *et al.*, 2001), the hypersensitive site pattern in J2eΔ*eklf* cells expressing the Δ253EKLF mutant is similar to that observed in J2eΔ*eklf* null cells. In contrast, cells expressing Δ221EKLF and Δ164EKLF show a hypersensitive site pattern similar to that observed with full-length EKLF (flEKLF). Therefore, the sequence between aa 221-253 is required and sufficient for an "open" configuration at the endogenous β-promoter (Brown *et al.*, 2002).

Based on prior structural analysis of EKLF, the activation domain of EKLF should reside in sequences upstream of the Δ221-253 regions. Our hypersensitive studies suggested that the transcriptional and chromatin remodeling domain activities of EKLF are separable. To test this hypothesis, transcriptional activity for each mutant was measured by RNA protection assay and correlated with its chromatin remodeling properties (Figure 1.3C). As anticipated, no significant level of the βmaj transcript was detected in Δ253EKLF cells. In contrast, expression of the Δ221EKLF polypeptide resulted in a small increase in gene transcripts. However, examination of cells expressing Δ164EKLF revealed levels of βmaj transcripts that paralleled flEKLF expression. Thus,

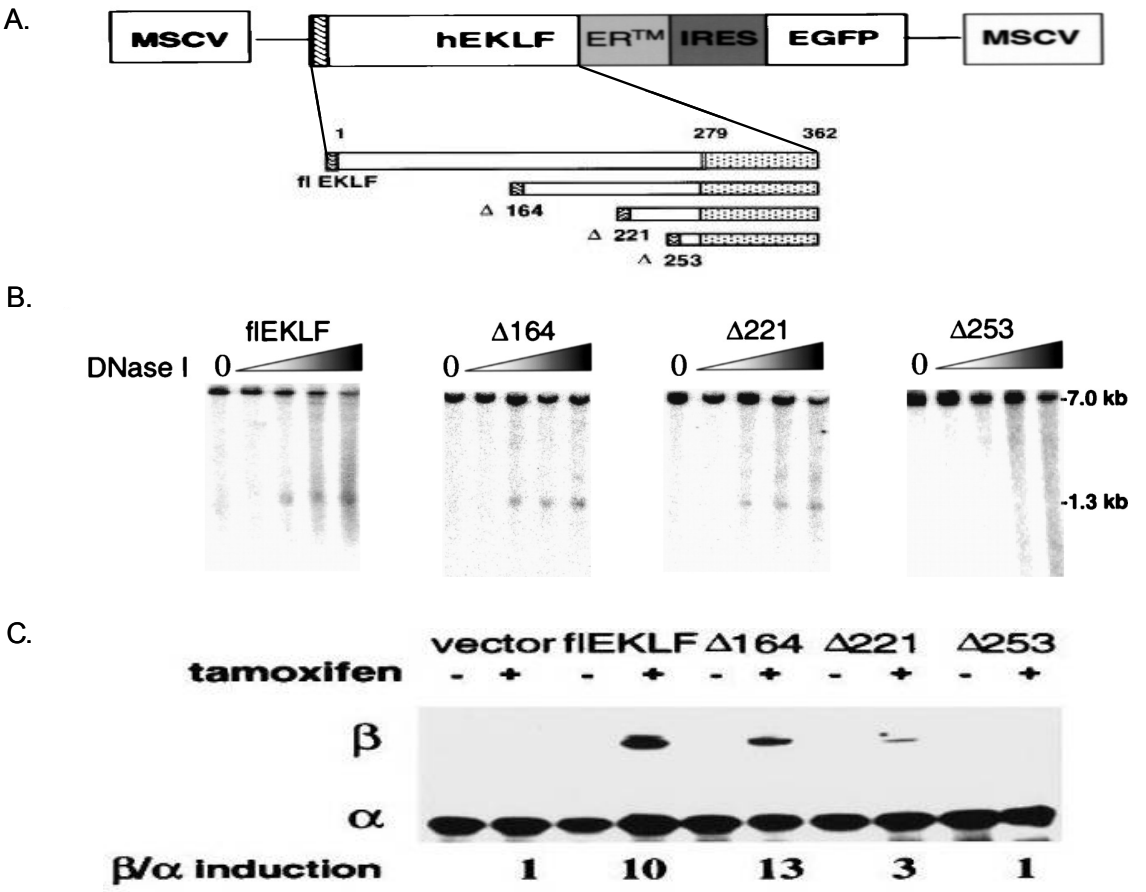


Figure 1.3. Separable chromatin remodeling and transactivation domains of EKLF. (A) Schematic diagram of flEKLF retrovirus and derivative mutants used to stably transduce J2e *eklf* cells. (B) The DNA binding domain (Δ253) is required but is not sufficient for chromatin remodeling at the ßmaj globin promoter. Cells expressing each EKLF mutant were cultured for 48 h in the presence of tamoxifen. Nuclei were isolated and exposed to increasing concentrations of DNase I as previously described. DNA was harvested, digested with *Eco*RI, and probed with a ßmaj-specific probe. The DNase I concentration is 0 in the first lane of each panel and increases as shown by the shaded triangle. (C) An internal domain of EKLF (Δ164) is sufficient to activate ß-globin gene expression to wild-type levels. RNA was harvested from J2e *eklf* clones expressing varying EKLF mutant moieties at 48 h post-tamoxifen induction RPA was performed utilizing ßmaj and riboprobes. The numbers underneath the panel represent the mean fold induction of the ß/α ratio for each construct assayed. Amended with permission from American Society for Microbiology. Brown, R. C., S. Pattison, R. J. van Ree, E. Coghill, A. Perkins, S. M. Jane, and J. M. Cunningham. 2002. Distinct domains of erythroid Krüppel-like factor modulate chromatin remodeling and transactivation at the endogenous beta-globin gene promoter. Mol. Cell. Biol. 22:161-170/DOI:10.1128.

an internal domain of EKLF is sufficient to activate β-globin gene expression to wild-type levels (Brown *et al.*, 2002). These observations are contrasted with previous studies in which the first 104 amino acids of EKLF activated GAL4-dependent transcription (Chen & Bieker, 1996). More importantly, our studies indicate EKLF contains separable chromatin remodeling and transactivation domains.

PROJECT OUTLINE

I joined Dr. Cunningham's laboratory with a strong interest in studying globin gene regulation to understand the molecular mechanism underlying one of the most common hematological disorders, β-thalassemia. Success in this endeavor would identify therapeutic targets that would reverse or delay the globin gene switch, thus ameliorating the β-thalassemic or sickle cell disease phenotype.

Based on the cellular studies by previous colleagues in the laboratory, I propose to examine the role of the newly defined chromatin remodeling domain of EKLF (Δ221EKLF) in a whole animal model that allows the analysis of modulation of gene expression and chromatin structure. My working hypothesis is that expression of the chromatin remodeling domain of EKLF, in the absence of the transactivation domain, is necessary and sufficient for altering the chromatin structure at the β-globin locus *in vivo.* A corollary hypothesis is that expression of the chromatin remodeling domain alone is not sufficient for activation of β-globin gene transcription *in vivo.* I plan to generate specific mutants of the EKLF gene at the endogenous locus in a murine knock-in model to address the following specific aims.

Specific aim 1: To characterize the cellular consequences on murine hematopoiesis of expression of the knock-in allele in vivo.

The goal of this specific aim is to provide an initial analysis of the cellular effects of Δ221EKLF expression in a murine model. First, matings between Δ221EKLF heterozygous male and female animals will be established to determine if Δ221EKLF homozygous animals are viable. If no adult Δ221EKLF homozygous animals are observed, I will dissect embryos at different time points during gestation to determine when and how Δ221EKLF animals die. I hypothesize that animals expressing Δ221EKLF die of a lethal anemia similar to the EKLF knock-out mice. Similarly, I predict that cells expressing Δ221EKLF fail to execute normal terminal differentiation. These studies should provide valuable information on the role of the chromatin remodeling domain of EKLF in transactivation of β-globin gene transcription *in vivo*.

Specific aim 2: To evaluate the functional consequences of expression of the knock-in allele on EKLF-dependent erythroid gene transcription.

I propose to explore the effects of Δ221EKLF expression on gene transcription *in vivo*. Utilizing real time RT-PCR, I will quantify the transcription of the β-globin gene and putative non-β-globin EKLF-target genes to determine whether any of these genes require the chromatin remodeling properties of EKLF alone for gene transcription. Based upon our laboratory's cellular studies, my overarching hypothesis is that expression of the chromatin remodeling domain in the absence of the transactivation domain is not sufficient to activate gene transcription. Together, these results will provide insights into the role of EKLF in coordinating gene transcription at the β-globin locus and other gene loci.

Specific aim 3: To explore the functional consequences of expression of the knock-in allele on chromatin structure at the β-globin locus.

The studies proposed in this aim will directly test my working hypothesis that expression of Δ221EKLF is sufficient for altering chromatin structure at the β-globin locus. First, I will analyze the nucleosomal remodeling of the β-globin promoter as measured by DNase I hypersensitivity. Subsequently, I will investigate the histone acetylation and methylation patterns at the β-globin locus utilizing quantitative chromatin immunoprecipitation (ChIP). The proposed studies should corroborate the observations in our EKLF-dependent erythroblast model and provide insight into the role of chromatin remodeling in transactivation of β-globin gene transcription.

In this dissertation, I describe the generation of the knock-in alleles of EKLF and the consequences of expression of the Δ221EKLF in erythroid and megakaryocytic differentiation. In Chapter 2, I will describe the construction of gene targeting vectors utilizing recombineering technology and the generation of Δ221EKLF heterozygous animals. In Chapter 3, I will report on the molecular consequences of expression of Δ221EKLF as it relates to chromatin remodeling and transcriptional activation of β-globin. In Chapter 4, I will report on the novel role for EKLF in megakaryocytic differentiation.

CHAPTER 2: GENERATION OF A KNOCK-IN ALLELE OF EKLF UTLIZING RECOMBINEERING TECHNOLOGY

INTRODUCTION

Genetically engineered mouse models have proven to be useful tools for many applications in research, medicine, and biotechnology. The approaches to generating these different mouse models have traditionally been based on the over-expression or ablation of a gene using transgenic and knock-out strategies, respectively (Roebroek *et al.*, 2002). More recently, by using a knock-in approach and placing the expression of an exogenous gene under the transcriptional control of *cis*-acting elements belonging to the endogenous gene, investigators are able to study the gene in a more subtle manner (Roebroek *et al.*, 2002).

Methods used for genetic engineering have relied mostly on a conventional approach. Restriction enzymes and DNA ligases are used to clone an appropriate piece of DNA sequences into a targeting vector. The major limitation of this strategy is the difficulty and time it takes to generate this vector utilizing large fragments of DNA (Copeland *et al.*, 2001; Liu *et al.*, 2003). At the time that I initiated my dissertation studies, a new and highly efficient method for manipulating the mouse genome had been developed. Termed recombineering, this method relies on the lambda phage-based homologous recombination in *Escherichia coli* to construct the targeting vector (Yu *et al.*, 2000; Liu *et al.*, 2003). Using this technology, it is possible to introduce large double-stranded DNA (dsDNA) fragments into DNA cloned on plasmids, bacterial artificial chromosomes (BACs), or P1 artificial chromosomes (PACs) via homologous recombination without the need for restriction enzymes or DNA ligases (Copeland *et al.*, 2001; Liu *et al.*, 2003). Additional advantages of using this new technology are speed, efficiency, and reliability.

Originally, recombineering has been utilized in yeast due to its efficient DNA double-stranded-break-and-repair recombination pathway, allowing the creation of recombinant DNA molecules by homologous recombination (Baudin *et al.*, 1993). These recombination pathways allow efficient recombination of transformed linear, double-stranded DNA (dsDNA) with homologous sites in the yeast genome. Moreover, proficient recombination occurs even with only short stretches of homologous sequence, thereby allowing recombinant DNA to be generated *in vivo* without the use of restriction enzymes and DNA ligases (Baudin *et al.*, 1993). Unlike in yeast, dsDNA is unstable in *E. coli* due to the presence of RecBCD, an ATP-dependent exonuclease that degrades dsDNA. However, *E. coli* strains that lack RecBCD can be transformed by linear dsDNA (Baudin *et al.*, 1993).

There are disadvantages to utilizing yeast and yeast artificial chromosomes (YACs) in recombineering. First, YACs are less stable in their yeast host, in which recombination is potent and always active. Thus, undesired deletions and gene rearrangements are a barrier to using this organism. In contrast, bacterial artificial

chromosomes (BACs) are stable in *E. coli* (Shizuya *et al.*, 1992; Copeland *et al.*, 2001). Moreover, YAC DNA is more difficult to purify compared to BAC DNA (Copeland *et al.*, 2001). A yeast cell may contain both wild-type and modified YACs (Peterson *et al.*, 1997), whereas a bacterial cell typically contains a single BAC (Copeland *et al.*, 2001). Finally, manipulating recombinant YACs that are generated in yeast can be laborious and usually requires the YACs to be transferred to *E. coli* for subsequent manipulation, whereas BAC modification occurs directly in *E. coli* (Copeland *et al.*, 2001).

Recombineering in *E. coli* can be accomplished by making use of lambda phage's homologous recombination proteins, called Red, which allow linear dsDNA fragments to be inserted via homologous recombination into DNA cloned on plasmids (Yu *et al.*, 2000; Cotta-de-Almeida *et al.*, 2003; Zhang & Huang, 2003). To generate a recombinogenic strain of bacteria, a defective lambda prophage which lacks lysis and replication functions but retains the Red proteins is inserted into the bacterial genome (Yu *et al.*, 2000). The phage genes of interest, *exo*, *bet*, and *gam*, are transcribed from the λ*P*L promoter. This promoter is repressed by the temperature-sensitive repressor *cI*857 at 32°C. In contrast, derepression, that is the repressor is inactive, occurs at 42°C (Yu *et al.*, 2000). At low temperatures (i.e., 32°C) no recombination proteins are produced. However, following a temperature shift to 42^{o}C for as little as 15 min, these proteins are expressed at high levels. The 5'-3' exonuclease, *exo,* creates single-stranded overhangs on introduced linear DNA; *bet* protects these overhangs and assists in the subsequent recombination process. Degradation of linear DNA is protected by *gam,* which inhibits the *E. coli Rec*BCD protein (Yu *et al.*, 2000).

Following induction of the recombination genes, linear dsDNA such as PCR products and oligonucleotides with sufficient homology in the 5' and 3' ends to a target DNA molecule already present in the bacteria (plasmid, BAC, or the bacterial genome itself) can be introduced into heat-shocked and electrocompetent bacteria using electroporation. The introduced DNA is modified by *exo* and *bet* and undergoes homologous recombination with the target molecule. The method is so efficient that co-electroporation of a supercoiled plasmid and a linear piece of DNA into heat-shocked, electrocompetent bacteria will work as well (Yu *et al.*, 2000; Copeland *et al.*, 2001; Liu *et al.*, 2003).

This chapter describes the generation of knock-in mutant alleles of EKLF using the recent recombineering technology that encompasses a phage-based *E. coli* homologous recombination system. Several groups have used this new form of genetic engineering to construct standard, conditional, and knock in gene targeting vectors to modify murine embryonic stem (ES) cells (Lui *et al.*, 2003; Zhou *et al.*, 2004). Utilizing this method, I have constructed three targeting vectors in which cDNA encoding three different truncation mutations of EKLF (Δ164EKLF, Δ221EKLF, and Δ253EKLF) have been inserted into the endogenous murine EKLF locus. The construction of all three targeting vectors was completed without the constraints of restriction sites and took a shorter time than a similar strategy utilizing traditional subcloning methods. Subsequently, the targeting vectors were used to target the endogenous murine EKLF locus in ES cells. The animals I have generated should prove to be useful tools for

analyzing the distinct molecular functions of EKLF *in vivo*.

Based upon recommendations from my graduate committee, I have chosen to focus my dissertation studies on characterizing one strain of mice while the other two strains are studied by my colleagues in the laboratory. I have chosen to focus on the Δ221EKLF strain for several reasons. First, previous studies by my laboratory colleagues have demonstrated that the Δ221EKLF domain is sufficient to alter local chromatin structure at the endogenous β-globin promoter; however this domain alone is insufficient to transactivate β-globin gene transcription to wild-type levels in a cellular model. Thus, the strain of mice expressing Δ221EKLF is the most ideal model to elucidate the role of EKLF in chromatin remodeling and gene activation *in vivo*.

MATERIALS AND METHODS

BAC transfer into recombinogenic strains

An EKLF BAC clone in DH10B was obtained from the BACPAC Resources Center at Children's Research Hospital Oakland Research Institute (CHORI). The EKLF BAC DNA (75kb) was purified using the Miniprep DNA kit (Qiagen) as previously described (Liu *et al.*, 2003). Briefly, *E. coli* cells encoding BACs were grown overnight in LB broth (5 mL) with chloramphenicol. Cells were collected by centrifugation at maximum speed (12,000 x g or 13, 000 rpm) and resuspended in buffer P1 (250 µL). Buffer P2 (250 µL) and buffer P3 (350 µL) were added to each tube, and the tubes were spun for 4 min at 12,000 x g. The supernatant was transferred to a new 1.5 mL tube and cleared by centrifugation for another 4 min. Isopropanol (750 µL) was added to the mixture, and DNA was precipitated at room temperature for 10 min. The DNA was collected by spinning the tube for 10 min at the maximal speed, washed once with 70% ethanol (1.0 mL), air dried, and resuspended in TE (50 µL). The purified EKLF BAC DNA (100-200 ng) was electroporated into the recombinogenic *E. coli* strain EL350 (a kind gift from Dr. Neal Copeland) using a BIO-RAD electroporator at 1.75 kV, 25 µF with the pulse controller set to 200 Ω and time constant between 4.3-4.7. Transformed colonies were recovered on LB agar with 40 µg/mL chloramphenicol. The EKLF BAC DNA prepared from the original DH10B and transformed EL350 bacteria was digested with *BamHI, EcoRI*, and *HindIII* and separated on agarose gels to confirm that no DNA rearrangements had occurred during the BAC transfer.

Plasmids

The EKLF BAC retrieval plasmid was generated by ligating PCR product AB (left arm, *EcoRI/BamHI*), PCR product XY (right arm, *BamHI/XbaI*), and *MC1-TK/polII-DT* (VP101, *EcoRI/XbaI*) using T4 DNA ligase (Promega). The mini-targeting plasmid was generated in two-steps. First, PCR product EF (*BglII/NotI*) was ligated with a floxed *Neo*-containing vector, a kind gift from Dr. Neal Copeland (PL452, *BamHI/NotI*). Then

PCR product CD (*SalI/HindIII*) and HA-Δ221EKLF cDNA-containing fragment (pspHA-EKLF-C3, *HindIII/EcoRI*) were ligated with the vector containing the *Neo* cassette and PCR product EF generated in the first step. The ligation product was transformed into chemically competent DH5α cells (Invitrogen) and plated on selective media containing both kanamycin (50 μg/mL) and carbenicillin (100 μg/mL). Only bacterial cells propagating the subcloned vector with the *Amp* and *Neo* resistance genes should grow under these conditions.

PCR products were amplified using ROCHE Expand High-Fidelity PCR System using 50 ng of BAC DNA following the manufacturer's recommendations. PCR was performed using an MJ Research PCR machine with the following settings: 94°C for 2 min, then 10 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 45 sec. This was followed by 15 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 45 sec, with an additional 5 sec extension time each cycle. To check the PCR reaction, 5 μL of the 50 μL PCR reaction mixture was loaded onto an agarose gel. The remaining 45 μL of PCR product was purified using the QIAGEN PCR Purification Kit.

Recombineering

Recombineering was performed as previously described (Liu *et al.*, 2003). To retrieve the gap-repaired plasmid, EL350 cells containing the EKLF BAC was grown at 32°C to an $OD_{600} = 0.5$ in LB broth with chloramphenicol. The cells were transferred to and shaken in a 42°C water bath for 15 min to induce expression of the λ recombination proteins, and quickly chilled in ice water for 10 min. Electrocompetent cells were prepared by washing the cells three times with ice cold water. Finally, the cell pellet was resuspended in ice cold water (50 μL) and electroporated with the *BamHI*-linearized retrieval vector (1-2 μL). After electroporation, 1 mL of LB medium was added to the cuvette, and the culture was incubated at 32°C for 1 h with shaking. The cells were then plated on agar plates with the appropriate antibiotic.

For targeting, frozen EL350 electrocompetent cells previously prepared in the laboratory were used. The frozen cells were thawed at room temperature and quickly put on ice. These cells were co-electroporated with the targeting cassette (100 ng) and the gap-repaired plasmid (10 ng) DNA as previously described (Liu *et al.*, 2001). The targeting cassette was excised from the mini-targeting vector with *NotI* and *SalI* digest and purified by the QIAGEN Gel Purification System.

Excision of the *Neo* cassette in bacteria

Frozen EL350 cells previously prepared in the laboratory and induced for *Cre* expression by prior growth in arabinose-containing medium were used to test the excision of the floxed *Neo* cassette. The EL350 strain of *E. coli* was previously engineered to harbor an arabinose-inducible C*re* gene (P_{BAD}-*cre)* (Lee *et al.*, 2001; Liu *et al.*, 2003). Plasmid DNA (10-50 ng) was electroporated into frozen electrocompetent cells (50 μL).

LB medium (1 mL) was added to the cuvette, and the culture was shaken at 32°C for 1 h. The cells were plated on ampicillin-containing media. DNA was extracted from selected ampicillin-resistant colonies and digested with restriction enzymes.

Gene targeting in ES cells and generation of Δ221EKLF mice

ES cells were obtained from Specialty Media and maintained following the manufacturer's recommendations. For gene targeting, the *NotI* linearized HA-Δ221EKLF-KI construct was electroporated into 129Sv ES cells (Specialty Media) and recombinants were selected in medium supplemented with G418 (Gibco) and ganciclovir (Syntex). Selection was continued for eight days and the surviving ES clones were picked and expanded for an additional four days. Genomic DNA was extracted from each clone and analyzed by Southern blotting to identify properly targeted ES clones. Sequences for 5' and 3' probes used in Southern blot analysis are available in Appendix Figure A.2.

Properly targeted ES clones with a normal karyotype were injected into C57BL/6 blastocysts and transferred into pseudopregnant females to generate chimeras (Transgenic Core Facility at St. Jude Children's Research Hospital). Male chimeras were mated with C57BL/6 wild-type females to generate F1 offsprings. To remove the floxed *Neo* cassette, Δ221EKLF heterozygous mice were mated with mice harboring the *cre* transgene diallelically expressed under the control of the adenovirus EIIa promoter that targets expression of Cre recombinase to the early mouse embryo (Jackson Labs).

Mouse genotyping

For Southern blotting, mouse tail DNA was digested with *NheI* (New England Biolabs), separated on 0.8% agarose gels, and transferred to GeneScreen Plus hybridization transfer membrane (Perkin Elmer). A 543 bp 3' external probe was used for hybridization. Genotyping by PCR was performed using a common WT and KI forward primer, (5'-ATGAGGCAGAAGAGAGAGAGGAG-3'), a WT reverse primer (96 bp; 5'-TGAGTGTACTGATGGAGGGTAAGA-3'), and a KI reverse primer (500 bp; 5'-CCGTGTGTTTCCGGTAGTG-3'). PCR primers for genotyping were designed using Primer Design in Clone Manager. PCR was performed on a BIORAD PCR machine with the following settings: 94°C for 3 min followed by 32 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min with a final extension at 72°C for 10 min. Products were visualized on agarose gel with ethidium bromide staining.

Husbandry of mice

The animals used in the studies were housed in the Animal Resources Center of St Jude Children's Research Hospital, Memphis, Tennessee, and the University of Chicago, Chicago, Illinois, according to approved Animal Care Use Protocols (ACUP).

Analysis of peripheral blood counts

Blood was harvested from the retro-orbital sinus into EDTA-coated microcapillary tubes and submitted to the St Jude Children's Research Hospital Animal Diagnostic Laboratory for complete blood count (CBC) analysis including a peripheral blood smear.

Primers

PCR primers for recombineering were designed using Primer Design in Clone Manager or Primer 3. The primer sequences used for generating the HA-Δ221EKLF-KI targeting vector are:
AB-fragment (540 bp): Primer A: 5'-CGGAATTCGGTGCTGGTGGTTGTCTAGG-3' Primer B: 5'-CGCGGATCCTGTGGTTGCTTGTAATTGA-3';
XY-fragment (640 bp): Primer X: 5'-CGCGGATCCTACATAGCCTCTGTGCATTC-3'; Primer Y: 5'-GCTCTAGACACCGTCTCTAGGTCAGGTA-3'; CD-fragment (596 bp): Primer C: 5'-ACGCGTCGACCAAAGGGTCCCAAAGACCTTTC-3'; Primer D: 5'-GGGTGGTGAGTGTACTGATG-3'; EF-fragment (541 bp): Primer E: 5'-GGAAGATCTGGCAGGTGGTCTTGCATAGG-3'; Primer F: 5'-ATAAGCGGCCGCAGTATTCAGGATGGGAGAAG-3'; Primers used to generate the fragment containing the HA-Δ221EKLF sequence were Forward: 5'-CATGCCATGGCCTATGACGTCCCAGATTACG-3'; Reverse: 5'-AGTCAGGATATCCTACTTAGTCAAAGGTGGCGCTTCATGTGC-3'.

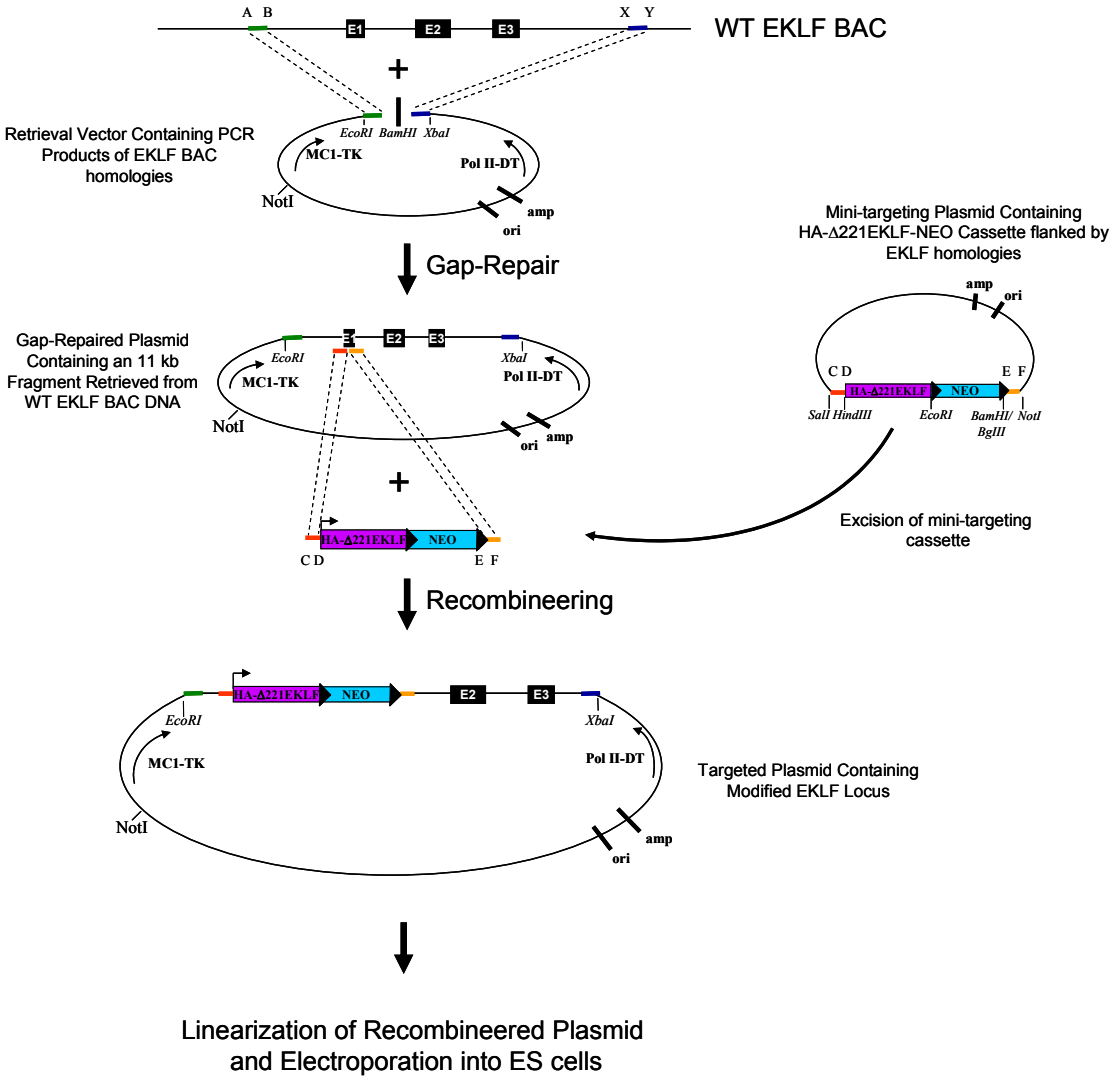
RESULTS

Generation and characterization of targeting vectors

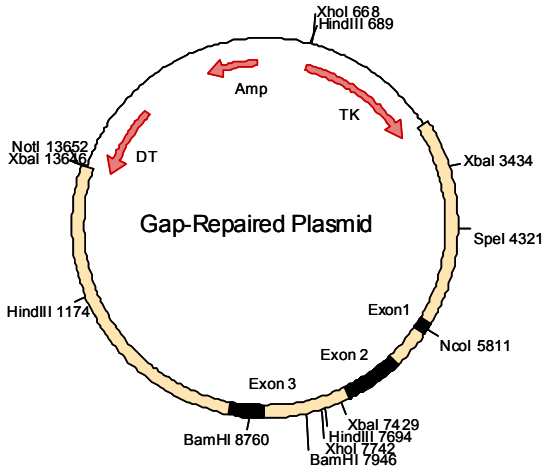
To elucidate the role of the functional domains of EKLF in primary mouse erythroblasts, I proposed to generate animals expressing distinct domains of EKLF identified in previous cellular assays (Brown *et al.*, 2002). Targeting vectors, in which cDNA encoding HA-tagged truncation mutant of EKLF linked in *cis* to a Neo selection cassette (HA-Δ221EKLF-Neo) were generated, utilizing recombineering methods. The overall strategy is illustrated in Figure 2.1.

First, to facilitate the manipulation of the EKLF genomic locus by recombineering, it was necessary to retrieve a region of the mouse genome containing the EKLF gene and its *cis*-regulatory sequences. To accomplish this, a vector capable of retrieving an 11 kb fragment spanning the EKLF locus was constructed by subcloning PCR amplified fragments AB and XY into an expression vector. These regions, AB and XY, mark the 5' and 3' ends of the fragment to be subcloned by gap repair, respectively. Next, to obtain the gap-repaired plasmid containing our genomic region of interest (Figure 2.2A), the *BamHI*-linearized retrieval vector was electroporated into the recombinogenic bacterial

Figure 2.1. Overview of the recombineering method used to generate vectors for targeting in ES cell. A BAC clone containing the EKLF genomic locus was transferred from the *E. coli* strain DH10B to the recombinogenic strain EL350. These cells were electroporated with a *BamHI* linearized retrieval plasmid containing PCR products, AB and XY, of homology to the EKLF BAC. By gap-repair, this plasmid rescued an 11 kb fragment containing the EKLF gene plus 3 kb of upstream sequences and 4.5 kb of downstream sequences. Cells containing the correct gap-repaired plasmid were electroporated with a fragment containing HA-Δ221EKLF-floxed Neo flanked by homology sequence from the EKLF genomic locus. This fragment was excised from the mini-targeting vector in which PCR products CD, EF, and HA-Δ221EKLF were subcloned into the PL452 plasmid containing a floxed Neo cassette. The resulting plasmid containing the modified EKLF locus with insertion of HA-Δ221EKLF-floxed NEO cassette into the ATG at amino acid 19 was linearized with NotI and electroporated into ES cells. EKLF contains three exons (E1-E3) as denoted by black boxes. AB (green) and XY (blue) are homologous to a region upstream and downstream of the EKLF locus, respectively. CD (red) and EF (orange) flank the fragment to be recombineered into the EKLF locus and are homologous to sequences in exon 1 of the EKLF gene. The CD region, containing the ATG start site, is placed in frame with HA-Δ221EKLF encoding sequences. Lox P sites are represented by black arrow heads.



A.



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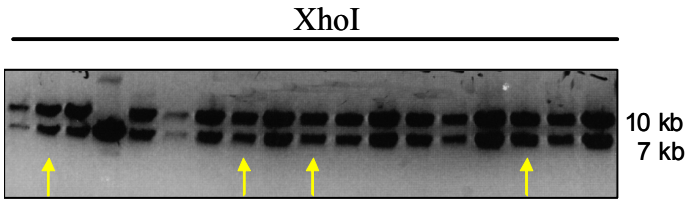

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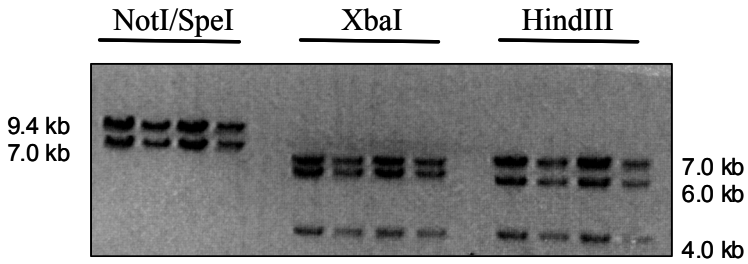


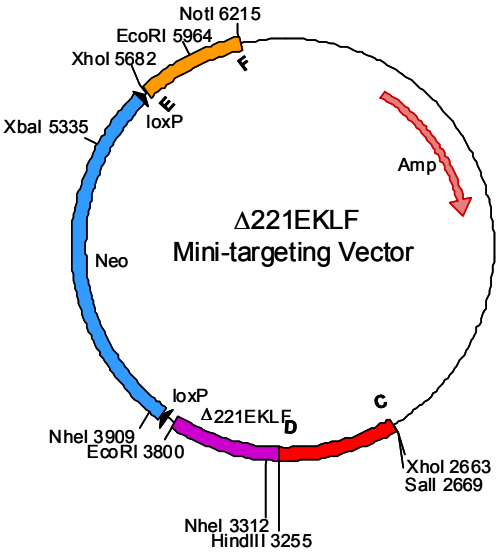
Figure 2.2. Mapping of the gap-repaired plasmid. (A) Diagram of the resultant gap-repaired plasmid obtained by electroporation of EL350 cells harboring EKLF BAC with a *BamHI*-linearized retrieval vector. DT = diphtheria toxin; TK = tymidine kinase; Amp = Ampicillin (B) To confirm the capture of the 11 kb EKLF BAC with appropriate flanking sequences, DNA was isolated from 18 colonies and digested with *XhoI* (10 kb and 7 kb). (C) Subsequently, DNA from 4 colonies (indicated by arrows from above) was digested with *NotI/SpeI* (9.4 kb, 7.7 kb), *XbaI* (6.8kb, 6.3 kb, 4.0 kb) *and HindII* (7.0 kb, 6.0 kb, 4.0 kb), respectively. The expected bands are in parentheses.

strain harboring the EKLF BAC. Since these bacterial cells had been engineered to express the λ recombination system with induction at 42°C, homologous recombination would occur resulting in the retrieval of the mouse genomic region flanked by the homology arms AB and XY. DNA from 18 ampicillin-resistant colonies were purified by the miniprep method and digested with *XhoI* to identify homologous recombinants. As shown in Figure 2.2B, DNA from 17 out of 18 colonies digested with *XhoI* showed the predicted 7 kb and 10 kb bands. The DNA in lane 4 appears to be undigested plasmid containing at least two topologically different forms of DNA, corresponding to supercoiled forms (bottom band) and nicked circles (upper band). DNA from four colonies (indicated by arrows) was selected for further restriction enzyme analysis. Digest with *NotI/SpeI* (9.4 kb / 7.7 kb), *Xba1*(7.0 kb / 6.0 kb / 4.0 kb), and *HindIII (*7.0 kb / 6.0 kb / 4.0 kb) produced fragments of the expected sizes (Figure 2.2C). Four DNA samples confirmed by restriction mapping were sent for sequencing at the Hartwell Center of St Jude Children's Research Hospital. One construct had numerous mutations and deletions near the junctions where homologous recombination took place while another construct had base pair substitutions within the EKLF gene. The gap-repaired plasmid with the correct sequences was selected for the next step in recombineering.

Next, a mini-targeting vector containing the knock-in fragment that will be used to target the gap-repaired plasmid was constructed by subcloning. Fragment EF which is homologous to the targeting site was generated by PCR, digested with *BglII/NotI*, and ligated with PL452 DNA digested with *NotI/BamHI*. The restriction enzymes *BglII* and *BamHI* have compatible ends (New England Biolabs). The floxed *Neo* gene in PL452 is expressed from a hybrid PGK-EM7 promoter (Liu *et al.*, 2003). Expression of *Neo* from the PGK promoter permits selection of *Neo* resistant mammalian ES clones. On the other hand, EM7 drives *Neo* expression in bacterial cells facilitating selection of *Neo* resistant colonies. PCR was used to generate the CD fragment with *SalI* and *HindIII* sites and a fragment containing HA-Δ221EKLF sequences with stop codons in all three reading frames and addition of *HindIII* and *EcoRI* restriction sites. Homology arm CD was designed carefully so that the HA-Δ221EKLF cDNA would be inserted into the endogenous ATG start site, placing the mutant gene under the *cis*-regulatory control of the endogenous locus.

To complete subcloning the mini-targeting vector (Figure 2.3A), *SalI/HindIII*-digested CD fragment and *HindIII/EcoRI*-digested HA-Δ221EKLF fragment were ligated with *SalI/EcoRI*-digested PL452-EF plasmid DNA. The ligation product was transformed into DH5α competent cells and plated on kanamycin/ampicillin selective media. Colonies propagating the subcloned plasmid with the *Amp* and *Neo* resistant genes should only grow under these conditions. Restriction analysis with *EcoRI* digest identified six out of eighteen colonies that were homologous recombinants (Figure 2.3B, as indicated by arrows). DNA from these six colonies was subjected to further restriction digestion analysis with *NotI/SalI* (3.5 kb, 2.9 kb), *NheI* (5.8 kb, 0.6 kb), *XhoI* (3.4 kb, 3.0 kb), and *HindIII/EcoRI* (3.7 kb, 2.2 kb, 0.6 kb) (Figure 2.3C). Three of the six colonies showed the correct restriction patterns. Before proceeding with the final step, the mini-targeting vector was extensively analyzed by restriction enzyme digestion (Appendix Figure A.1; Table A.2). Furthermore, sequence analysis of the junctions between CD-

A.



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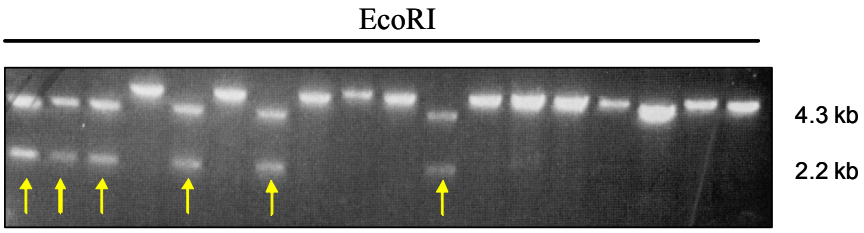

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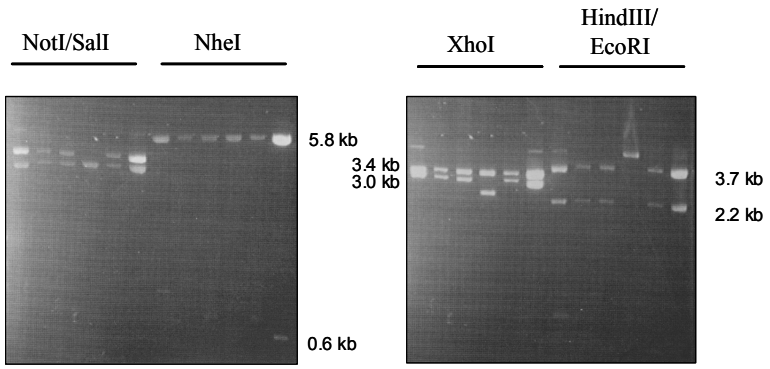


Figure 2.3. Analysis of Δ221EKLF mini-targeting vector. (A) Plasmid map of HA-Δ221EKLF mini targeting vector with restriction enzyme sites. CD, EF = homologies to EKLF gene; Amp = Ampicillin; Neo = Neomycin (B) DNA from 18 kanamycin resistant colonies were prepared and digested with *EcoRI* (4.3 kb, 2.2 kb). (C) DNA from the six correctly identified colonies (in lanes 1, 2, 3, 5, 7, and 11 panel B) was further digested with *NotI/SalI* (3.5 kb, 2.9 kb), *NheI* (5.8 kb, 0.6 kb), *XhoI* (3.4 kb, 3.0 kb), *and HindIII/EcoRI* (3.7 kb, 2.2 kb, 0.6 kb), respectively. The predicted fragments for the correctly targeted vector are in parentheses

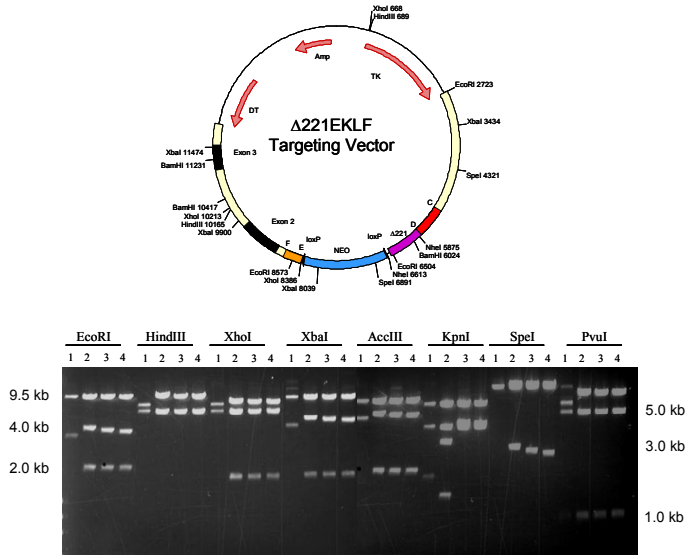
HA-Δ221EKLF-*Neo*-EF confirmed that the predicted sequence was present.

Finally, to obtain the final targeting vector (Figure 2.4A) that will be used for targeting in ES cells, the complete mini-targeting cassette was introduced into the gap-repaired plasmid by recombineering. The mini-targeting cassette containing CD-HA-Δ221EKLF-*Neo*-EF was first excised from PL452 by *NotI/SalI* digest and gel-purified. Then, the purified cassette (300 ng) was co-electroporated along with the gap-repaired plasmid DNA (50 ng) into EL350 cells, which had been induced for Red recombination at 42^{o}C. Subsequently, the bacterial cells were plated on ampicillin/kanamycin selective media. No growth was visible after 24-48 hr incubation at 32^{o}C. Co-electroporation was then attempted with varying amounts of targeting cassette (100-500 ng) and gap-repaired DNA (10-100 ng). Several ampicillin/kanamycin-resistant colonies were analyzed, however, there were no correct recombinants. I then decided to perform sequential electroporation. First, the gap-repaired DNA was electroporated into EL350 cells. The colonies were selected and the DNA digested with restriction enzymes to identify the bacterial colony containing the gap-repaired plasmid. Next, the mini-targeting cassette was electroporated into EL350 cells containing the gap-repaired plasmid. These cells had been induced for the Red recombination genes by a short incubation at 42^{o}C to allow homologous recombination to occur. This resulted in modification of the EKLF genomic locus contained in the gap-repaired plasmid by targeting the mini-cassette to the start site of EKLF.

Prior to gene targeting in ES cells, the final targeting vector was characterized structurally and functionally. First, to screen for aberrant gene rearrangements, DNA from the gap-repaired plasmid (denoted 1), Δ164EKLF targeting vector (denoted 2), Δ221EKLF targeting vector (denoted 3), and Δ253EKLF targeting vector (denoted 4) were digested with numerous restriction enzymes. Shown in Figure 2.4A are the restriction enzyme patterns after digestion with *EcoRI, HindIII, XhoI, XbaI, AccIII, KpnI, SpeI,* and *PvuI*, respectively. All samples displayed the expected digestion pattern by agarose gel electrophoresis. The predicted fragments are listed in Table 2.1. Next, to evaluate for mutations that may have occurred during recombineering, DNA from each targeting vector was submitted to the Hartwell Center at St Jude Children's Research Hospital for sequence analysis and confirmed that the targeting vectors had the correct sequences.

Finally, to ensure that the loxP sites in the targeting vectors would properly recombine in the presence of *cre* recombinase thereby removing the *Neo* cassette, the targeting vectors were electroporated into EL350 cells, which had been induced for *cre* expression by prior growth in arabinose-containing medium. Ampillicin-resistant colonies were picked and the DNA from these colonies was digested with *SpeI, HindIII, BamHI*, and *NheI*, respectively (Figure 2.4B, data shown for Δ221EKLF only). After *cre* induction, there is a loss of *SpeI* and *NheI* sites resulting in only one band. Digestion with *HindIII* and *BamHI* leads to a smaller product due to excision of the *Neo* cassette as demonstrated by one of the bands being shifted lower (*i.e.*, lower molecular weight).

A.



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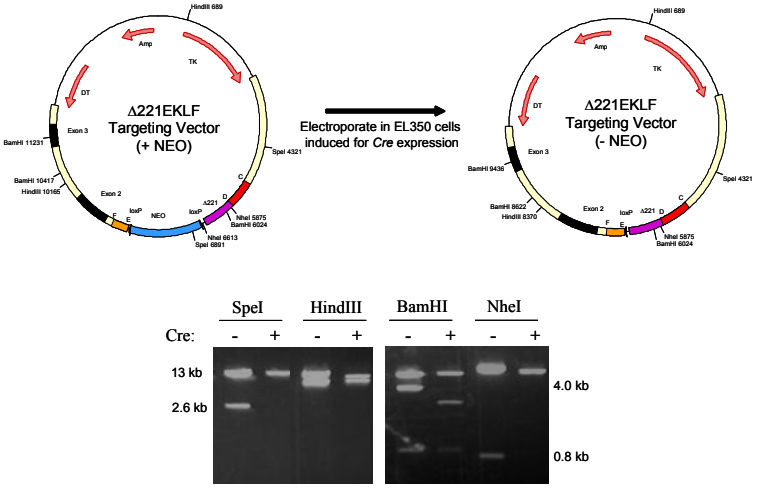

Figure 2.4. Verification of the final targeting vector using restriction enzyme analysis. (A) Plasmid map of the HA-Δ221EKLF final targeting vector. DNA was prepared from ampicillin/kanamycin-resistant colonies and subjected to digestion with various enzymes. Lane 1 = retrieval vector containing 11 kb EKLF BAC; lane 2 = HA-Δ164EKLF; lane 3 = HA-Δ221EKLF; lane 4 = HA-Δ153EKLF. (B) Plasmid maps of the HA-Δ221EKLF final targeting vector with and without Neo. The final targeting vector was electroporated in EL350 cells induced for *cre* expression and ampicillin-resistant colonies were picked. DNA from the colonies was digested with several restriction enzymes. CD, EF = EKLF homologies; DT = diphtheria toxin; TK = thymidine kinase; Amp = Ampicillin; Neo = Neomycin.

Table 2.1. Predicted fragment size with restriction enzyme digestion.

Enzyme	Retrieval (1) plasmid	Δ164 (2)	Δ221 (3)	Δ253 (4)
EcoRI	9.5 3.4	9.6 4.0 2.1	9.5 3.8 2.1	9.4 3.7 2.1
HindIII	7.0 5.9	9.6 5.9	9.5 5.9	9.4 5.9
XhoI	7.3 5.8	7.9 5.8 1.8	7.7 5.8 1.8	7.6 5.8 1.8
XbaI	7.3 5.6	8.9 4.8 1.9	8.9 4.6 1.9	8.9 4.5 1.9
AccIII	8.0 4.9	8.0 5.5 2.0	8.0 5.4 2.0	8.0 5.3 2.0
KpnI	7.1 4.0 1.8	7.1 4.0 3.0 1.4	7.1 4.3 4.0	7.1 4.2 4.0
SpeI	12.9	12.8 2.7	12.8 2.6	12.8 2.5
PvuI	6.6 5.2 1.0	9.3 5.2 1.0	9.1 5.2 1.0	9.0 5.2 1.0

Targeting of the murine EKLF genomic locus

A targeting vector was generated to introduce the Δ221EKLF encoding cDNA at the start ATG of the endogenous EKLF gene (Figure 2.5A). EKLF has only 3 exons (Miller & Bieker, 1993). In my targeting strategy, exons 2 and 3 of the endogenous gene were left intact. Stop codons were placed in all three reading frames to ensure proper translation of the knock-in allele. The 5' and 3' homology regions were 3 kb and 4 kb, respectively. MC1-thymidine kinase and PolII-diptheria toxin cassettes were included for double negative selection in the ES cells. This selection strategy has been successfully utilized in our laboratory to enrich for ES clones that have undergone homologous recombination, as opposed to random integration (V.P. personal communication). The data shown here are specific to HA-Δ221EKLF, however, targeting for HA-Δ164EKLF and HA-Δ253EKLF were completed in a similar fashion.

To replace the wild-type EKLF gene with HA-Δ221EKLF in ES cells, the targeting vector was linearized by *NotI* digestion and electroporated into 129/SvEv ES cells. After eight days of selection in G418 and ganciclovir, ES cell clones were picked and expanded. Genomic DNA from these clones was digested with *EcoRV* or *NheI* and analyzed by Southern blot analysis with 5' and 3' probes, respectively. The wild-type EKLF allele is detected as an 8.7 kb fragment with the 5' probe and as an 11.1 kb fragment with the 3' probe. The recombinant allele is detected as a 9.1 kb fragment with the 5' probe and as a 6.8 kb fragment with the 3' probe because of the introduction of novel *EcoRV* and *NheI* sites into the 5' and 3' regions of the genes, respectively, in the targeting vector. As shown in Figure 2.5B, ES DNA in lanes 3-7 of the top panel contain the 8.7 kb wild-type allele and the 6.8 kb knock-in allele with the 5' probe, whereas lanes 1-2 are wild-type ES cell DNA containing only an 8.7 kb fragment. Similarly, ES cell DNA in lanes 3-6 of the bottom panel contain the 11.1 kb wild-type allele and the 9.1 kb knock-in allele with the 3' probe, and lanes 1-2 are wild-type ES cell DNA containing only an 11.1 kb fragment. A total of 223 ES clones were screened, and 17 positive clones were identified (~ 8% recombinants).

Δ221EKLF heterozygous mice are phenotypically normal

ES cells properly targeted with Δ221EKLF were injected into C57Bl/6 wild-type blastocysts to generate Δ221EKLF chimeras. Male chimeras whose genetic composition is derived from the ES cells and donor blastocysts were mated with female C57Bl/6 mice to transmit the knock-in allele. To remove the *Neo* cassette, F1 offspring were then crossed with mice harboring a *cre* transgene under the control of the adenovirus EIIa promoter that targets the expression of *cre* recombinase to the early mouse embryo (Jackson Laboratory). Our studies utilized mice in which the *Neo* cassette has been removed, thereby eliminating the possibility of *Neo* gene expression interfering with our observed results. To identify animals with the transmitted allele, tail DNA was extracted and digested with *NheI* and hybridized with a 3' probe. As shown in Figure 2.6A, DNA from wild-type animals contains a single fragment at 11.1 kb, whereas heterozygous animals contain two fragments, an 11.1 kb wild-type fragment and a 9.2 kb knock-in

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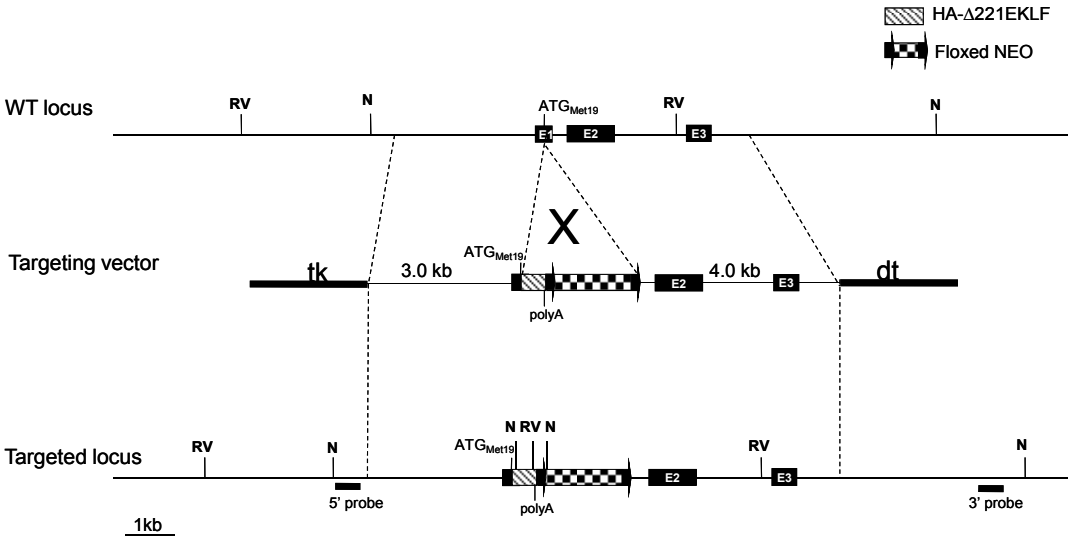

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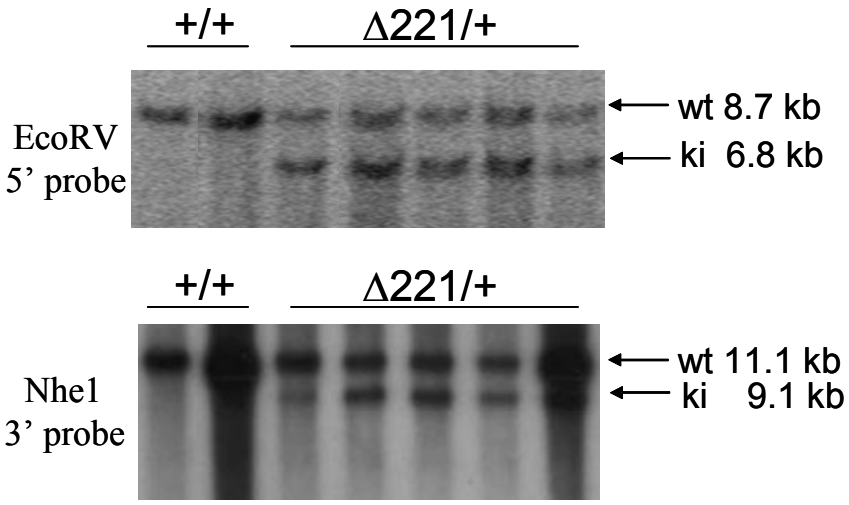
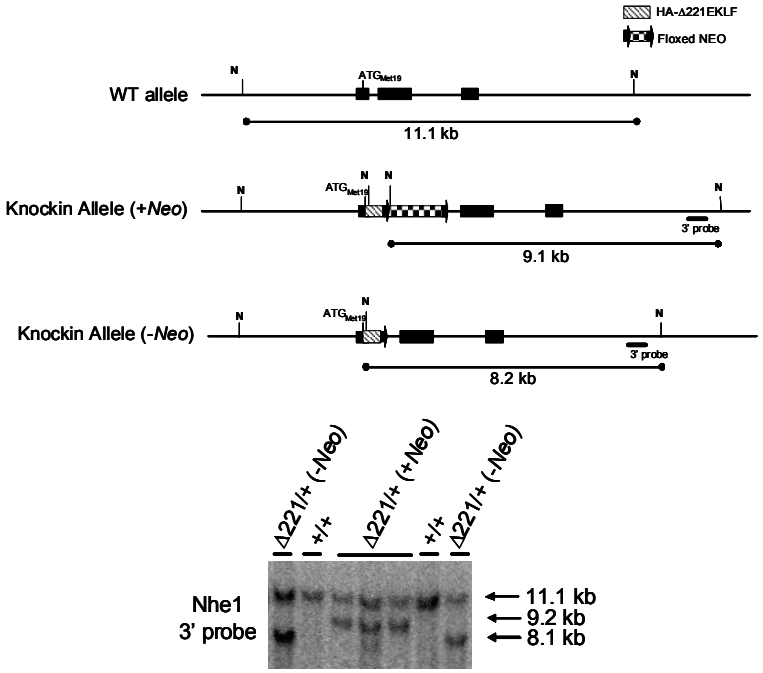


Figure 2.5. Targeting of cDNA encoding HA-Δ221EKLF into the endogenous EKLF locus. (A) Targeting strategy showing the wild-type EKLF locus, the targeting vector used for gene targeting in ES cells, and the targeted locus in which HA-Δ221EKLF was inserted utilizing the endogenous ATG start site thereby placing the knock-in mutant gene under the control of the *cis*-regulatory elements. Shaded rectangles indicate the location of the external probes used for Southern blotting. N = NheI, RV = EcoRV, tk = thymidine kinase; dt = diphtheria toxin (B) Southern blot analysis of ES clones using *EcoRV* digest with 5' probe and *NheI* digest and 3' probe. The wild-type allele is detected as an 8.7 kb and an 11.1 kb band, respectively. The knock-in allele is detected as a 6.8 kb and 9.1 kb band, respectively.

A.



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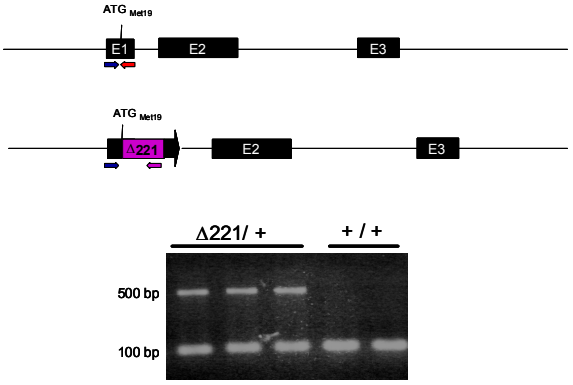


Figure 2.6. Identification of heterozygous mice expressing HA-Δ221EKLF. (A) Top panel: A schematic diagram of the wild-type allele and knock-in allele with and without *Neo* cassette with the predicted fragment s shown. Bottom panel: Southern blot analysis of mouse tail DNA digested with *NheI* and hybridized with 3' probe. The wild-type band is 11.1 kb; knock-in bands 9.2 kb (with *Neo*) and 8.1 kb (without *Neo*). (B) Top panel: A diagram of the wild-type and knock-in alleles. Colored arrows indicate the location of primers used in multiplex PCR. Dark blue = common forward primer; red = wild-type reverse primer; purple = knock-in reverse primer. Bottom panel: A representative photograph of agarose gel electrophoresis. PCR products amplified with DNA from wild-type animals and heterozygous knock-in animals. The wild-type product is 96 bp and the knock-in product is 500 bp.

fragment. Mice in which the *Neo* cassette has been excised have an 8.1 kb fragment, due to loss of a *NheI* site. Subsequently, a multiplex PCR method utilizing a common forward primer with two different reverse primers was developed to facilitate genotyping of the knock-in animals (Figure 2.6B). PCR of DNA from a wild-type animal amplified only a 96 bp fragment with the primers, whereas DNA from a heterozygous animal amplified a wild-type fragment of 96 bp and a knock-in fragment of 500 bp with the same primers.

Animals heterozygous for the EKLF gene have no discernable phenotype (Nuez *et al.*, 1995; Perkins *et al.*, 1995). There are, however, exceptions where deletion of a single gene copy leads to an abnormal phenotype (Johnson *et al.*, 1988; Muroya *et al.*, 2001; Deutschbauer *et al.*, 2005) whereby the heterozygous mutation on one allele blocks the activity of the wild-type protein is still encoded by the normal allele. To determine whether the knock-in mutant protein encoding the Δ221EKLF domain resulted in an unexpected phenotype, I evaluated Δ221EKLF heterozygous adult mice. First, analysis of the Mendelian ratio from heterozygous matings demonstrated that Δ221EKLF heterozygous animals survive to adulthood without any visible defects (Table 2.2). As expected, these studies also revealed that Δ221EKLF homozygous animals are not viable (see Chapter 3).

To evaluate the effects of the heterozygous knock-in allele on mouse hematopoiesis, I examined the peripheral blood obtained from Δ221EKLF heterozygous, EKLF-null heterozygous, and wild-type mice. Complete blood count (CBC) analysis revealed no abnormalities in the hematological parameters measured, including white blood cell (WBC) number, red blood cell (RBC) number, hemoglobin (Hgb), hematocrit (Hct), and platelet (Plt) count (Table 2.3). Similarly, Wright-Giemsa staining of the peripheral blood smear demonstrated that the circulating erythrocytes in Δ221EKLF mice are morphologically similar to those found in wild-type and EKLF-null heterozygous animals (Figure 2.7).

DISCUSSION

To generate the knock-in alleles of EKLF, I have utilized a lambda phage-based recombination method called recombineering. This method relies on homologous recombination in *E. coli* instead of the traditional subcloning method utilizing restriction enzymes and DNA ligases for vector construction, thus eliminating the time and difficulty encountered in traditional subcloning methods (Yu *et al.*, 2000; Copeland *et al.*, 2001; Liu *et al.*, 2003). The basic steps of recombineering are outlined in Figure 2.1. The targeting vectors have been designed in such a way that the cDNA encoding the truncation mutation of EKLF (i.e. Δ221EKLF) would be expressed under the endogenous EKLF promoter and *cis*-regulatory elements. Hence, it is anticipated that the expression of the mutant genes, Δ164EKLF, Δ221EKLF, and Δ253EKLF, would resemble that of the wild-type endogenous EKLF protein. This approach ensures the proper expression of the mutant factor during development and eliminates some of the obstacles in using cell lines and artificial rescue constructs (Tsai *et al.*, 1998). This approach has been used to

Table 2.2. Genotyping of adult animals from heterozygous matings.

Genotype	Number of adult animals[a]
WT/WT	213 / 154
Δ221EKLF/WT	402 / 307
Δ221EKLF/ Δ221EKLF	0 / 154
Total[b]	615

[a] numbers are expressed as observed / expected
[b] total number of animals analyzed

Table 2.3. Complete blood count analysis of Δ221EKLF heterozygous adult mice.

Parameter	WT/WT	WT/Δ221EKLF	(*p*-value)	WT/EKLF^{null}	(*p*-value)
WBC (x10^3/μL)	6.22 ± 1.73	10.67 ± 2.48	(0.54)	11.40 ± 1.94	(0.13)
RBC (x10^6/μL)	9.09 ± 0.68	8.48 ± 0.51	(0.49)	9.10 ± 0.34	(0.74)
Hgb (g/dL)	13.40 ± 0.70	12.83 ± 0.23	(0.28)	13.43 ± 0.42	(0.97)
Hct (%)	39.60 ± 1.73	38.30 ± 2.44	(0.25)	39.07 ± 1.88	(0.95)
Plt (x10^3/μL)	824 ± 148	1005 ± 73.5	(0.13)	1048 ± 83	(0.13)

Note: Animals were age and sex matched with 3 adult mice analyzed per cohort. Data shown are mean ± standard deviation. Data were analyzed by the student's t-test; *p*-values are listed in parentheses compared to WT.

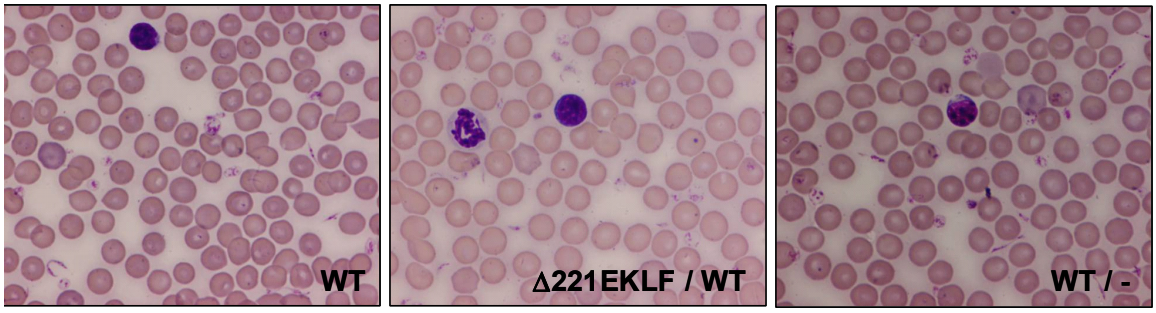


Figure 2.7. Normal circulating erythrocytes in Δ221EKLF heterozygous adult animals. Peripheral blood smear (Wright-Giemsa) of adult animals obtained by retro-orbital bleeds. Erythrocytes from Δ221EKLF heterozygous animals are morphologically similar to erythrocytes from wild-type and EKLF heterozygous animals. Magnification x100.

demonstrate that myogenin can replace Myf5 in rib cage development (Hanks *et al.*, 1998) and that En-2 can replace the function of En-1 in mid-hindbrain development (Wang *et al.*, 1996). Similarly, knock-in mutation of GATA-3 into the GATA-1 locus can partially replace the highly homologous factor in erythroid cell development (Tsai *et al.*, 1998).

Utilizing the recombineering method, I found it difficult to co-electroporate the gap-repaired plasmid and the mini-targeting cassette as originally described (Liu *et al.*, 2001). Instead of co-electroporation, I electroporated the mini-targeting cassette into electrocompetent EL350 cells which harbored the correct retrieval vector as determined by restriction enzyme and sequence analysis. This alternative method required an intermediate step to make the retrieval vector-containing EL350 cells electrocompetent before electroporation with the mini -targeting cassette. In contrast, the previously described co-electroporation method required a single step in which the retrieval vector and mini-targeting cassette DNA were co-electroporated into previously frozen electrocompetent EL350 bacterial cells. The time required for my altered approach was not significant and did not greatly affect the total time required to obtain the final targeting vectors.

Although the major step of vector construction relied on homologous recombination in bacteria by taking advantage of the lambda phage recombination genes, certain intermediate steps such as construction of a plasmid to retrieve an 11 kb of sequence spanning the EKLF gene from the EKLF BAC and the mini-targeting vectors utilized traditional subcloning methods. As a result, I encountered obstacles common to traditional subcloning. First, I found it difficult to perform a triple ligation (fragments AB, XY, and vector) to construct the retrieval vector. Very few colonies grew on the selection plates, and the selected colonies did not harbor the correct plasmid. To circumvent this problem, I increased the ratio of vector to insert in the ligation reaction from 1:3 to 1:5 which yielded more colonies to screen. Similarly, I first tried to construct the mini-targeting vector in a single reaction by ligating fragments CD, HA-Δ221EKLF, *Neo*-cassette, and EF into a pBlueScript vector. This yielded no growth on the agar plates. An alternative method was to sequentially subclone each fragment into a vector until a complete mini-targeting vector was constructed. To attempt this altered strategy, I had to design new PCR primers for fragments CD and EF with different restriction sites to facilitate subcloning. To my advantage, I used the PL450 vector which already contained the *Neo* cassette to subclone in fragment EF. Next, I performed a triple ligation using increased ratio of vector to inserts to subclone fragments CD and HA-Δ221EKLF into PL450-EF. Overall, construction of the mini-targeting vectors utilizing traditional subcloning methods proved to be the most time consuming portion of generating the knock in alleles of EKLF. However, I was not hindered by the restriction sites as the site for the restriction enzyme of interest was conveniently included in the PCR amplification primers.

In my targeting strategy, I have included a *Neo* cassette adjacent and downstream of the Δ221EKLF cDNA to facilitate selection during recombineering in bacteria and targeting in ES cells. Previous studies of knock-out mice have demonstrated the

unpredictable phenotypes that can be caused by the retention of *Neo* cassettes (Olson *et al.*, 1996; Pham *et al.*, 1996). For example, studies in which several specific mutations were generated within the human or murine β-globin LCR by insertion of PGK-*Neo* led to abrogation of the expression of multiple globin genes downstream from the cassette. However, deletion of the selectable marker cassette resulted in restoration of LCR activity, suggesting that the cassette interfered with normal interactions between the LCR and downstream regulatory elements in the gene promoters (Pham *et al.*, 1996). To improve Δ221EKLF expression that could be altered by an adjacent downstream *Neo* transgene and to rule out any toxic effects of *Neo* expression at this locus, the *Neo* cassette in Δ221EKLF heterozygous mice was removed by breeding to a *cre* transgenic mouse line (Lasko *et al.*, 1996). Subsequent studies in this dissertation have been completed in mice in which the *Neo* cassette has been removed.

Analysis of the Δ221EKLF heterozygous animals provides an early glimpse into the functionality of the mutant knock-in protein *in vivo*. Given that adult Δ221EKLF heterozygous animals develop normally with no discernible phenotype, I speculate that the mutant knock-in allele does not exhibit a dominant negative or haploinsufficient phenotype. This observation is supported by my results from the CBC analysis and peripheral blood smear which are consistent with a normal hematological phenotype. Although the results are not significant (p-value > 0.05), it should be noted that these studies were performed with three animals in each cohort. To minimize the possibility of prematurely rejecting a dominant negative phenotype in Δ221EKLF animals, studies in Δ221EKLF heterozygous animals should be repeated with a larger sample size. Indeed, it is not uncommon for this phenotype to occur with loss-of-function studies such as been described for the transcription factors *TWIST* (Johnson *et al.*, 1988), *GATA3* (Muroya *et al.*, 2001), and *STAT6* (Bürgis & Gessner, 2007). For instance, mutant TWIST protein is expressed and acts as a dominant negative protein by binding to target promoters and then blocking gene transcription (Johnson *et al.,* 1998). I will demonstrate in Chapter 3 that the mutant Δ221EKLF protein is expressed in fetal liver and possesses chromatin remodeling function *in vivo.*

In summary, I have constructed knock-in targeting vectors in which cDNAs encoding three different amino terminal truncation mutants of EKLF have been engineered into the initiation site of the endogenous murine EKLF gene utilizing the recombineering method. Using this method, I have constructed all three targeting vectors in a relatively short time. Subsequently, the vectors have been used to target the endogenous EKLF locus in ES cells via homologous recombination in order to generate Δ221EKLF heterozygous animals. Following the recommendations of my research committee, I have chosen to focus my dissertation studies on one strain of mice, the Δ221EKLF expressing animals. This domain of EKLF appears to be the most interesting to study *in vivo* as it has been previously identified in cellular assays to possess chromatin remodeling properties, however, lacks the ability to transactivate β-globin gene transcription (Brown *et al.*, 2002). This animal model will be useful to test my hypothesis that expression of Δ221EKLF domain is sufficient to reorganize the local chromatin architecture at the β-globin promoter but is insufficient to transcriptionally activate β-globin *in vivo.* In the following chapter, I will describe the functional consequences of

expression of Δ221EKLF mutant protein in mice homozygous for the knock-in locus.

CHAPTER 3: CHARACTERIZATION OF THE MOLECULAR CONSEQUENCES OF Δ221EKLF EXPRESSION *IN VIVO*

INTRODUCTION

The β-globin locus serves as a paradigm for studying the regulation of a multigene locus. The murine β-globin locus, located on chromosome 7, encodes four genes (5'-εy-βh1-β^{maj}-β^{min}-3') which are arranged in the order of their expression during ontogeny. Appropriate regulation of the globin genes in a tissue- and developmental-specific manner resides in DNA elements located proximal and distal to the genes. The β-globin locus control region (LCR) is a powerful DNA element located far upstream of the globin genes. The LCR is required for high level expression of all the genes during development (Grosveld *et al.*, 1987; Bender *et al.*, 2000). The precise mechanism of how the LCR enhances globin gene transcription is still unsettled; however, it is thought to involve some method of physical communication between the LCR and the globin genes (Bulger & Groudine, 1999; Engel & Tanimoto, 2000).

The murine LCR consists of a series of DNase I hypersensitive sites (HS1-6) that contain binding sites for transcriptional factors. A current hypothesis regards the LCR as a holocomplex that enhances globin gene expression through recruitment of chromatin remodeling, coactivator, and transcriptional complexes (Wijgerde *et al.*, 1995; Bungert *et al.*, 1999; Tolhuis *et al.*, 2002). Indeed, it has been shown that RNA polymerase II (PolII) is recruited to the LCR (Johnson *et al.*, 2003). Similarly, GATA-1, NF-E2, and EKLF have been shown to bind the HS of the LCR (Pevny *et al.*, 1995; Fujiwara *et al.*, 1996; Forsberg *et al.*, 2000; Zhou *et al.*, 2006). Furthermore, both GATA-1 and EKLF are required for β-globin LCR/promoter interaction (Drissen *et al.*, 2004; Vakoc *et al.*, 2005).

The β-globin promoter contains several *cis*-acting elements that modulate the expression of the β-globin gene. Of these, the CACC motif is of the most interest to the present study. Mutations of this consensus sequence lead to a thalassemic state (Orkin *et al.*, 1984) and specifically disrupt the binding of EKLF (Miller & Bieker, 1993; Feng *et al.*, 1994). Disruption of the EKLF gene by homologous recombination has demonstrated its non-redundant role during erythroid development. EKLF-null embryos die of a lethal anemia by embryonic day 14-15 of gestation (E14-15), as definitive erythroid cells failed to produce β-globin transcripts *in vivo* (Nuez *et al.*, 1995; Perkins *et al.*, 1995). Further analysis of these EKLF null embryos revealed loss of a developmentally specific DNase I hypersensitivity site in the proximal β-globin promoter of E14 fetal liver erythroblasts derived from EKLF null embryos (Wijgerde *et al*., 1996). Because the degree of DNase I hypersensitivity of a given locus correlates with nucleosomal remodeling (Steger & Workmann, 1996; Pazin *et al.*, 1997), these findings are consistent with the idea that EKLF is required for chromatin reorganization at the β-globin promoter in definitive erythroid cells.

Studies to elucidate the molecular mechanisms by which EKLF modulates

chromatin remodeling at the β-globin promoter have relied heavily on *in vitro* chromatin reconstitution assays (Armstrong *et al.*, 1998; Kadam *et al.*, 2000). These studies have identified a SWI/SNF-containing multiprotein complex, E-RC1, which interacts with the zinc finger DNA binding domain of EKLF. This interaction results in chromatin remodeling and transcriptional activation of a chromatinized β-globin template. Additional evidence supporting this interaction comes from studies demonstrating that EKLF interacts with BRG1, a subunit of E-RC1 (Zhang *et al.*, 2001). Contrary to *in vitro* chromatin assays (Kadam *et al.*, 2000), our laboratory has shown that the zinc finger domain alone is not sufficient to remodel chromatin at the β-globin promoter in erythroid cell lines (Brown *et al.*, 2002). Additional sequences in the amino terminus are required for EKLF-dependent chromatin remodeling and EKLF interaction with BRG1. However, evidence for a direct interaction between EKLF and SWI/SNF components *in vivo* is still lacking.

To test my hypothesis that expression of Δ221EKLF alone is sufficient to alter the local chromatin structure at the β-globin locus *in vivo,* I will explore the consequences of expression of Δ221EKLF and analyze the determinants required for chromatin remodeling and β-globin transcription *in vivo.* Despite the advances in our understanding of the function of EKLF as a chromatin modifier and transcriptional activator, knowledge about the direct interaction of EKLF and SWI/SNF components and how EKLF modulates chromatin *in vivo* remains elusive. Utilizing the newly generated Δ221EKLF animals, I show that expression of Δ221EKLF results in embryonic lethality similar to mice lacking EKLF. In stark contrast to lack of EKLF, expression of Δ221EKLF completely restores the local chromatin structure at the β-globin promoter. However, this domain is unable to rescue β-globin gene transcription *in vivo* and fails to recruit GATA-1 and NF-E2 to the β-globin promoter. My results suggest that the Δ221EKLF domain is sufficient for chromatin reorganization at the β-globin promoter, thus priming the β-globin gene for transcriptional activation. Furthermore, I show that nucleosomal remodeling and histone modifications at the β-globin promoter are insufficient to activate high-level β-globin transcription *in vivo.*

MATERIALS AND METHODS

RNA analysis

Total RNA isolated from fetal liver of E14.5 embryos was subjected to RT-PCR analysis. RNA was extracted using the Trizol method and cDNA was prepared using the Superscript III First Strand System (Invitrogen) following the manufacturer's protocols. Briefly, fetal livers from E14.5 embryos were homogenized in Trizol (200-500 μL) reagent (Invitrogen). RNA (10 μg) was treated with Turbo DNase (2 μL)(Ambion) in a 50 μL reaction to remove genomic DNA contamination. Following DNase treatment, DNase-treated RNA (11 μL) was used for first strand DNA synthesis.

For RT-PCR of EKLF gene expression, cDNA (2 μL) was analyzed on a BIO-RAD PCR machine with the following settings: 94°C for 3 min, then 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, followed by 72°C for 10 min. PCR products were analyzed on agarose gels. RT-PCR primers were generated using the Primer 3 program and are as follows: Exon 1: forward: 5'-ATGAGGCAGAAGAGAGAGGAG-3'; reverse: 5'-TGAGTGTACTGATGGAGGGTAAGA -3'; Exon 2: forward: 5'-CACAGTACCAAGGCCACTTC -3'; reverse: 5'-GTCCCAGGTCCAAGACAATTC-5'; Exons 2/3: forward: 5'-GGGAAGAGCTACACCAAGAGC-3'; reverse: 5'-GAAGGGACGATGTCCAGTGT-3'; HA-Δ221EKLF: forward: 5'-TATGCTAGCCTCCCGGGTTA-3'; reverse: 5'-GGTCTCGGCTATCACACCTG-3'; HPRT: forward: 5'- GCAGTACAGCCCCAAAATGG-3'; reverse: 5'-AACAAAGTCTGGCCTGTATCCAA-3' (Hodge *et al.*, 2006).

β-globin mRNA and primary transcripts were measured by real time RT-PCR utilizing SYBR green fluorescence on an Applied Biosystems Prism 7000 machine. The amount of product was determined relative to a standard curve generated from a titration of the cDNA. Dissociation curves after amplification showed that primer pairs generated single products. The primers used for real time RT-PCR are shown in Appendix Table A.5. Each sample was run in duplicate with at least five independent experiments performed.

Protein analysis

Whole cell extracts were prepared from fetal liver of E14.5 embryos using the P-TER reagent (Pierce) according to the manufacturer's recommendations. Protein concentration was measured by the Bradford Assay reagent (BioRad). One hundred micrograms of protein was mixed with loading dye and denatured by boiling for 5 min. The proteins were resolved on a 15% SDS-PAGE gel followed by wet transfer. The blots were blocked in 5% milk in 0.1% PBS/Tween-20 for 1 h at room temperature with constant rocking. Primary antibody at 1:200 dilution or 1:400 dilution for HA and EKLF, respectively, was added and the blots were probed overnight in the cold room with constant rocking. Blots were washed once for 15 min and twice for 5 min times with 0.1% PBS/Tween-20. Secondary antibody conjugated with horseradish peroxidase (HRP) was added at a dilution of 1:1000 and incubated for 1 h at room temperature. The blots were washed three times as before with 0.1% PBS/Tween-20. Peroxidase activity was visualized by enhanced chemiluminescence (ECL) using Western blotting detection reagents from Amersham Biosciences. The Western blots were probed with HA-antibody (Santa Cruz), Cunningham laboratory generated EKLF-antibody directed toward the zinc fingers (J.M.C unpublished), and actin-HRP (Abcam) as loading control.

Cytospin, sections, and staining

Fetal liver erythroblasts from E14.5 embryos were made into single cell suspension in D10 (DMEM + 10% FBS) and cytocentrifuged onto glass slides. For immuno-localization studies, the cells were fixed in 4% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100-PBS, and blocked in 10% normal donkey serum (Jackson ImmunoResearch) for 1 hr at room temperature. Anti-HA antibody was added to the slides and incubated at 4°C overnight. A secondary antibody, conjugated to FITC (Molecular Probes), was applied for another 2 h of incubation at room temperature. DNA staining was carried out using DAPI (4', 6'diamidino-2-phenylindole) (Vector Shield). Slides were visualized with a Leica DM IRB fluorescence microscope.

Staining with benzidine-Giemsa (Sigma) was performed according to the manufacturer's recommendations. Briefly, cytospin preparations were fixed in cold methanol for 2 min, allowed to air dry, and then stained in benzidine for 20 min. After 20 min, the slides were rinsed with distilled water and placed in Giemsa stain for another 20min. Slides were rinsed with water, air dried, and mounted with cover slips.

To prepare sections of the fetal liver, wild-type, Δ221EKLF, and EKLF-null E14.5 fetal livers were immersion fixed in 4% formalin overnight, or longer, before submission to the Core Pathology Department at the University of Chicago. The fetal livers were processed for sectioning and stained with H&E and Prussian blue. Slides were viewed under a Nikon Eclipse E400 microscope fitted with a camera.

DNase I sensitivity assays

DNase I sensitivity assays were performed essentially as described by McArthur *et al.* (2001) with slight modifications. Briefly, fetal livers were harvested from E14.5 mouse embryos and made into single cell suspension by passing through a 70 µm cell strainer. Cells (6×10^6) were washed once with cold PBS and resuspended with 5 ml of buffer A (15 mM Tris HCl (pH 7.6), 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.15 mM sperminine). Cells were lysed in the presence of 0.5 % (v/v) NP40, and nuclei were collected and resuspended in 1.2 mL of digestion buffer (buffer A supplemented with 3 mM $CaCl_2$, 75 mM NaCl). 200 µL of the nuclei mixture was added to Eppendorf tubes containing 50 µL of the digestion mixture containing 0-18 units of DNase I (ROCHE). Digestions were carried out at 37 °C for five minutes before being stopped by the addition of an equal volume of stop buffer (0.1 M NaCl, 0.1 % (w/v) SDS, 50 mM Tris-HCl (pH 8.0), 100 mM EDTA). The samples were treated with proteinase K (500 µg/mL) at 55°C overnight and DNA recovered by extraction with phenol/chloroform and precipitation in ethanol. The DNA was then dialyzed against two changes of TE buffer, re-precipitated and diluted in water to a concentration of 10 ng/µL as measured by spectrophotometry.

Real time PCR was performed on the DNA samples using SYBR green mix in a total reaction volume of 25 µL. To quantify the amount of DNA remaining following

DNase I treatment, a standard curve was generated from titration of undigested genomic DNA. The values at each concentration of DNase I was normalized to a previously determined DNase I insensitive gene, Nf-M (McArthur *et al.*, 2001). Dissociation curves after amplification showed that a single product was formed for each primer pair. The primer pairs used were: βmaj promoter forward: 5'-CAGGGAGAAATATGCTTGTCATCA-3' and βmaj promoter reverse: 5'-GTGAGCAGATTGGCCCTTACC-3'; Nf-M forward: 5′-GCTGGGTGATGCTTACGACC-3′ and Nf-M reverse: 5′-GCGGCATTTGAACCCTCTT-3′ (McArthur *et al.*, 2001).

Chromatin immunoprecipitation assays

ChIP assays were performed as previously described (Forsberg *et al.*, 2000; Kiekhaefer *et al.*, 2002), with slight modifications. Fetal livers from E13.5 mouse embryos were harvested and passed separately through a 70 μm cell strainer into 20 ml of DMEM-based medium supplemented with 10% FBS. Protein-DNA cross-linking was performed by incubating suspensions of fetal liver (at a concentration of 10^6 cells/mL) with formaldehyde at a final concentration of 0.8% for 10 min at room temperature with gentle agitation. Glycine (0.125 M) was added to quench the reaction. Cells were then collected by centrifugation at 1000 rpm for 5 min, washed in cold PBS and a second wash in cold PBS supplemented with Roche complete EDTA-free protease inhibitor and PMSF (Fluka). Cross-linked cells were snap frozen and stored at -70°C until further processing.

Immunoprecipitation was performed with 1 x 10^7 cells per IP condition. The buffer composition used in the ChIP assay is listed in the Appendix Table A.3. Nuclei were isolated by incubation in cell lysis buffer for 10 min on ice followed by centrifugation at 600 × *g* for 5 min. Nuclei were lysed in nuclei lysis buffer for 10 min on ice. The lysate was sonicated for 20 minutes total using the Bioruptor sonicator (Diagenode, Belgium) set on High power and cycles of 30 seconds ON and 30 seconds OFF to obtain an average size of 500 bp. Soluble chromatin was diluted 1:4 with IP dilution buffer and precleared by addition of 5 μL/mL preimmune serum followed by 50 μL of salmon sperm DNA/Protein A agarose (Millipore). An aliquot ($1/10^{th}$ of the total volume) of precleared chromatin was removed (input) and used in the subsequent PCR analysis. The remainder of the chromatin was incubated with and without primary antibodies for 4 hours at 4°C. Immune complexes were collected by incubation with 75 μL salmon sperm DNA/Protein A agarose (Millipore) for 2 h at 4°C. A control sample was prepared in all experiments in which IP wash buffer 1 was added instead of chromatin. Protein A agarose pellets were washed twice with 500 μL aliquots of IP wash buffer 1, once with IP wash buffer 2, and twice with TE buffer. Immune complexes were eluted twice with 100 μL of IP elution buffer made fresh and NaCl (0.4 M) were added, and cross-links were reversed by incubation overnight at 65°C. Samples were digested with Proteinase K (0.3 mg/mL) for 2 h at 45°C. DNA was purified by Qiaquick PCR purification kit (QIAGEN) and eluted twice with 50 μL elution buffer each time.

Immunoprecipitated DNA (2 μL) was analyzed by real-time PCR (Applied

Biosystems Prism 7000) with the appropriate primer pairs. Samples from at least three independent immunoprecipitations were analyzed. Product was quantified by SYBR green fluorescence in 25 μL reactions, and the amount of product was determined relative to a standard curve generated from a titration of input chromatin. Dissociation curves after amplification showed that primer pairs generated single products.

Primer pairs used in ChIP analysis are listed in Appendix Table A.4. The antibodies used in ChIP analysis were anti-HA antibody (Santa Cruz, sc-805x), anti-acetylated histone H3 antibody (Upstate, 06-599), anti-RNA PolII (Santa Cruz, sc-899x), anti-GATA-1 (Abcam, ab11963), and anti-NF-E2 (Santa Cruz, sc-291x). Normal rabbit IgG (Santa Cruz, sc-2027) was used as a control.

RESULTS

Δ221EKLF homozygous animals die *in utero* and exhibit ineffective erythropoiesis

Initial studies analyzing the matings of Δ221EKLF heterozygous animals (described in Chapter 2) revealed that Δ221EKLF homozygous adult mice are not viable. Of 615 adult mice genotyped, no live born Δ221EKLF homozygous animals were observed. This observation is consistent with an embryonic lethal phenotype.

To determine when the animals succumb *in utero*, timed pregnancies were established. These studies revealed that Δ221EKLF homozygous animals died by day 15.5 (E15.5) of gestation, a similar timepoint to EKLF-null embryos (Nuez *et al.*, 1995; Perkins *et al.*, 1995) (Table 3.1). Furthermore, gross examination of the Δ221EKLF embryos at day 15.5 of gestation revealed a pale embryo with a smaller and paler fetal liver as compared to a wild-type littermate control (Figure 3.1). These observations are consistent with the phenotype previously described in EKLF-null embryos of similar chronology (Perkins *et al.*, 1995).

The Δ221EKLF embryos appeared to lack any blood in the circulatory system, consistent with defective erythropoiesis *in vivo*. This observation was supported by benzidine-Giemsa staining of cytospun preparations of fetal liver erythroblasts and Prussian blue staining of fetal liver sections. As shown in Figure 3.2, there are very few benzidine-positive cells in fetal liver from Δ221EKLF embryo. To quantify the difference, the number of benzidine-positive cells was manually counted under low magnification. As shown in Table 3.2, similar to EKLF-null embryos, Δ221EKLF embryos had 33% fewer benzidine-positive cells when compared to wild-type embryos. Benzidine stains for hemoglobin (i.e. brown color), a hallmark of terminal erythroid differentiation (Zhang *et al.*, 2003). These observations, thus, suggest a failure of the Δ221EKLF-expressing erythroblasts to properly accumulate hemoglobin and execute terminal erythroid differentiation as previously demonstrated in EKLF-null cells (Drissen *et al.*, 2005; Pilon *et al.*, 2008). Furthermore, the Δ221EKLF erythroblasts, like EKLF-null erythroblasts, are misshapen as evident by the irregularly shaped cell membrane

Table 3.1. Genotyping of embryos at different stages of gestation from heterozygous matings.

Genotype	E10.5 [a]	E13.5	E14.5	E15.5	Adult
WT/WT	12/12	30/27	109/89	17/29	213/154
Δ221EKLF/WT	22/23	57/55	175/179	29/27	402/307
Δ221EKLF/Δ221EKLF	13/12	22/27	73/89	4/13	0/154
Total[b]	47	109	357	53	615

[a] numbers are expressed as observed/expected
[b] total number of animals analyzed

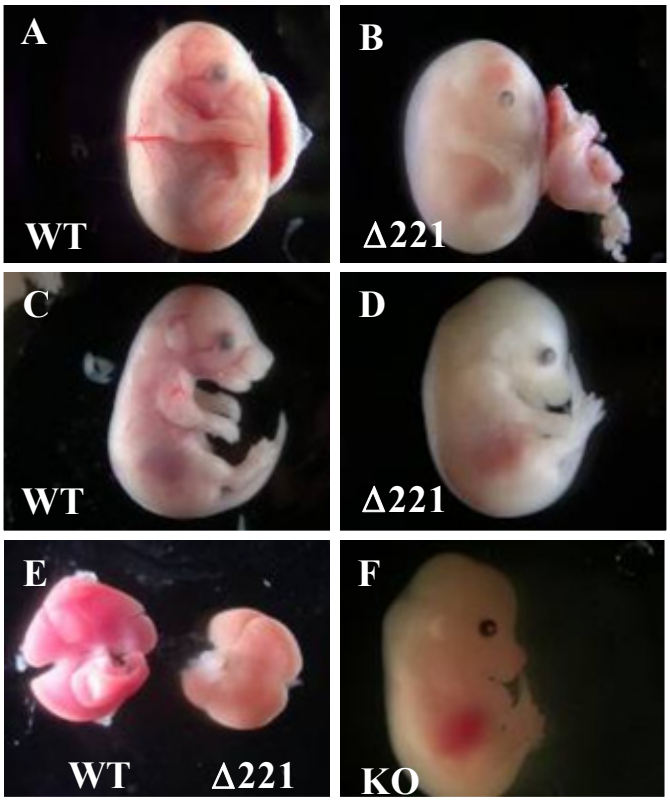


Figure 3.1. Δ221EKLF embryos are anemic. (A-D) Photographs of wild-type and Δ221EKLF embryos at E15.5. The homozygous Δ221EKLF embryo is pale and slightly smaller compared to wild-type littermate. A marked deficit of circulating red cells is evident. (E) Fetal liver from E15.5 wild-type and Δ221EKLF embryos. The Δ221EKLF fetal liver is smaller and paler than fetal liver from a littermate control. (F) Photograph of E15.5 EKLF-null embryo. The EKLF-null embryo is pale.

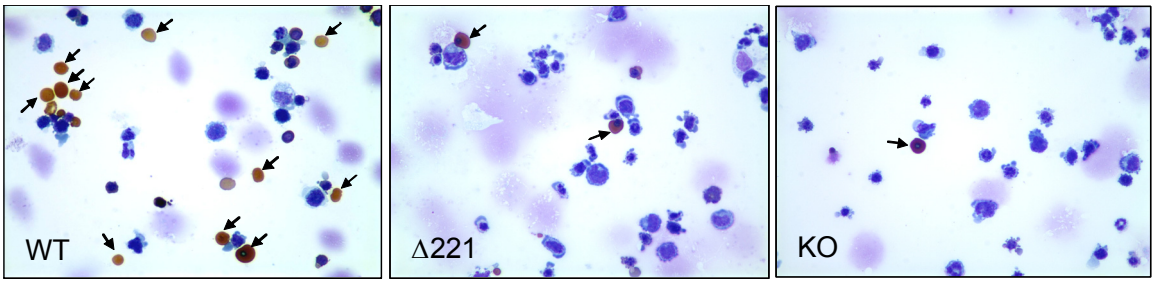


Figure 3.2. Defects in cell shape and content in Δ221EKLF fetal liver erythroblasts. Cytospin preparations of E14.5 fetal liver erythroid cells were stained with benzidine-Giemsa. There are fewer benzidine-positive cells (denoted by arrowheads) from Δ221EKLF fetal liver. Defective erythroid cell membrane is noted in Δ221EKLF. Original magnification x 100.

Table 3.2. Quantitative analysis of benzidine-positive E14.5 fetal liver erythroblasts.

Genotype	**# benzidine-positive cells/0.24mm^2 field**
WT	25 ± 4.2
Δ221EKLF / Δ221EKLF	8 ± 2.7
KO	7 ± 1.3

Note: Data shown as average number of benzidine-positive cells counted from three different fields (± SD). Fetal liver from 2-3 embryos of each genotype were analyzed.

compared to wild-type erythroblasts. This observation is consistent with the idea that Δ221EKLF erythroblasts have membrane defects as has been described in EKLF-null cells (Drissen et al, 2005; Hodge *et al.*, 2006). Similarly, Prussian blue staining of fetal liver sections revealed excess iron deposits (i.e. blue particles) in the fetal liver of Δ221EKLF embryos (Figure 3.3, bottom panel). This observation is consistent with the idea that Δ221EKLF expressing erythroblasts fail to properly accumulate hemoglobin during terminal erythroid differentiation similar to that described in erythroblasts lacking EKLF (Perkins *et al.,* 1995; Drissen *et al.,* 2005). Together, these observations support the idea that Δ221EKLF mice exhibit a phenotype consistent with ineffective erythropoiesis, comparable to EKLF-null mice (Nuez *et al.*, 1995; Perkins *et al.*, 1995).

Δ221EKLF is expressed in fetal liver

To evaluate the expression of the mutant knock-in allele *in vivo,* RNA from fetal liver erythroblasts derived from E14.5 embryos was analyzed by RT-PCR. As shown in Figure 3.4A, the mutant transcript is detected only in RNA extracted from Δ221EKLF fetal liver erythroblasts. Furthermore, the downstream endogenous exons 2 and 3 of EKLF, which were left intact in the targeting strategy, are not transcribed in these mice. This observation is expected given that a polyadenylation signal was included in the targeting strategy to prevent interference from the endogenous downstream exons. Thus, it appears that the mutant knock-in allele is indeed transcribed *in vivo.*

Because mRNA expression does not necessarily correlate with protein abundance (Greenbaum *et al.*, 2003), Western blotting analysis was performed to detect the Δ221EKLF mutant protein *in vivo*. The Δ221EKLF encoding cDNA included a HA epitope tag at the amino terminus to facilitate immunological detection. Using HA-specific anti-sera, the mutant protein was detected in the fetal liver (Figure 3.4B). However, the results do not compare the level of mutant protein expression to that of wild-type EKLF protein. Anti-EKLF antibodies are commercially available, however, the epitopes are directed to sequences outside of the Δ221EKLF domain. Previously, our laboratory had generated an antibody directed against the zinc finger DNA-binding domain of EKLF (J.M.C. unpublished). Using this antibody, the mutant Δ221EKLF protein was detected in the fetal liver of Δ221EKLF heterozygous and homozygous embryos (Figure 3.4C) albeit at a lower level than wild-type EKLF protein. The lower level of knock-in protein could result from instability or altered regulation of the knock-in protein. However, I have shown that Δ221EKLF protein retains full chromatin remodeling properties in a similar manner to wild-type EKLF protein (see below). More importantly, the endogenous wild-type EKLF protein is not detected in the fetal liver of homozygous knock-in animals. The subcellular localization of the mutant protein was analyzed by immuno-staining. As shown in Figure 3.5 (panels j-l), the mutant protein stained with anti-HA antibody co-localizes in the nucleus as indicated by DAPI staining. Taken together, these results demonstrate that the mutant knock-in protein is expressed in the fetal liver and is localized to the nucleus.

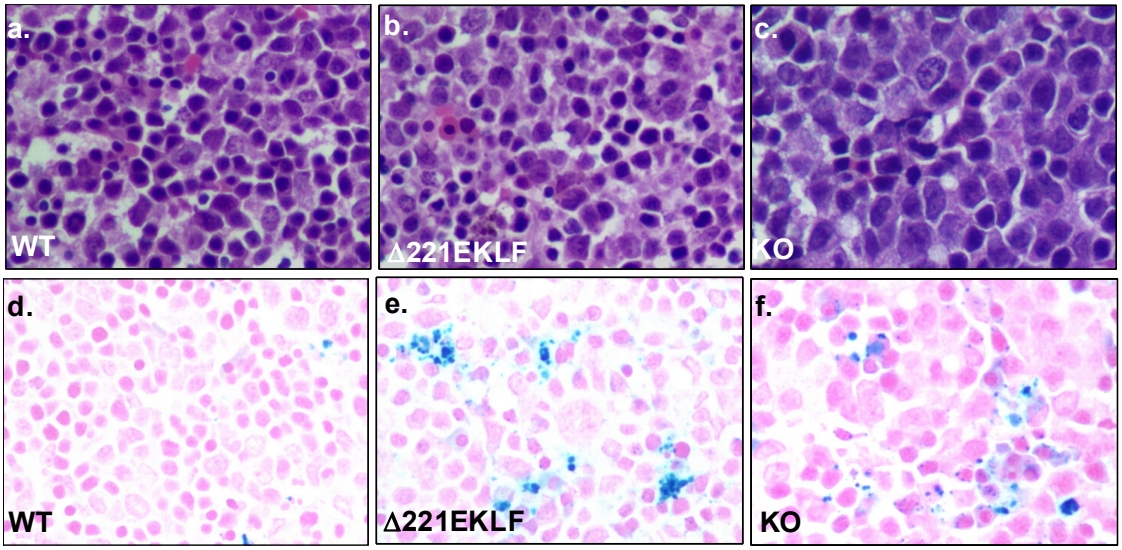
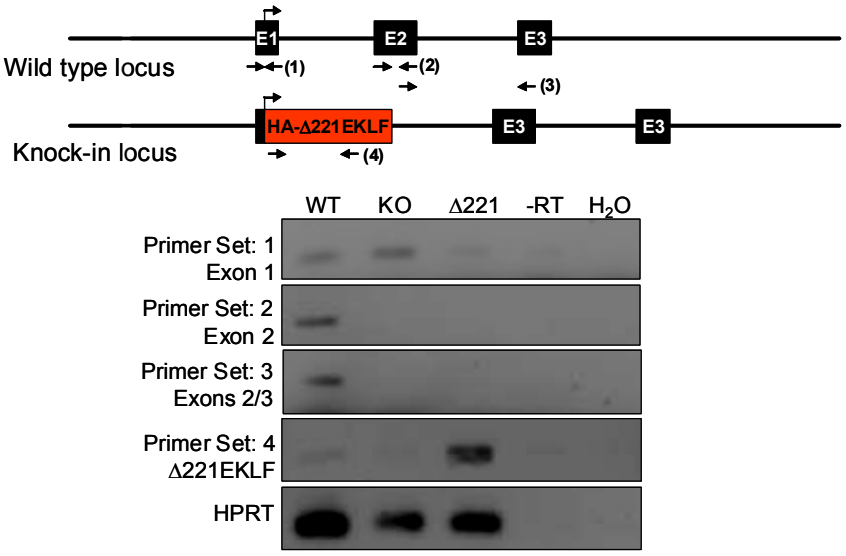


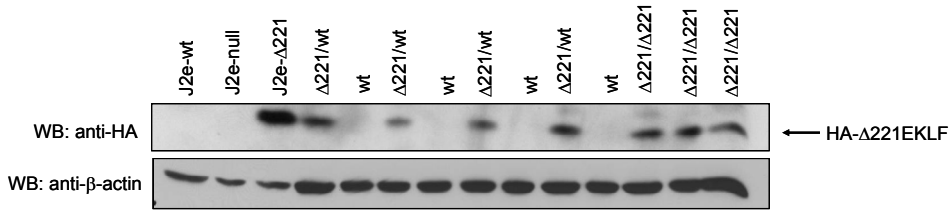
Figure 3.3. Accumulation of hemosiderin in fetal liver of mice expressing Δ221EKLF. Paraffin-embedded sections of fetal liver from E14.5 embryos: (a-c) hematoxylin and eosin stain and (d-f) Prussian blue stain. Hemosiderin is detected as blue deposits in the fetal liver. Original magnification x 400.

Figure 3.4. Expression of Δ221EKLF in fetal liver. (A) Top Panel: Schematic of the wild-type and knock-in alleles of EKLF. Arrows indicate the primers used in RT-PCR. Bottom Panel: Representative picture of agarose gel demonstrating that Δ221EKLF transcripts are amplified only with RNA extracted from fetal liver of homozygous Δ221EKLF embryos at E14.5. Additionally, downstream exons 2 and 3 of the endogenous EKLF locus are not transcribed in these animals. HPRT transcripts are detected as loading control. (B-C) Western blot analysis demonstrating that Δ221EKLF knock-in protein is expressed in fetal liver. Whole cell extracts were prepared from fetal liver of E14.5 wild-type, Δ221EKLF heterozygous, and Δ221EKLF homozygous embryos. Whole cell extracts from J2e cell lines were included as controls. (B) An anti-HA antibody was used to detect the HA-tagged mutant protein (HA-Δ221EKLF). (C) Anti-EKLF antibody generated in our laboratory (J.M.C. unpublished) was used to detect wild-type EKLF and mutant Δ221EKLF proteins. β-actin expression was detected as loading controls.

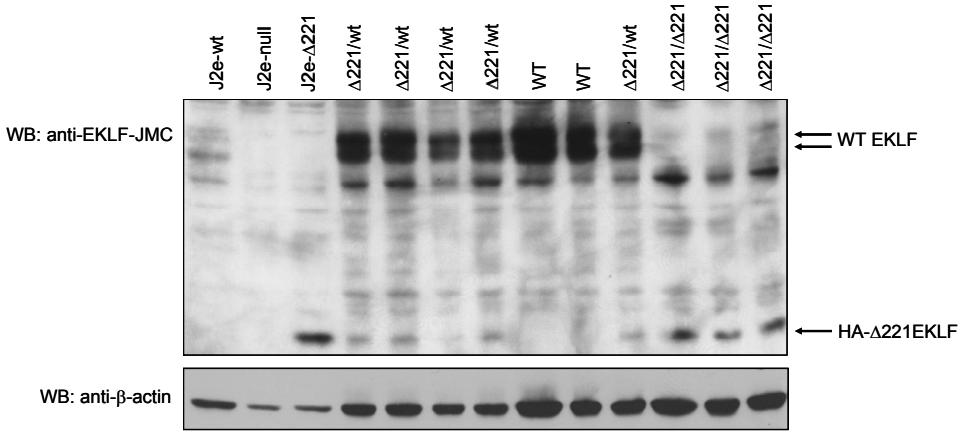
A.



B.



C.



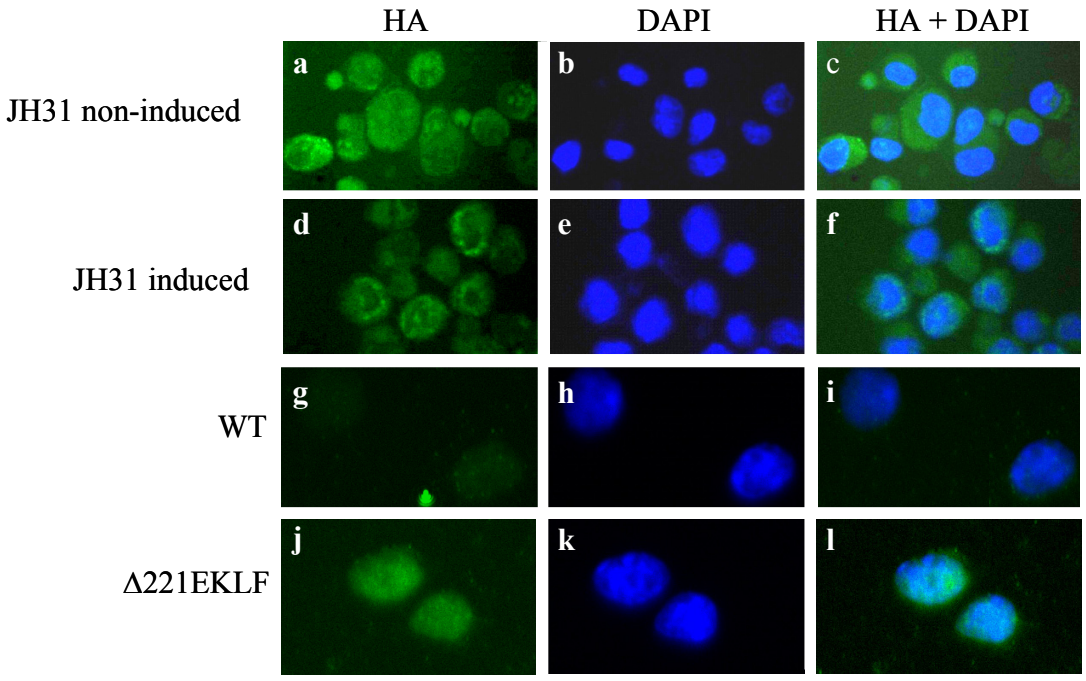


Figure 3.5. Localization of Δ221EKLF knock-in protein. Cytospin preparations of fetal liver erythroblasts from E14.5 Δ221EKLF homozygous embryos were stained with anti-HA antibody (j-l). The slides were mounted with VECTASHIELD® mounting medium containing DAPI. Wild-type fetal liver erythroblasts, which lack the HA tag, served as a negative controls (g-i) while the inducible JH31 cellular system containing a HA-EKLF-ER fusion protein served as positive controls (a-f). In the absence of the ER ligand, HA-EKLF-ER protein is bound to chaperone proteins in the cytoplasm but can be translocated inside the nucleus in the presence of Tamoxifen.

Expression of Δ212EKLF is sufficient for ß-globin promoter remodeling

To determine the functional consequences of Δ221EKLF on the chromatin structure *in vivo*, nucleosomal remodeling at the β-globin promoter was determined by a real time PCR-based DNase I hypersensitivity assay (McArthur *et al.*, 2001). Chromatin prepared from fetal liver nuclei was incubated with increasing amounts of the DNase I enzyme. DNA was purified and analyzed by real time PCR. The sensitivity is plotted as a percent of DNA remaining at different concentrations of DNase I and normalized to an internal control region from the neurofilament Nf-M gene located on murine chromosome 14. Nf-M is relatively resistant to DNase I digestion, although at higher concentrations of DNase I and longer incubation time period, the DNA at this region is digested eventually by DNase I. As shown in Figure 3.6, chromatin extracted from Δ221EKLF fetal liver erythroblasts is sensitive to treatment with increasing amounts of DNase I at a level comparable to chromatin from derived from wild-type erythroblasts. In contrast, and consistent with previous observations (Wijgerde *et al.*, 1996), chromatin derived from EKLF-null erythroblasts are relatively insensitive to DNase I treatment up to 18 U of enzyme. The amount of sensitivity is erythroid cell-dependent, and the fetal liver has previously been determined to be greater than 20% hepatocytes at this stage (McArthur *et al.*, 2001).

Expression of Δ221EKLF induces histone modifications at the β-globin promoter

To further explore the role of Δ221EKLF in reorganizing the chromatin architecture at the β-globin gene promoter, the acetylation and methylation patterns of histone H3 (H3) across the β-globin locus was determined by ChIP assays. Extending the results from the DNase I HS assay, I hypothesized that the Δ221EKLF domain is sufficient to induce acetylation of histone H3 and methylation at K4. As shown in Figure 3.7, histones were acetylated at wild-type levels at the β-globin promoter in Δ221EKLF erythroblasts. Interestingly, the level of acetylation was comparable to EKLF-null erythroblasts at the 3' coding region of the β-globin gene in Δ221EKLF erythroblasts. This observation is consistent with the idea of a lack of significant β-globin elongation in Δ221EKLF erythroblasts (Sawado *et al.*, 2003). By contrast, the acetylation pattern at the β-globin HS2 was comparable in wild-type, Δ221EKLF, and EKLF-null erythroblasts, suggesting acetylation of HS2 of the LCR occurs independently of EKLF. Similarly, analysis of the trimethylation pattern of lysine 4 of histone H3 revealed comparable levels of trimethylation at the β-globin HS2; however, trimethylation at the β-globin promoter was increased in Δ221EKLF erythroblasts to a comparable level observed in wild-type erythroblasts (Figure 3.8). This modification, trimethylated H3K4, has been associated with active genes *in vivo* (Bernstein *et al.*, 2005). Taken together, the data suggest that expression of the Δ221EKLF domain is sufficient for full and appropriate chromatin remodeling activity *in vivo.*

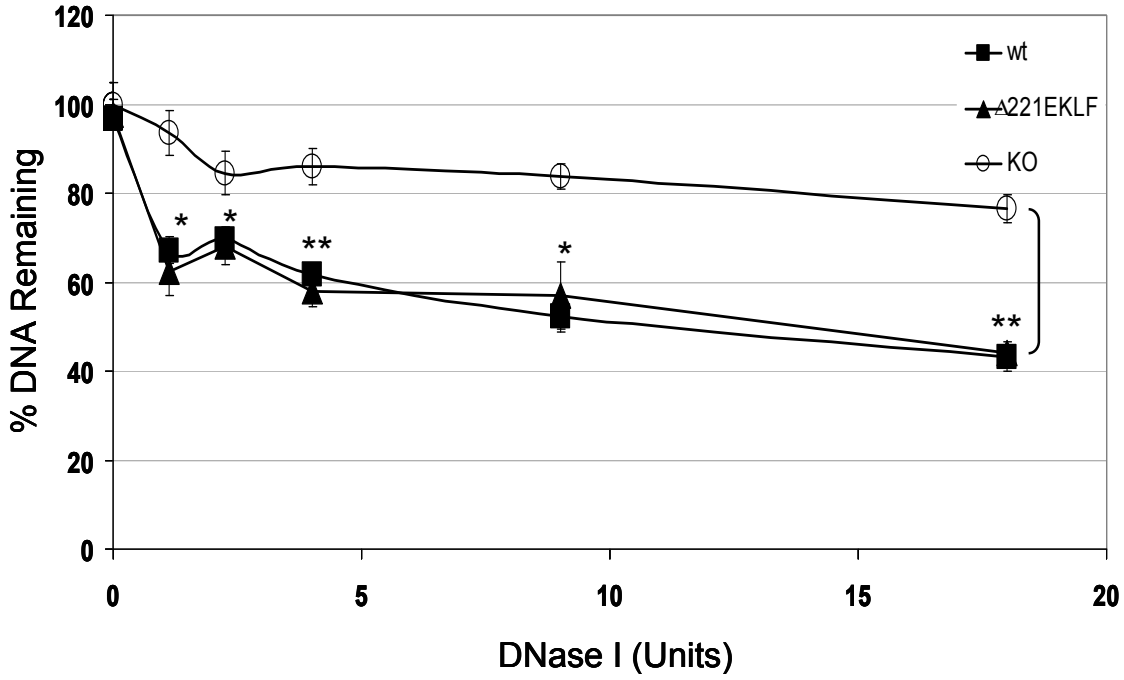


Figure 3.6. DNase I sensitivity at the β-globin promoter in Δ221EKLF fetal liver erythroblast. Plot of a quantitative real time PCR based DNase I hypersensitivity assay used to analyze the region of murine βmaj promoter in fetal liver from E14.5 wild-type, Δ221EKLF, and EKLF-null embryos. The results are plotted as the percent of DNA remaining after 5 min incubation with increasing amounts of DNase I enzyme normalized with the relatively insensitive Nf-M gene. Data shown are averages of at least three independent experiments. Error bars indicate the standard error of the mean. Asterisks denote *p*-value compared to KO. * = *p*-value <0.05; ** = *p*-value <0.001.

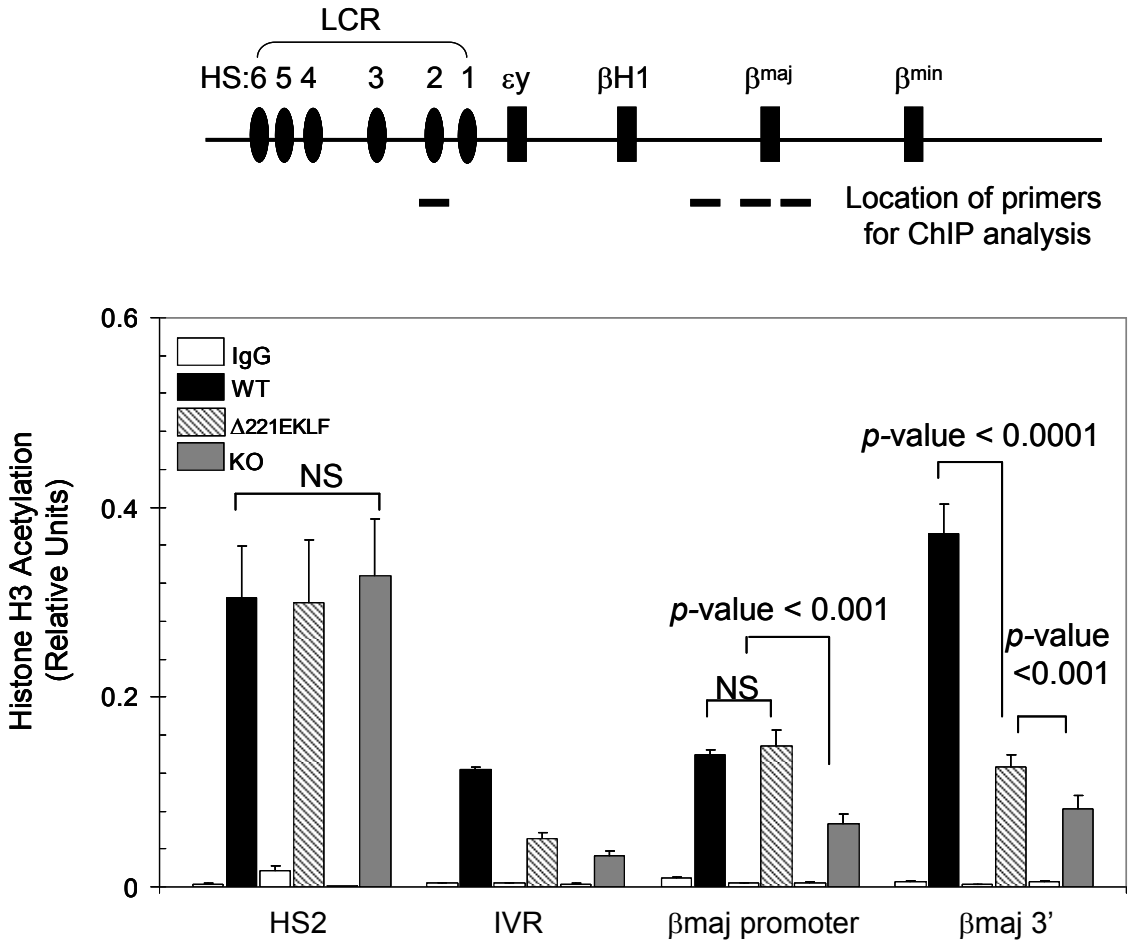


Figure 3.7. Global acetylation of histone H3 across the β-globin locus. Top panel: schematic of murine β-globin locus with location of primer pairs used in ChIP analysis denoted by solid lines. Bottom panel: pattern of histone H3 acetylation across the β-globin locus was analyzed by ChIP assay using anti-acetylated histone H3 (AcH3) antibody. Data shown are averages of at least five independent experiments performed in duplicate (mean ± SEM). NS = not significant. IVR = intervening region in the β-globin locus.

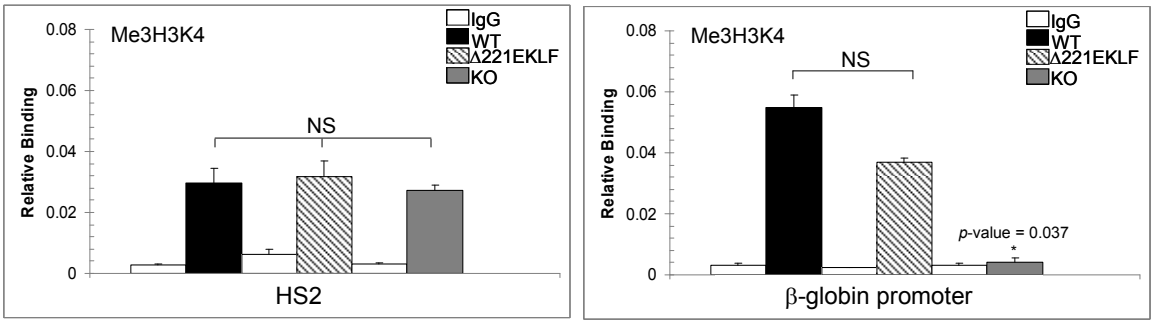


Figure 3.8. Trimethylation pattern at lysine 4 of histone H3. The trimethylation at lysine 4 of histone H3 (Me3H3K4) at the β-globin HS2 and promoter sequences was analyzed by ChIP assay. Data shown are averages of at least five independent experiments performed in duplicate (mean ± SEM). NS = not significant.

Δ221EKLF binds to erythroid-specific gene loci

The zinc finger DNA binding domain of EKLF recognizes and binds to the consensus sequence CCNCNCCCN found at many erythroid gene loci. To explore whether the mutant Δ221EKLF protein was capable of binding to known EKLF regulatory sequences *in vivo,* chromatin immunoprecipitation (ChIP) was performed with a HA-specific anti-serum. In contrast to erythroid cells lacking EKLF, fetal liver erythroblasts expressing the Δ221EKLF protein demonstrated appropriate binding of the chromatin remodeling encoding domain to known-EKLF regulatory sequences at the β-globin locus, but not to non-erythroid gene promoters (i.e. RPII215). As shown in Figure 3.9, Δ221EKLF protein binds to the β-globin HS2, the β-globin gene promoter as well as to the promoters of two erythroid genes, AHSP (Pilon *et al.*, 2006) and α-globin (Shyu *et al.*, 2005). In contrast, the mutant protein did not bind to regions that do not contain EKLF binding sites, including an intervening region in the β-globin locus (IVR), downstream region of the β-globin gene, and the non-erythroid gene RPII215 (Im *et al.*, 2005). The RPII215 gene, located on murine chromosome 11, encodes the largest subunit of RNA polymerase II (Pravtcheva *et al.*, 1986). These results confirm that the mutant protein expressed in fetal liver is capable of binding to the EKLF regulatory sequences *in vivo*.

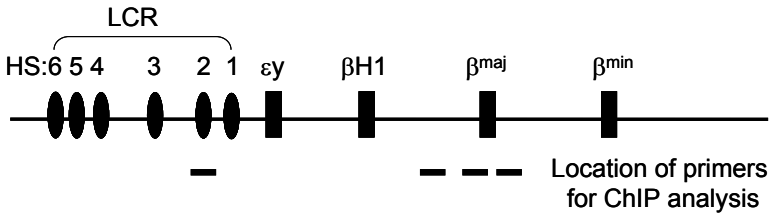
Expression of Δ221EKLF is insufficient for high-level β-globin transcription

EKLF is essential for β-globin gene transcription. Although previous cellular studies have demonstrated that a larger domain of EKLF is required and sufficient to activate β-globin gene transcription (Brown *et al.*, 2002), it is not clear what determinants are required *in vivo*. To determine if expression of Δ221EKLF was sufficient for activation of gene transcription *in vivo,* the expression level of the adult β-globin gene in Δ221EKLF fetal liver erythroblasts from E14.5 embryos were analyzed by real time RT-PCR. Similar to complete loss of EKLF, there was minimal gene expression, or transcription as assayed by primary transcript analysis, of the β-globin gene in Δ221EKLF erythroblasts compared to wild-type erythroblasts (Figure 3.10). These results suggest that additional amino terminal sequences are required for EKLF-dependent gene activation *in vivo.* Moreover, the lack of β-transcripts suggests that these animals succumbed *in utero,* at least in part, from a severe anemia in a similar manner to EKLF-null animals (Nuez *et al.*, 1995; Perkins *et al.*, 1995).

Differential binding of factors at the β-globin LCR and promoter

The failure of Δ221EKLF to activate β-globin transcription, despite nearly normal promoter remodeling as determined by DNase I HS formation and hyperacetylation of histones at the β-globin promoter, led me to explore the role of Δ221EKLF in factor recruitment to the β-globin LCR and promoter. First, occupancy of PolII at the β-globin locus was examined by ChIP assay with anti-PolII antibody capable of detecting both phosphorylated and unphosphorylated forms of PolII. Phosphorylation of PolII serine 5

A.



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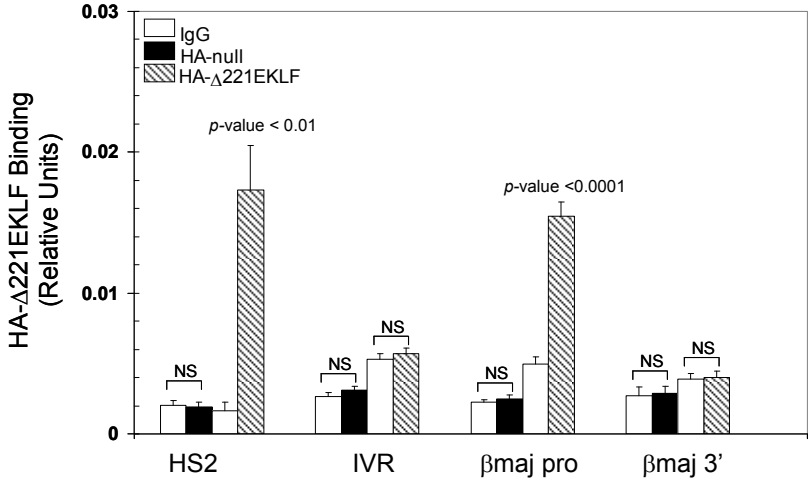

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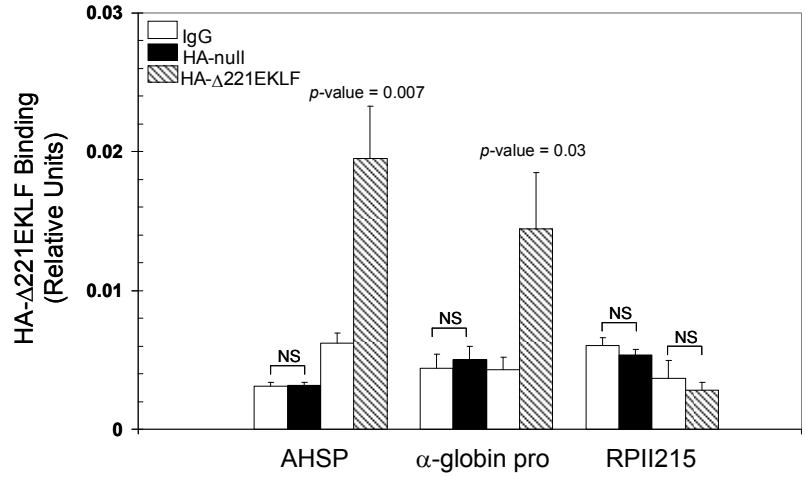


Figure 3.9. Differential binding of Δ221EKLF to erythroid-specific gene loci. (A) Schematic of murine β-globin locus with location of primer pairs used in ChIP analysis denoted by solid lines. (B) The binding of Δ221EKLF across the β-globin locus was examined using ChIP assays with anti-HA specific anti-serum. (C) The binding of Δ221EKLF at other erythroid-specific gene promoters (positive controls) and a non-erythroid gene promoter (negative control). Data shown are averages of at least five independent experiments performed in duplicate (mean ± SEM). NS = not significant.

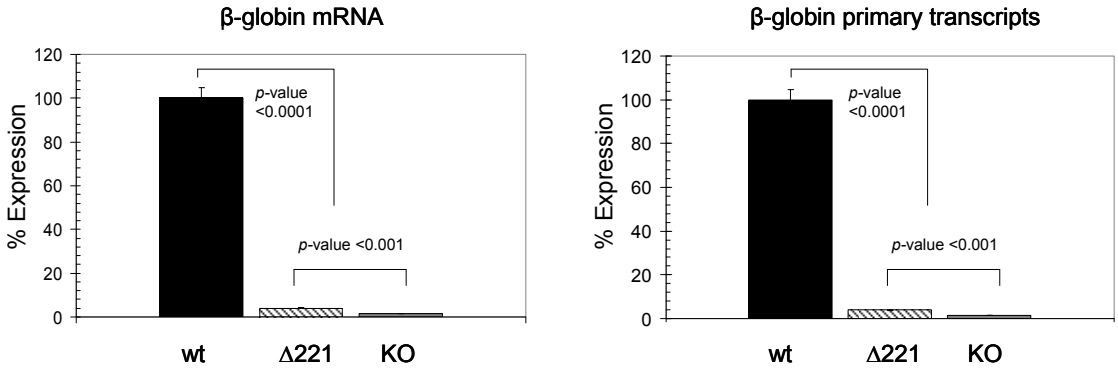


Figure 3.10. Lack of β-globin transcripts in Δ221EKLF fetal liver erythroblasts *in vivo*. RNA was extracted from fetal liver of E14.5 wild-type, Δ221EKLF, and EKLF-null embryos and treated with Turbo DNase prior to cDNA synthesis. β-globin gene expression (as determined by mRNA levels) and transcription (as assayed by primary transcripts) were analyzed by real time RT-PCR. Data shown are averages of at least five independent experiments (mean ± SEM).

is an essential step in transition from a stalled to an elongating polymerase (Phatnami &Greenleaf, 2006). Not surprisingly, there was increased occupancy of PolII at the β-globin promoter and 3' of the gene in wild-type erythroblasts (Figure 3.11), consistent with active β-globin transcription. In contrast, minimal RNA polymerase II occupancy was observed at the β-globin promoter in Δ221EKLF expressing cells of a similar chronology. Furthermore, the amount of PolII at the 3' region of the β-globin gene was not significantly increased, consistent with the idea of a paused PolII complex at the β-globin promoter (Phatnami & Greenleaf, 2006). This observation is supported by the β-globin transcript data, demonstrating a significant reduction in β-globin transcripts in Δ221EKLF erythroblasts as compared with wild-type erythroblasts (Figure 3.10). When compared to EKLF-null cells, occupancy of PolII at the β-globin promoter was approximately 3-fold higher in Δ221EKLF cells (p-value <0.0001). By contrast, similar levels of PolII were recruited to HS2 of the LCR in wild-type, Δ221EKLF, and EKLF-null cells, consistent with the idea that EKLF does not mediate this step. Furthermore, this result is not surprising given previous studies have demonstrated that recruitment of PolII to the LCR occurs independent of recruitment to and activation of the β-globin promoter (Johnson *et al.*, 2003). Importantly, my results support a role for EKLF in recruitment of PolII to the β-globin promoter, as has been previously described for GATA-1 and NF-E2 (Johnson *et al.*, 2001).

The β-globin promoter contains numerous binding sites for erythroid-specific and ubiquitous transcription factors that regulate appropriate high-level expression of the β-globin gene. EKLF, GATA-1, and NF-E2 are among the best characterized transcriptional activators and have been shown to bind the β-globin promoter sequences and DNase HS sites (HS) at the LCR (Mahajan *et al.*, 2007). To elucidate the role of Δ221EKLF in EKLF-mediated transactivation of the β-globin gene, I analyzed the recruitment GATA-1 and NF-E2 to the HS2 of the LCR and β-globin promoter in Δ221EKLF expressing fetal liver erythroblasts (Figure 3.12). The occupancy of NF-E2 and GATA-1 at an intervening region (IVR) on the β-globin locus and the downstream region of the β-globin gene were included as negative controls. Chromatin immunoprecipitation analysis revealed a comparable level of NF-E2 occupancy at the β-globin HS2; however, NF-E2 occupancy at the β-globin promoter was significantly reduced in Δ221EKLF erythroblasts as compared with wild-type erythroblasts (Figure 3.12A). These results are consistent with previous reports that demonstrate NF-E2 recruitment to HS2 of the LCR and β-globin promoter is essential for activation of β-globin transcription (Forsberg *et al.*, 2000; Sawado *et al.*, 2003). Similarly, GATA-1 occupancy at the β-globin HS2 was comparable in wild-type, Δ221EKLF, and EKLF-null erythroblasts (Figure 3.12B). In contrast, GATA-1 occupancy at the β-globin promoter was significantly reduced in Δ221EKLF as compared to wild-type erythroblasts. These observations are consistent with the role for GATA-1 at HS2 of the LCR in co-activator and PolII recruitment to the β-globin promoter (Cho *et al.*, 2008). Additionally, my results in Δ221EKLF-expressing cells suggest that a larger amino terminal domain of EKLF is required and necessary for recruitment of GATA-1 and NF-E2 to the β-globin promoter.

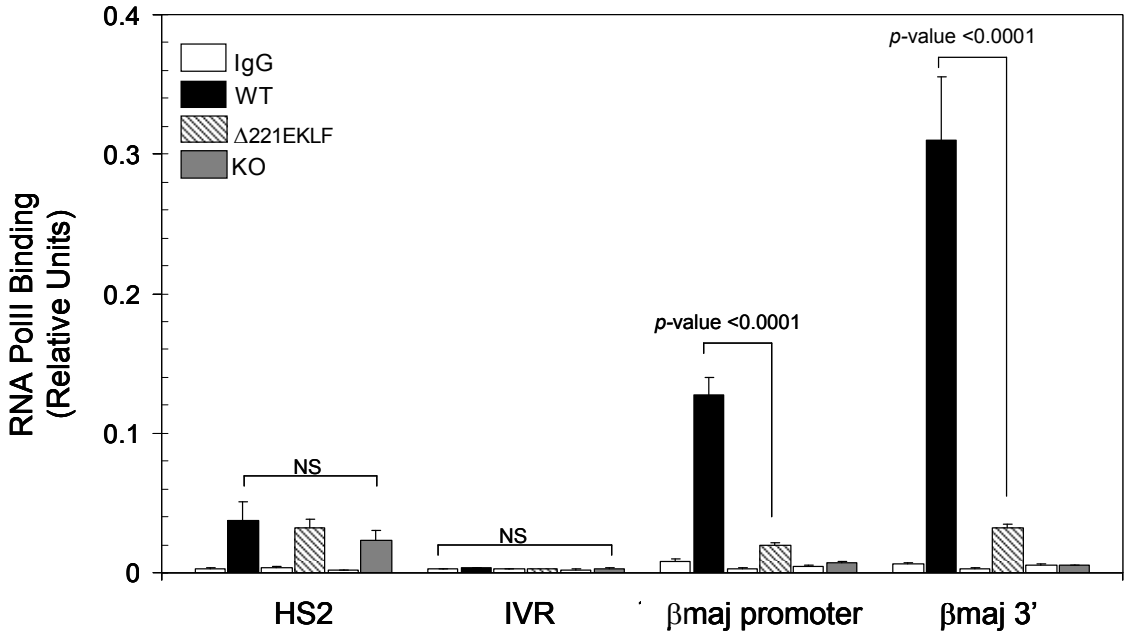
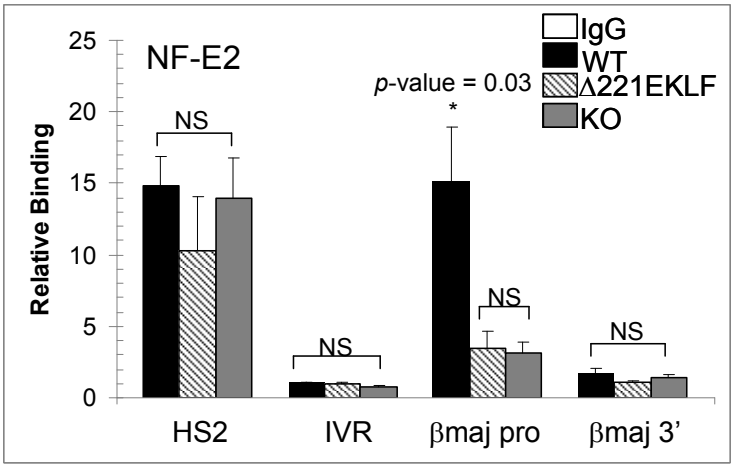


Figure 3.11. Lack of RNA polymerase at the β-globin promoter in Δ221EKLF expressing cells. The amount of total PolII occupancy across the β-globin locus was examined by ChIP analysis with the polyclonal anti-PolII antibody. Data shown are averages of at least five independent experiments performed in duplicate (mean ± SEM). NS = not significant.

A.



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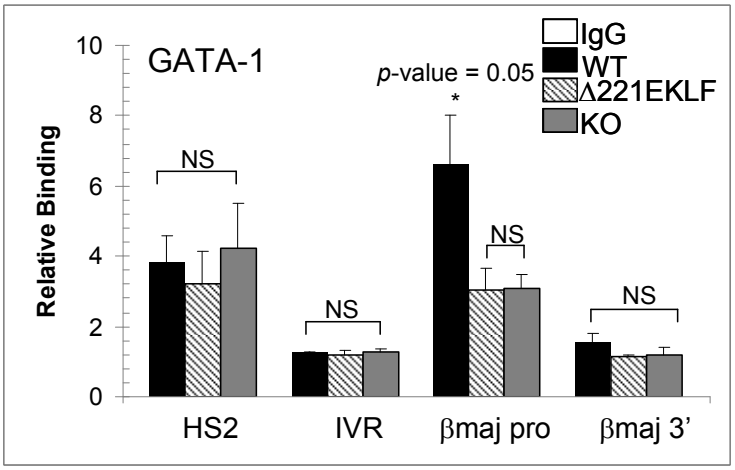


Figure 3.12. Binding of erythroid-specific transcription factors at the β-globin promoter *in vivo*. Binding of (A) NF-E2 and (B) GATA-1 across the β-globin locus was analyzed by ChIP assay in fetal liver from E14.5 wild-type, Δ221EKLF, and EKLF-null embryos. Data shown are averages of at least three independent experiments performed in duplicate (mean ± SEM). NS = not significant.

DISCUSSION

The studies in this chapter are aimed at exploring the hypothesis that expression of Δ221EKLF alone is sufficient for altering the local chromatin structure at the β-globin promoter *in vivo*. Furthermore, these studies have provided significant insights into EKLF-mediated activation of β-globin transcription *in vivo*.

Expression of Δ221EKLF is not sufficient to rescue the EKLF-null phenotype

I have demonstrated in the present study that expression of Δ221EKLF *in vivo* cannot rescue the EKLF null phenotype. Like mice lacking EKLF (Nuez *et al.*, 1995; Perkins *et al.*, 1995), Δ221EKLF mice exhibit a phenotype consistent with ineffective erythropoiesis and die by E15.5 of gestation. The Δ221EKLF embryo is anemic with few hemoglobin-containing erythroblasts in the fetal liver, consistent with a block in erythroid differentiation. Expression of Δ221EKLF protein has been confirmed *in vivo*. Although the mutant Δ221EKLF protein appears to be expressed at lower levels than wild-type EKLF protein, this alone cannot account for the observed phenotype of Δ221EKLF mice given that the mutant protein retains full and appropriate chromatin remodeling properties (see discussion below). Previous studies in our laboratory have shown that this domain of EKLF cannot activate β-globin gene transcription in erythroid cell lines (Brown *et al.*, 2002). Thus, it was anticipated that expression of Δ221EKLF in a whole animal model would result in embryonic lethality reminiscent of the EKLF-null mice.

Expression of Δ221EKLF is sufficient for nucleosomal remodeling and histone modifications *in vivo*

Previous studies analyzing the distinct domains of EKLF in erythroid cell lines demonstrated that chromatin remodeling and transcriptional activation functions of EKLF are separable (Brown *et al.*, 2002). Conclusions from these studies are that sequences between amino acid 221-253 are necessary and sufficient for chromatin remodeling at the β-globin promoter while additional N-terminal amino acid sequences are required for transactivation of β-globin gene transcription. Moreover, expression of Δ221EKLF in an erythroid cell line results in DNase I HS formation at the β-globin promoter to wild-type levels.

My studies expand on these conclusions and provide novel results on the chromatin remodeling property of EKLF *in vivo*. Indeed, DNase I hypersensitivity assays reveal that chromatin derived from Δ221EKLF fetal liver erythroblasts is as sensitive to DNase I treatment as wild-type chromatin, whereas chromatin from EKLF-null erythroblasts is relatively insensitive (Figure 3.6). Because the degree of DNase I sensitivity corresponds with nucleosomal remodeling (Steger & Workman, 1996; Pazin *et al.*, 1997), the data strongly suggest that expression of Δ221EKLF is sufficient for nucleosomal remodeling at the β-globin promoter *in vivo*. Previous studies analyzing the role of EKLF in chromatin remodeling have relied on *in vitro* chromatin reconstitution

assays. These studies have identified a SWI/SNF-containing multiprotein complex, E-RC1, which interacts with the zinc finger DNA binding domain of EKLF (Armstrong *et al.*, 1998; Kadam *et al.*, 2000). This interaction results in chromatin remodeling and transcriptional activation of a chromatinized β-globin template. Additional evidence supporting this interaction comes from studies demonstrating that EKLF interacts with BRG1, a subunit of E-RC1 (Zhang *et al.*, 2001). However, evidence for a direct interaction between EKLF and SWI/SNF components *in vivo* is still lacking; however, BRG1 is a highly attractive candidate to explore.

Similarly, I show that expression of Δ221EKLF is sufficient to induce histone modifications *in vivo.* First, global acetylation of histone H3 (AcH3) at the β-globin promoter is increased in Δ221EKLF erythroblasts to an equivalent level as observed in wild-type erythroblasts (Figure 3.7). Similarly, trimethylation at lysine 4 of histone H3 at the β-globin promoter is increased to wild-type levels in Δ221EKLF erythroblasts (Figure 3.8). It is known that higher acetylation particularly of histones H3 and H4 are associated with the 'opening' of chromatin for transcription (Yan & Boyd, 2006). Furthermore, trimethylation at lysine 4 of histone H3 has been associated with promoters of active genes (Bernstein *et al.*, 2005). My results not only extend these observations to include EKLF, but also provide evidence that the Δ221EKLF domain alone is sufficient for high-level H3 acetylation at the β-globin promoter. EKLF has previously been shown to interact with the histone acetyltransferase, CREB binding protein (CBP) (Zhang & Bieker, 1998; Zhang *et al.*, 2001), which in addition to acetylation of EKLF protein can also increase acetylation of histones (Blobel, 2002). Thus, it is highly tempting to speculate that Δ221EKLF induces CBP recruitment and increases the acetylation of histone H3 at the β-globin promoter in the absence of β-globin transcription.

NF-E2 and GATA-1 have been implicated in mediating H3 acetylation and methylation at the β-globin promoter (Kiekhaefer *et al.*, 2002; Kiekhaefer *et al.*, 2004; Demers *et al.*, 2007). Using cell lines, these studies have demonstrated that GATA-1 induces H3 acetylation (Kiekhaefer *et al.*, 2002) while NF-E2 induces H3K4 trimethylation at the β-globin promoter (Kiekhaefer *et al.*, 2004; Demers *et al.*, 2007). In contrasts, my study in primary erythroblasts suggests EKLF, and more importantly the Δ221EKLF domain, is required and sufficient for H3 acetylation and H3K4 trimethylation at the β-globin promoter *in vivo*. As shown in Figure 3.12, there is modest NF-E2 and GATA-1 occupancy at the β-globin promoter in Δ221EKLF and EKLF-null cells when compared to wild-type erythroblasts. However, in contrast to complete loss of EKLF, expression of Δ221EKLF results in H3 acetylation and H3K4 trimethylation to a level comparable to wild-type in Δ221EKLF erythroblasts. While it is not possible to discount entirely the role of GATA-1 and NF-E2 in histone modifications at the β-globin promoter, my results are consistent with the idea that Δ221EKLF is a critical mediator of this process. It is possible the basal levels of GATA-1 and NF-E2 at the β-globin promoter require Δ221EKLF to induce H3 acetylation and H3K4 trimethylation *in vivo.*

Nucleosomal remodeling and histone modification are not sufficient for recruitment of erythroid-specific transcription factors to the β-globin promoter and activated β-globin transcription

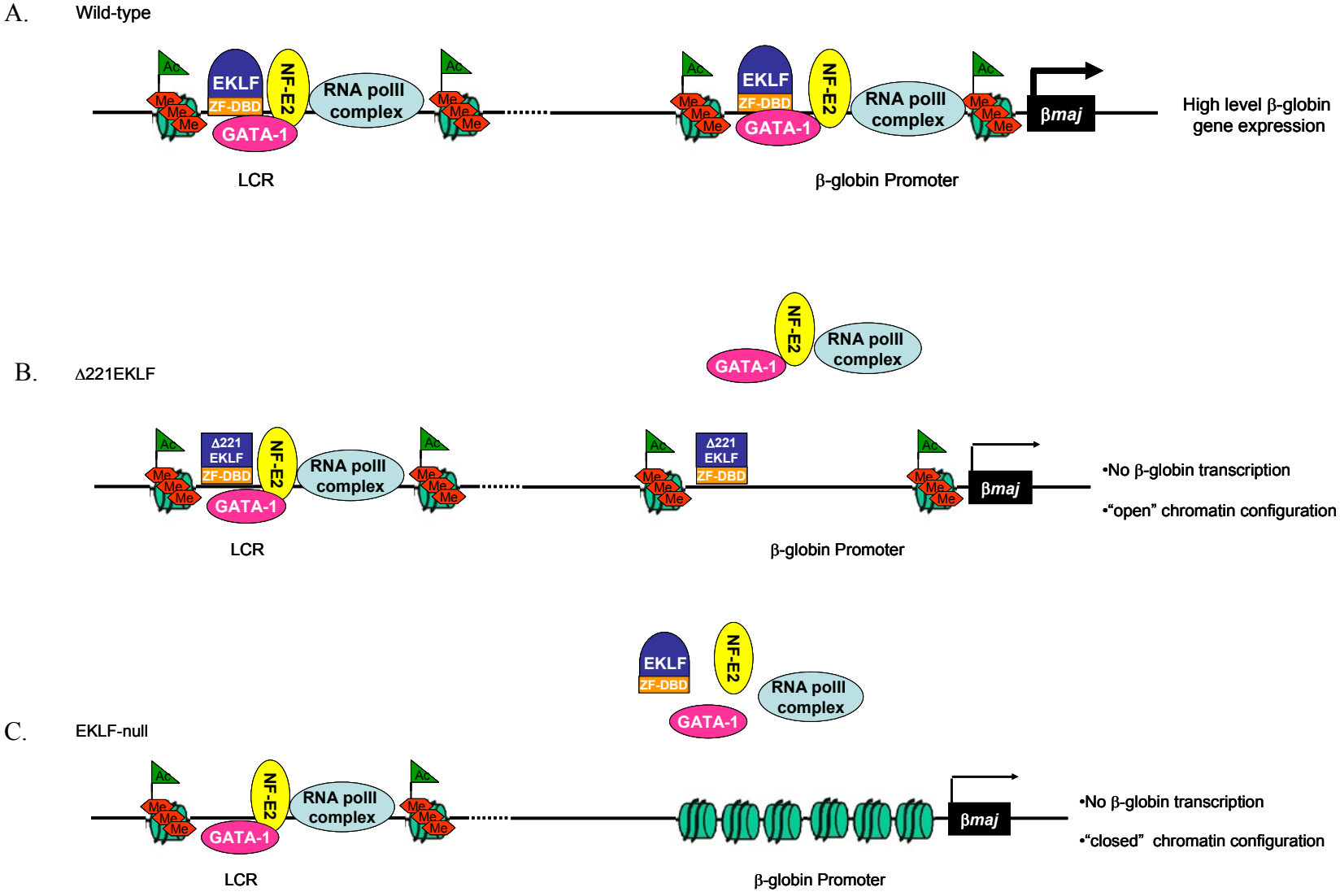
In the present studies, I show that the level of β-globin transcripts in the Δ221EKLF mice is less than 5% of wild-type mice (Figure 3.10). These results are consistent with our previous analysis of Δ221EKLF expressed in an erythroid cell line (Brown *et al.*, 2002). Furthermore, the low levels of β-globin gene transcription despite high levels of H3 acetylation and nucleosomal remodeling at the β-globin promoter is highly reminiscent of previous studies in mice homozygous for a targeted deletion of the LCR (Bender *et al.*, 2000). These studies have demonstrated that deletion of the LCR resulted in a severe reduction of β-globin transcription with concomitant decreased PolII occupancy at the β-globin promoter (Bender *et al.*, 2000; Sawado *et al.*, 2003). However, the β-globin promoter remains hyperacetylated and nuclease sensitive in LCR deletion and wild-type mice. Taken together, these observations suggest that nucleosomal remodeling and modification of histones are not sufficient to achieve high level β-globin transcription *in vivo*, consistent with lack of LCR recruitment to the promoter. Furthermore, the reduced level of PolII occupancy in Δ221EKLF mice is consistent with the idea of a long range transfer of PolII from the LCR to the promoter to stimulate transcription as has been previously described by Johnson *et al.* (2001).

GATA-1 and NF-E2 have both been implicated in β-globin gene activation (Kim & Bresnick, 2007). The present studies demonstrate that EKLF is essential for recruitment of these factors to the β-globin promoter but not HS2 of the LCR. Using ChIP analysis, both GATA-1 and NF-E2 are recruited normally to HS2 of the LCR in Δ221EKLF and EKLF-null erythroblasts. However, there is significantly lower GATA-1 and NF-E2 occupancy at the β-globin promoter in Δ221EKLF and EKLF-null erythroblasts as compared to wild-type erythroblasts. My results are novel and unique to Δ221EKLF-expressing cells, providing strong support for an essential role for EKLF in recruitment of GATA-1 and NF-E2 to the β-globin promoter. These results are contrasted with previous studies that have demonstrated GATA-1 and NF-E2 occupy the β-globin promoter in erythroid cells from mice lacking the LCR (Sawado *et al.*, 2003; Vakoc *et al.*, 2005), suggesting that recruitment of GATA-1 and NF-E2 to the β-globin promoter occur independent of the LCR. The results in the present study are consistent with two possible mechanisms of transcriptional factor recruitment to the β-globin promoter. The first mechanism proposes that recruitment of GATA-1 and NF-E2 to the β-globin promoter is EKLF-dependent, requiring a larger amino terminal domain of EKLF. An alternative mechanism is that GATA-1 and NF-E2 are first recruited to the β-globin LCR (EKLF-independent) and then transferred to the β-globin promoter (EKLF-dependent). Studies to examine LCR/β-globin promoter interaction would provide additional insights into the mechanism of transcriptional factor recruitment to the β-globin promoter to stimulate gene transcription.

A model for Δ221EKLF in β-globin promoter remodeling

Although *in vitro* and cellular studies have analyzed the coordinated recruitment of transcription factors and coactivators required for high level β-globin transcription, much knowledge remains to be gained from studying EKLF-mediated chromatin remodeling and β-globin transcription *in vivo.* The results from the present studies in Δ221EKLF mice indicate that chromatin remodeling and histone modifications are not sufficient to achieve high-level β-globin transcription *in vivo*. Furthermore, these studies suggest that Δ221EKLF is sufficient for β-globin promoter remodeling but additional amino terminal sequences are necessary for recruitment of transcriptional factors to the promoter. A proposed model for the role of Δ221EKLF in promoter remodeling is shown in Figure 3.13. First, H3 acetylation and H3K4 trimethylation at HS2 of the β-globin LCR occurs independent of EKLF. Furthermore, GATA-1, NF-E2, and PolII are recruited normally in the absence of EKLF. In contrast, these events at the β-globin promoter are EKLF-dependent. Similar to the observation in wild-type erythroblasts, expression of Δ221EKLF results in an open chromatin configuration with histone modifications consistent with an active gene promoter. However, recruitment of GATA-1, NF-E2, and PolII to stimulate transcription at the β-globin promoter requires additional amino terminal sequences of EKLF not included in the Δ221EKLF domain. In contrast, the β-globin promoter in EKLF-null erythroblasts remains in a closed chromatin configuration consistent with a repressed β-globin promoter. It is unclear at this juncture what role Δ221EKLF plays in LCR/β-globin promoter interaction. Thus, further dissection of the role of Δ221EKLF in modulation of the LCR/β-globin promoter interaction and recruitment of other factors at the β-globin promoter will provide additional fundamental insights into EKLF-mediated chromatin remodeling and β-globin transcription *in vivo.*

Figure 3.13. Models of β-globin promoter remodeling in erythroid cells. Histone modifications and recruitment of GATA-1, NF-E2, and PolII to the β-globin LCR does not require EKLF. In contrast, the events at the β-globin promoter are EKLF-dependent. (A) In wild-type erythroblasts, EKLF induces H3 hyperacetylation and H3K4 trimethylation. Transcriptional factors (GATA-1, NF-E2, PolII) are recruited to the β-globin promoter to stimulate β-globin gene transcription. (B) In Δ221EKLF-expressing erythroblasts, Δ221EKLF alone is sufficient to induce H3 hyeracetylation and H3K4 trimethylation. However, additional amino terminal sequences of EKLF are required for recruitment of GATA-1, NF-E2, and PolII to stimulate β-globin gene transcription. (C) In the absence of EKLF, the β-globin promoter remains in a closed chromatin configuration consistent with a repressed gene promoter.



CHAPTER 4: EXPRESSION OF Δ221EKLF RESULTS IN ALTERED ERYTHROID MATURATION AND BLOCKED MEGAKARYOCYTIC EXPANSION

INTRODUCTION

Hematopoiesis is a complex developmental process in which hematopoietic stem cells (HSCs) continuously generate all blood cell lineages. The current model of hematopoeisis proposes that HSCs proceed through multiple steps of committed progenitors to give rise to mature blood cells in each lineage (Weissman *et al.*, 2001, Orkin & Zon, 2008) (see Figure1.1 in Chapter 1). This model is based historically on the results from *in vitro* assays developed to quantify multi-potential progenitors and lineage-restricted progenitors of bone marrow cells cultured in semi-solid media (Nakorn *et al.*, 2003). Erythroid and megakaryocytic lineages are believed to be derived, at least in part, from a common bipotential progenitor. Termed the megakaryoctye-erythrocyte progenitor (MEP) this cell generates unipotential erythroid or megakaryocytic progenitors (Debili *et al.*, 1996; Nakorn *et al.*, 2003). These precursors express many hematopoietic transcription factors that are essential for both erythropoiesis and megakaryopoiesis. Although many of these factors are essential, the precise mechanism of action/pathways directing the fate of the MEP towards one lineage over another remains obscure.

Two factors, EKLF and Fli-1, have emerged in recent studies as key players in the erythroid-megakaryocytic differentiation switch. Both are expressed in the bipotential MEP (Frontelo *et al.*, 2007); however, the levels of these genes are differentially expressed depending on the commitment fate of the MEP. During early MEP differentiation, EKLF and Fli-1 have contrasting levels of expression. In erythroid progenitors, EKLF expression is high, whereas in megakaryocytic progenitors Fli-1 expression is more than 60-fold greater compared with erythroid progenitors (Frontelo *et al.*, 2007).

EKLF and Fli-1 protein-protein interactions have also been noted (Starck *et al.*, 2003). These *in vitro* studies suggest that EKLF and Fli-1 do not inhibit each other's binding activity and Fli-1 mediated activation of a megakaryocytic gene promoter construct (*i.e.*, GpIX) can be repressed by EKLF zinc finger domain alone (Starck *et al.*, 2003). Supporting these observations, Bouilloux *et al.* (2008) have shown that knock-down of EKLF by shRNA results in an increase in megakaryocytic gene expression in differentiated MEL cells. Furthermore, ChIP analysis revealed increased acetylation of histone H3 and occupancy of PolII, GATA-1, and Fli-1 at megakaryocytic gene promoters with EKLF knock-down in MEL cells. By contrast, studies by Siatecka *et al.* (2007) propose that sumoylation of lysine 74 of EKLF is critical for repression of megakaryopoiesis. Taken together, these studies suggest three potential, and non-exclusive, models of EKLF-mediated repression of megakaryopoiesis (Figure 4.1): (i) EKLF inhibits Fli-1 gene transcription; (ii) EKLF inhibits Fli-1 binding to its target promoters (i.e. GpIX) by protein-protein interaction; and (iii) EKLF binds to Fli-1-

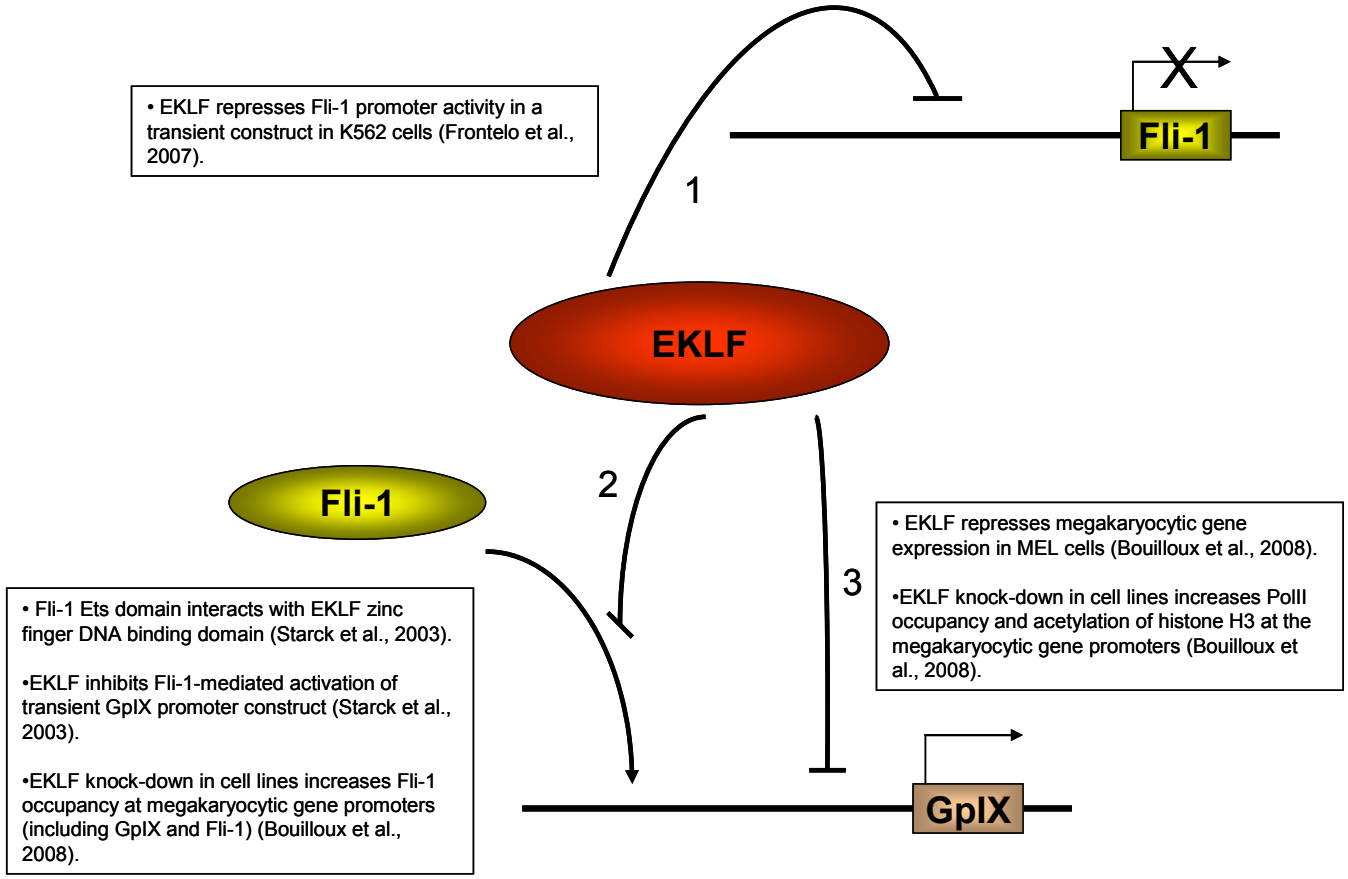


Figure 4.1. Potential models of EKLF-mediated repression of megakaryopoiesis. Shown here is a simplified diagram depicting three possible mechanisms for EKLF-mediated repression of megakaryopoiesis with support for each model from the current literature. (1) EKLF inhibits Fli-1 gene transcription. (2) EKLF inhibits Fli-1 binding to its targets (i.e. GpIX) by protein-protein interaction. (3) EKLF binds to Fli-1-targeted regulatory sequences and blocks Fli-1-medication activation.

targeted regulatory sequences and blocks Fli-1 mediated activation.

While previous studies have provided significant insights into the role of EKLF in the erythroid-megakaryocytic differentiation program, it is important to note that these studies have been performed in cell lines and may not recapitulate events *in vivo*. The studies in this chapter seek to expand our current knowledge on the role of EKLF in MEP differentiation utilizing the newly generated mice expressing Δ221EKLF. I report here that, similar to complete loss of EKLF, expression of Δ221EKLF results in a block of terminal erythroid differentiation at the proerythroblast stage, due in part, to decreased expression of genes essential for erythroid development. However, proliferation of erythroid progenitors is unaltered when compared to wild-type erythroblasts.

In contrast, expression of Δ221EKLF inhibits the expansion of megakaryocytic progenitors observed with complete loss of EKLF. Molecular analysis of the Fli-1 promoter is consistent with the idea that inhibition of megakaryocytic expansion is achieved, in part, by Δ221EKLF repression of Fli-1 through recruitment of a Sin3A-containing repressor complex. Taken together, my results suggest that the Δ221EKLF domain alone is sufficient for EKLF-dependent inhibition of megakaryocytic differentiation *in vivo.*

MATERIALS AND METHODS

Mice

EKLF-null mice were previously generated via gene targeting by Perkins *et al.* (1995). Δ221EKLF mice were generated via gene targeting as described in Chapter 2 of this dissertation. Genotyping of wild-type, EKLF-null, Δ221EKLF embryos were performed by PCR method as described in Chapter 3.

Colony-forming assays

Fetal livers cells were obtained by passing E14.5 fetal liver through a 70 μm cell strainer in D10 (DMEM + 10% FBS). To evaluate the erythroid colonies (burst forming units- erythroid; BFU-E), granulopoietic colonies (colony forming units-granulocyte-macrophage; CFU-GM), and colonies containing multiple lineages of cells (colony-forming unit-granulocyte-erythroid-monocyte-megakaryocyte; CFU-GEMM), fetal liver cell suspensions were plated (1 x 10^4 cells/mL) on methylcellulose medium (MethoCult 3434; StemCell Technologies) supplemented with stem cell factor, IL-3, IL-6, and erythropoietin as recommended by the manufacturer. Cells were plated in 35mm dishes and maintained at 37°C in a humidified atmosphere at 5% CO_2 for 10–12 days. The colonies were then counted under an inverted microscope and expressed as average number of CFU colonies per culture. Colonies were identified as described in the technical manual for Mouse Colony-Forming Assay using Methocult

(http://www.stemcell.com/technical/28405_methocult%20M.pdf).

To evaluate the colony forming units-megakaryocytes (CFU-Mk), fetal liver cell suspensions were plated (1 x10^5 cells/mL) on collagen medium (Megacult-C, StemCell Technologies) containing collagen (1.1 mg/mL), recombinant thrombopoietin (50 ng/Ml), recombinant IL-3 (10 ng/mL), recombinant IL-6 (20 ng/mL), and recombinant IL-11 (50 ng/mL). Cells were plated in double chamber slides in a 100 mm petri dish and maintained at 37°C in a humidified atmosphere at 5% CO_2 for 14 days as recommended by the manufacturer (StemCell Technologies). The slides were fixed with methanol-acetone solution after removing the double chamber and stored at -20°C until staining could be performed. Histochemical staining was performed with acetylthiocholiniode (Sigma). The colonies were then counted under an inverted microscope and expressed as average number of CFU-Mk colonies per culture. CFU-Mk colonies were identified as a group of 3 to 50 megakaryocytes per colony with acetyl cholinesterase activity, evident by brown granules in the cytoplasm. Each experiment was performed a minimum of four times, and the data were analyzed using a Student *t*-test.

FACS analysis

Fetal livers from E14.5 embryos were passed through a 23-gauge needle three times, filtered through a 70 μM cell strainer, washed with D10 medium (DMEM + 10% FBS), and submitted on ice to the Flow Cytometry and Cell Sorting Shared Resource Center at St Jude Children's Research Hospital. Cells were washed in PBS + 5% FBS and incubated on ice in human gamma gobulin solution (100 mg/mL in PBS-5%) for blocking of nonspecific staining. Cells were then washed in PBS-5% and incubated on ice for 30 min with 0.5 μg of phycoerythrin (PE)-conjugated anti-mouse CD71 and allophycocyanin (APC)-conjugated anti-mouse TER119 antibodies (BD PharMingen). Results were analyzed using Flow-Jo software (Tree Star).

Globin gene expression by real time RT-PCR

Real time RT-PCR to analyze the expression of EKLF target genes were performed as described in Chapter 3. The primers used for real time RT-PCR are shown in Appendix Table A.5. Each sample was run in duplicate with at least five independent experiments performed.

Chromatin immunoprecipitation assays

ChIP assay was performed as described in Chapter 3 with addition of anti-Sin3A (Santa Cruz, sc-994x) antibody. Primers for ChIP analysis at Fli-1 promoter are: forward: CGTGGACCCCGTCATTGTT and reverse: GCACTGCGCACACAGGATACT (Frontelo *et al.*, 2007). Primers for β-globin promoter are the same as those described in Chapter 3.

RESULTS

Expression of Δ221EKLF alters erythroid differentiation and inhibits expansion of megakaryocytic progenitors

To assess the maturation and differentiation potential of fetal liver cells from Δ221EKLF embryos, I used a flow cytometry assay that allows quantitative evaluation of erythroid differentiation on the basis of expression of the erythroid-specific TER119 and nonerythroid-specific CD71 (transferrin receptor) cell surface markers. Cells were flow sorted, and a density plot of relative logarithmic fluorescence units for CD71-expressing versus TER19-expressing was generated (Figure 4.2A). According to previous studies, cells are defined by a characteristic pattern of CD71 and TER119 staining such that $CD71^{med}TER119^{low}$ cells are predominantly progenitor cells, $CD71^{high}TER119^{low}$ are early basophilic proerythroblasts, $CD71^{high}TER119^{high}$ are early and late basophilic erythroblasts, $CD71^{med}TER119^{high}$ are chromatophilic and orthochromatophilic erythroblasts, and $CD71^{low}TER119^{high}$ are late orthochromatophilic erythroblasts and recticulocytes (Socolovsky *et al.*, 2001; Zhang *et al.*, 2003). Using this assay, I validated previous studies that demonstrated TER119 was absent in EKLF-null fetal liver cells while CD71 expression at the cell surface was normal (Figure 4.2A, top panels) (Hodge *et al.*, 2005), suggesting that loss of EKLF resulted in a block of erythroid maturation at the proerythroblast stage (Pilon *et al.*, 2008). As shown in Figure 4.2A (bottom panels), a comparable pattern of CD71/TER119 staining was observed in fetal liver cells from Δ221EKLF embryos of a similar chronology. Fetal liver cells from EKLF heterozygous and Δ221EKLF heterozygous littermates demonstrated proper execution of the terminal erythroid differentiation program. Thus, it appears that expression of the Δ221EKLF domain alone is insufficient for execution of the terminal erythroid differentiation program *in vivo,* resulting in a block at an early stage of erythroid differentiation.

A block in terminal erythroid differentiation as a consequence of Δ221EKLF expression led me to speculate that Δ221EKLF affected the proliferation of hematopoietic progenitors *in vivo*. To quantify the number of erythroid colonies, fetal liver cells from wild-type, Δ221EKLF, and EKLF-null embryos were cultured in a methylcellulose-based medium supplemented with cytokines to promote the growth of BFU-E, CFU-GM, and CFU-GEMM colonies. As shown in Figure 4.2B, there was no statistical difference in the number of BFU-e and CFU-GM colonies cultured from fetal liver of wild-type, Δ221EKLF, and EKLF-null embryos. Analysis of the BFU-E colonies revealed colonies of similar size (Appendix Figure A.3). These observations are in agreement with previous studies in EKLF-null mice (Perkins *et al.,* 1995), consistent with the idea that expression of Δ221EKLF does not affect the proliferation of erythroid progenitors. On the other hand, these studies revealed comparable numbers of CFU-GEMM colonies in Δ221EKLF and wild-type animals. In contrast, there is an expansion of CFU-GEMM colonies in EKLF-null animals. This observation was quite interesting given that recent studies by Frontelo *et al.* (2007) and Bouilloux *et al.* (2008) proposed a novel role for EKLF in megakaryopoiesis. Subsequently, I speculated whether the Δ221EKLF domain alone is sufficient to inhibit the expansion of megakaryocytic

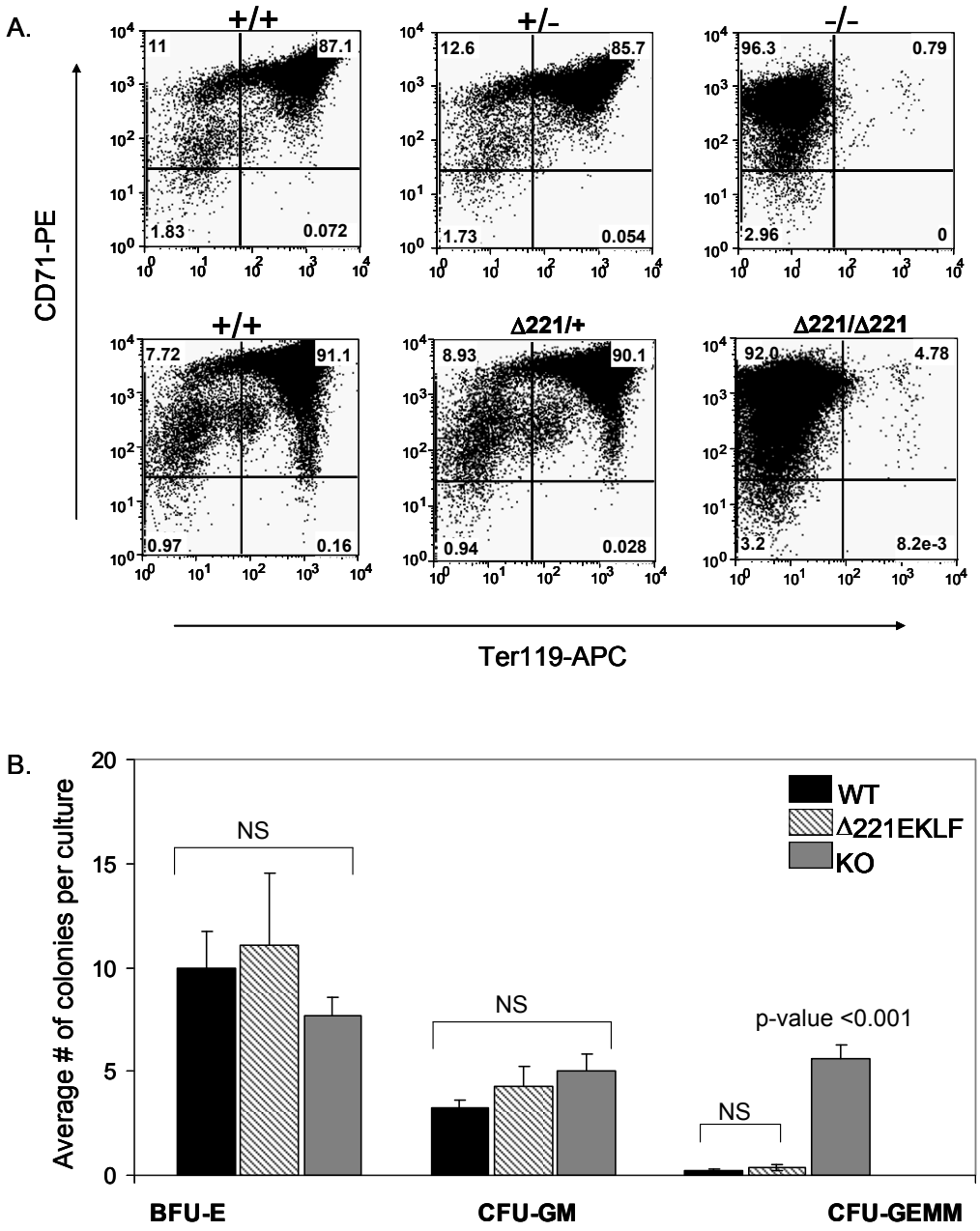


Figure 4.2. Effects of Δ221EKLF expression on terminal erythroid differentiation and proliferation of fetal liver progenitor cells. (A) Fluorescence-activated cell sorting analysis of E14.5 fetal liver cells from wild-type, EKLF-null heterozygous and EKLF-null embryos (top panel). In a separate experiment, FACS analysis of E14.5 fetal liver from wild-type, Δ221EKLF heterozygous, and Δ221EKLF embryos. Cells were stained with anti-CD71 and anti-TER119 antibodies. (B) Analysis of hematopoietic colony-forming cells in E14.5 fetal liver cells from wild-type, Δ221EKLF, and EKLF-null embryos. Data shown are averages of at least three independent experiments performed in duplicate (mean ± SEM). NS = not significant; *p*-value for significant difference shown.

progenitors observed with complete loss of EKLF.

Δ221EKLF alters expression of non-β-globin-like erythroid-specific genes

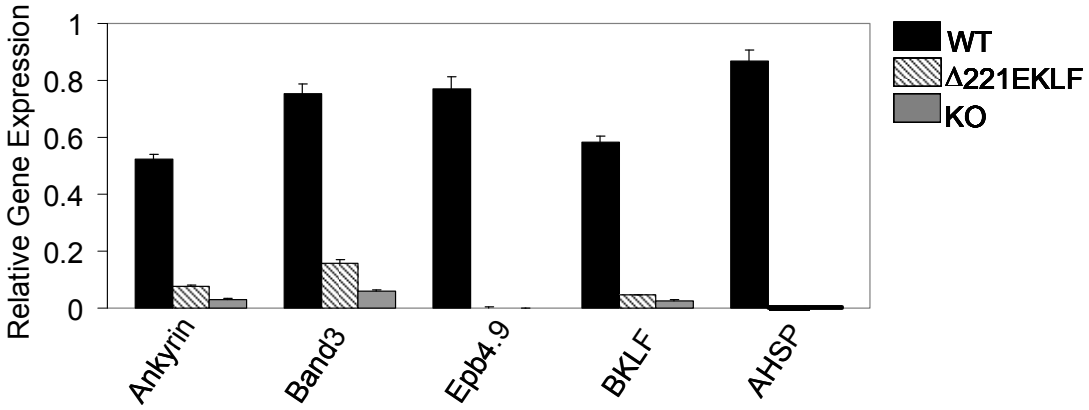
To determine if the failure of terminal erythroid differentiation in Δ221EKLF mice was due in part to lack of expression of non-β-globin erythroid-specific genes, I examined the expression of genes that have been shown to be direct targets of EKLF activation and those that are putative targets (Figure 4.3). Real time RT-PCR analysis revealed that expression of known EKLF target genes, Epb4.9 (Hodge *et al.*, 2005) ankyrin and band 3 (Nilson *et al.*, 2006), BKLF (Funnell *et al.*, 2006), and AHSP (Pilon *et al.*, 2006) were significantly reduced in fetal liver erythroblasts from Δ221EKLF embryos as compared to wild-type erythroblasts of similar chronology (Figure 4.3A). Similarly, expression of Δ221EKLF could not rescue high level expression of genes involved in heme biosynthesis (Figure 4.3B). Interestingly, these genes have not been shown to be direct EKLF target genes. These results are consistent with earlier observations at the β-globin locus (see Figure 3.10), confirming that a larger amino terminal domain of EKLF is required for EKLF-dependent gene transcription *in vivo.*

A role for Δ221EKLF in megakaryopoiesis and regulation of Fli-1

While it is interesting that Δ221EKLF fails to rescue expression of other non-β-globin-like erythroid-specific genes *in vivo* and the local chromatin structure at these gene promoters remains to be analyzed, I have decided to shift my focus to explore further the novel observation that Δ221EKLF inhibits expansion of megakaryocytic progenitors *in vivo*. Around the time that I made this observation in Δ221EKLF mice, two interesting studies utilizing cell lines concluded that EKLF represses megakaryocytic differentiation, at least in part, through Fli-1 repression (Frontelo *et al.*, 2007; Bouilloux *et al.*, 2008). Extending these studies, Siatecka *et al.* (2007) proposed a critical role for the sumoylation of lysine 74 in repression of megakaryopoiesis in a transgenic mouse model. These studies are reminiscent of earlier studies in which overexpression of EKLF transgene resulted in reduced platelet counts in adult mice (Tewari *et al.*, 1998). While these studies have significantly expanded our knowledge on the role of EKLF in megakaryopoiesis, they have all been studied by overexpression of EKLF protein. Thus, understanding EKLF repression of Fli-1 is of crucial importance, and the Δ221EKLF mice provide a unique opportunity in which to explore this novel role for EKLF *in vivo.*

To confirm that the blocked expansion of CFU-GEMM, a mixed cell colony containing granulocyte-erythrocyte-monocyte-megakaryocyte progenitors, was indeed due to inhibition of megakaryocytic progenitors, I performed a more specific assay to identify only CFU-MK by staining for acetylcholinesterase activity. Acetylcholinesterase activity is a marker for murine megakaryocytes (Saleque *et al.*, 2002) and is evident by dark-brown to dark-red granules. As expected, expression of Δ221EKLF *in vivo,* resulted in fewer megakaryocytic colonies as compared to complete loss of EKLF (Figure 4.4). More importantly, the number of MK progenitors in Δ221EKLF fetal liver is equivalent.

A.



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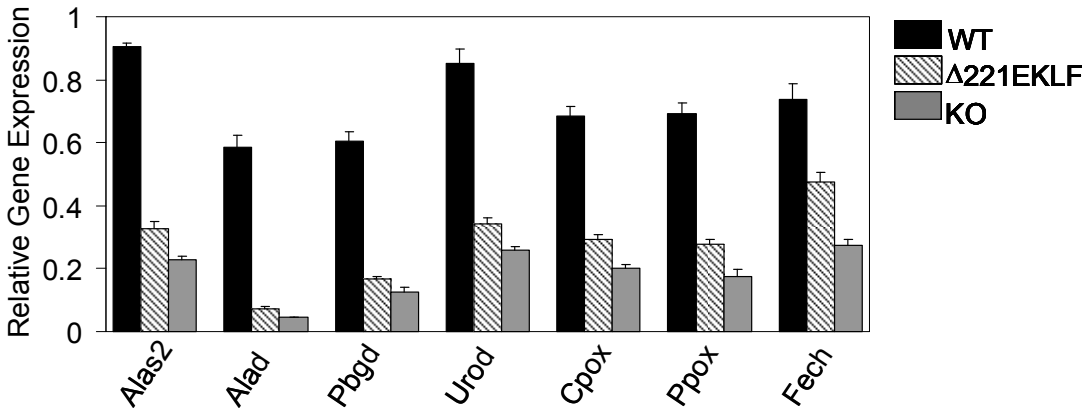


Figure 4.3. Altered pattern of erythroid-specific gene expression in Δ221EKLF fetal liver erythroblasts. cDNA was synthesized from RNA extracted from fetal liver of E14.5 wild-type, Δ221EKLF, and EKLF-null embryos and analyzed by real time RT-PCR. (A) Expression level of genes previously shown to be direct targets of EKLF-mediated activation. (B) Expression level of genes involved in the heme biosynthesis and not known to be direct targets of EKLF-mediated activation. Data shown are averages of at least five independent experiments performed in duplicate (mean ± SEM).

to that found in the wild-type fetal liver, consistent with the idea that expression of Δ221EKLF alone is sufficient to restore megakaryocytic progenitors to wild-type levels. Similarly, the MK colonies obtained from Δ221EKLF fetal liver are similar in appearance and size to those from wild-type fetal liver (Figure 4.4, bottom panel). Together, these observations are consistent with the idea that expression Δ221EKLF alone is sufficient to block the expansion of MK progenitors observed with complete loss of EKLF

To explore the role of Δ221EKLF in regulating the Fli-1 promoter, real time RT-PCR was used to determine the expression of Fli-1 in fetal liver erythroblasts derived from wild-type, Δ221EKLF, and EKLF-null embryos (Figure 4.5). This was contrasted with expression of β-globin in the same samples (Figure 4.5A). As shown in Figure 4.5B., there was no statistically significant difference in Fli-1 expression as measured by real time RT-PCR. This observation did not agree with previous expression array analysis (Frontelo *et al.*, 2007) and my earlier findings that expansion of the MK progenitors observed with complete loss of EKLF was inhibited by Δ221EKLF expression (see Figure 4.2B). It is also possible that I did not observe repression of Fli-1 as expected because I had not analyzed the mRNA levels in the context of MK progenitor cells or for other reasons (see Discussion below). To test if Fli-1 levels were differentially expressed during early murine development, Fli-1 mRNA was determined in erythroid cells derived from yolk sac of E9.5 embryos. Interestingly, a 2-fold decrease in Fli-1 mRNA was noted in Δ221EKLF and wild-type yolk sac cells (Figure 4.5C) when compared with EKLF-null cells, although the difference in expression only approached statistical significance (p-value = 0.07).

To further explore the role of Δ221EKLF in regulation of Fli-1, chromatin immunoprecipitation (ChIP) assay was utilized to analyze Δ221EKLF binding, PolII occupancy, and histone H3 acetylation at the Fli-1 promoter contrasting these observations with studies of factor occupancy at the β-globin promoter (Figure 4.6A-C). Analysis of factor binding at the promoters revealed a distinct pattern of binding. Δ221EKLF occupied the β-globin and Fli-1 promoters in a similar fashion (Figure 4.6A), an observation consistent with previous data for wild-type EKLF (Zhou *et al.*, 2006; Frontelo *et al.,* 2007). While binding of PolII protein at the β-globin promoter was enriched in wild-type fetal liver erythroblasts, there was minimal PolII binding noted in Δ221EKLF and EKLF-null erythroblasts (Figure 4.6B, left panel). By contrast, there was a 2-fold decrease in PolII recruitment at the Fli-1 promoter in wild-type and Δ221EKLF erythroblasts (Figure 4.6B, right panel), consistent with the idea that Fli-1 levels are reduced in these cell types. Similarly, the β-globin promoter is acetylated to comparable levels in wild-type and Δ221EKLF erythroblasts (Figure 4.6C, left panel). By contrast, the Fli-1 promoter is hypoacetylated in these cell types and hyperacetylated in EKLF-null cells (Figure 4.6C, right panel).

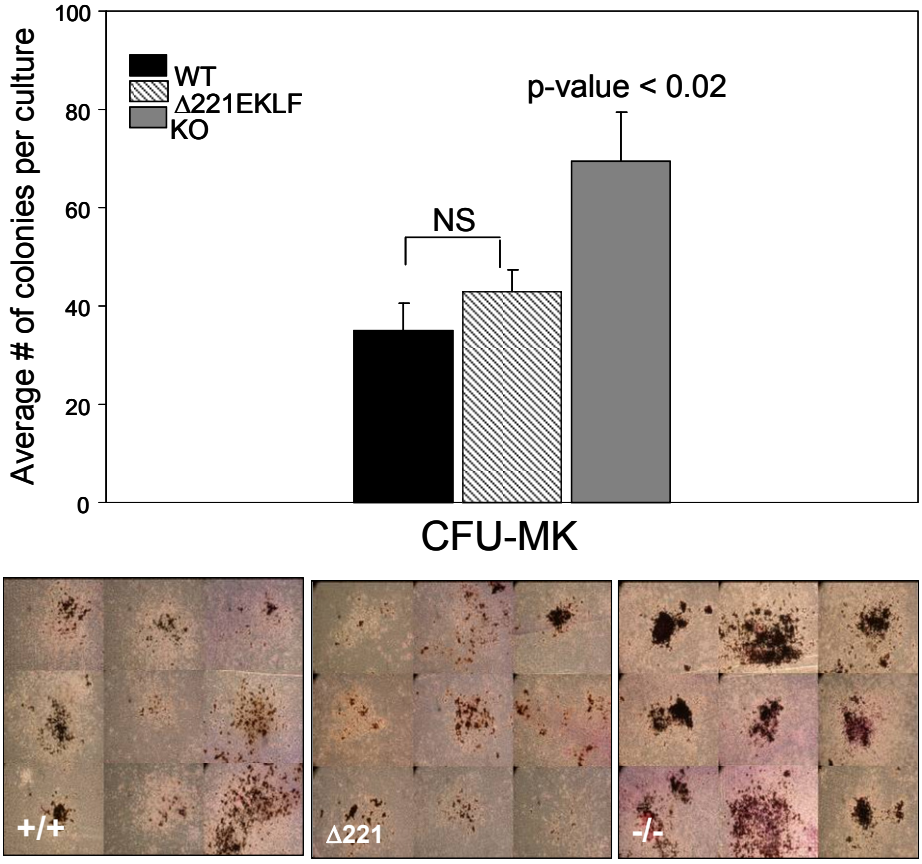
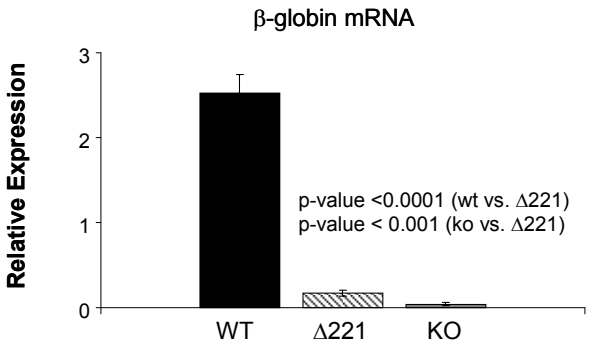


Figure 4.4. Blocked expansion of megakaryocytic progenitors in Δ221EKLF mice. Analysis of megakaryocytic colony-forming cells (CFU-MK) in E14.5 fetal liver cells from wild-type, Δ221EKLF, and EKLF-null embryos. Top panel: frequency of CFU-MK colonies counted per culture condition (1 x 10^5 cells plated). Cells were plated in double chamber slides, maintained at 37°C in a humidified atmosphere at 5% CO_2 for 14 days, after which slides were fixed and stained for acetylcholinesterase activity, and CFU-MK counted. Data shown are averages of at least three independent experiments performed in duplicate (mean ± SEM). NS = not significant; *p*-value for significant difference shown. Bottom panel: photographs of representative CFU-MK colonies from wild type, Δ221EKLF, and EKLF-null fetal liver cells after staining. Acetylcholinesterase is evident by the dark-brown granules.

A.



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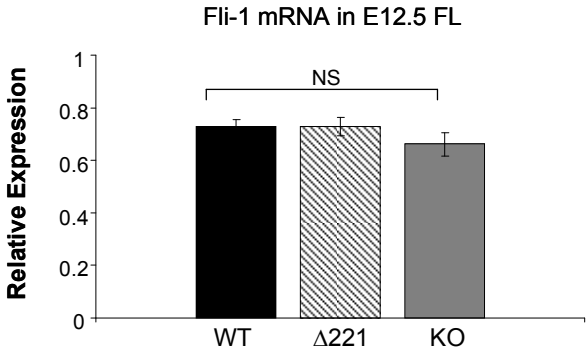

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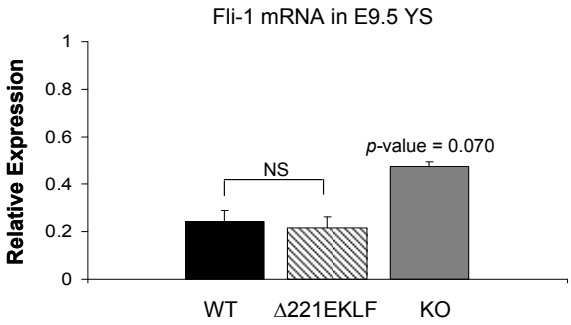

Figure 4.5. Differential expression of β-globin and Fli-1 gene in Δ221EKLF expressing cells. cDNA prepared from RNA extracted from E14.5 fetal liver (FL) and E9.5 yolk sac (YS) of wild-type, Δ221EKLF, and ELF-null embryos were analyzed by real time RT-PCR for (A) β-globin mRNA, (B) Fli-1 mRNA in cells derived from fetal liver, and (C) Fli-1 mRNA in cells derived from yolk sac. Data shown as average from at least five independent experiments performed in duplicate (mean ± SEM). *p*-value shown for significant difference. NS = not significant.

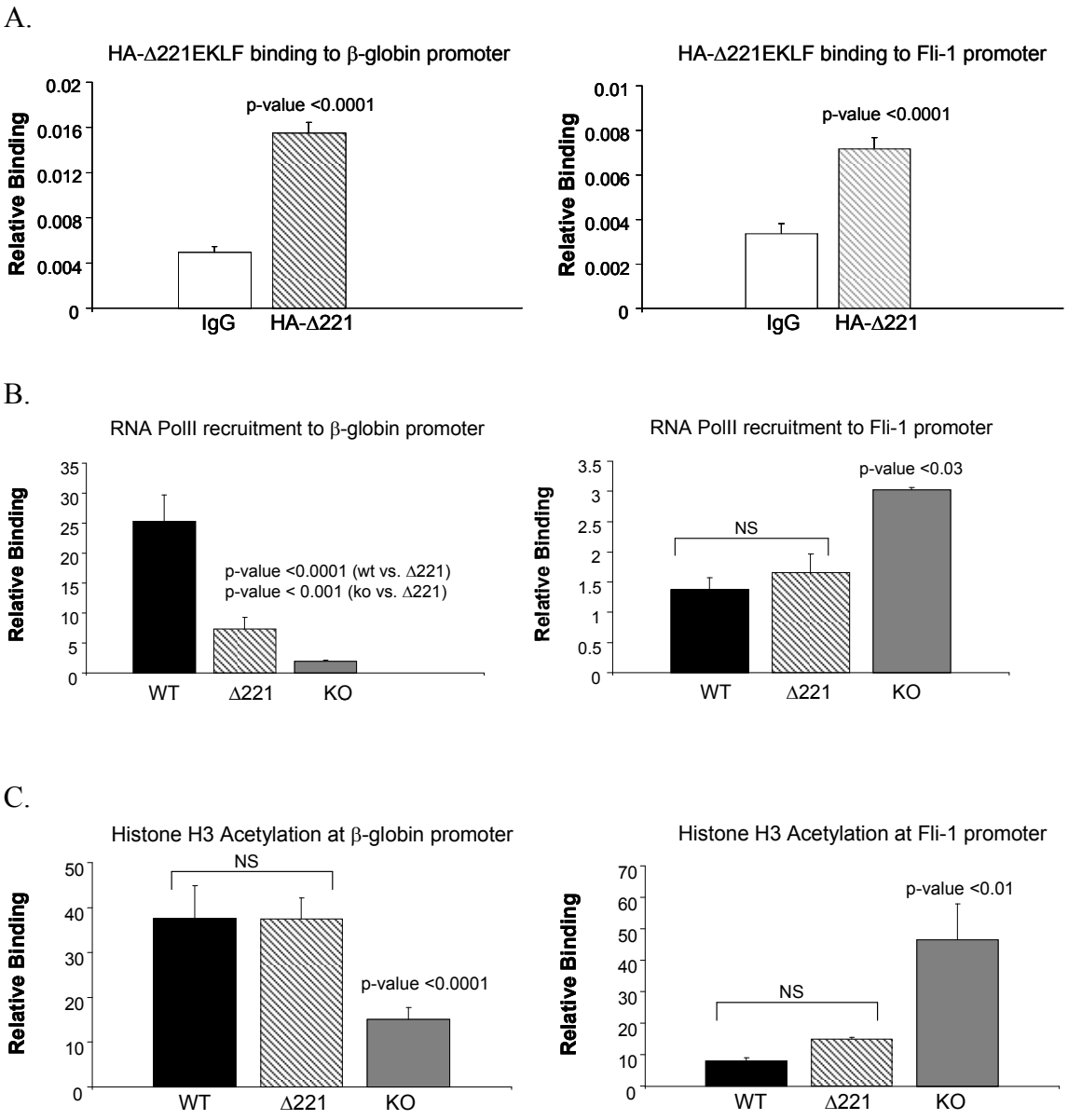

Figure 4.6. Distinct regulation of β-globin and Fli-1 promoters in Δ221EKLF fetal liver erythroblasts. ChIP assays were utilized to examine factor binding at the β-globin and Fl-1 promoters, respectively. (A) Binding of Δ221EKLF at the β-globin and Fli-1 promoters in fetal liver erythroblasts from E13.5 Δ221EKLF embryos was detected by anti-HA-specific antiserum. (B) Occupancy of total PolII at the β-globin and Fli-1 promoters in fetal liver erythroblasts from E13.5 wild-type, Δ221EKLF, and EKLF-null embryos. (C) Acetylation of Histone H3 at the β-globin and Fli-1 promoters in fetal liver erythroblasts from E13.5 wild-typeΔ221EKLF, and EKLF-null embryos. Data shown as averages from at least five independent experiments performed in duplicate (mean ± SEM). *p*-value shown for significant difference. NS = not significant.

A repressor complex is recruited to the Fli-1 promoter in Δ221EKLF cells

To explore the mechanism by which EKLF and Δ221EKLF might regulate Fli-1 transcription *in vivo*, ChIP assays were utilized to examine the recruitment Sin3A to the Fli-1 promoter. Previous studies have shown that EKLF exhibits transcriptional repression properties by interacting with Sin3A through its zinc finger DNA binding domain (Chen & Bieker, 1996; Chen & Bieker, 2004). Sin3A binding at the Fli-1 promoter was increased 2-fold in fetal liver erythroblasts derived from wild-type and Δ221EKLF embryos as compared to EKLF-null erythroblasts (Figure 4.7, right panel), thus suggesting that the Fli-1 promoter is repressed. In contrast, no significant binding of Sin3A was noted at the β-globin promoter (Figure 4.7, left panel), consistent with the idea that a repressor complex has no role in regulation of the β-globin gene. In summary, these results suggest that EKLF-mediated repression of Fli-1 is achieved in part by recruitment of a Sin3A-containing complex and that expression of Δ221EKLF alone is sufficient for this recruitment.

DISCUSSION

Studies in this chapter suggest that expression of Δ221EKLF alone is insufficient to support erythroid terminal differentiation; however, this domain of EKLF is sufficient to block expansion of megakaryocytic progenitors *in vivo.* This novel observation in mice expressing Δ221EKLF confirms the importance of generating these animals as valuable tools to elucidate the role of EKLF-mediated activation and repression at erythroid- and megakaryocytic-specific gene loci *in vivo.*

Failure of erythroid differentiation in Δ221EKLF-expressing cells

EKLF was first characterized as a transcriptional activator that modulated β-globin gene expression during erythropoiesis. Subsequent studies extended the targets of EKLF-mediated activation to include α-hemoglobin stabilizing protein (AHSP), cytoskeleton proteins (ankyrin, Epb4.9), and transcription factors (BKLF) (Pilon *et al.*, 2006; Nilson *et al.*, 2006; Funnell *et al.*,2007). Understandably, these are critical genes for erythroid development and differentiation. The studies in this chapter not only confirm that EKLF is essential for erythroid gene expression and terminal erythroid differentiation, but also a domain of EKLF (Δ221EKLF) that is capable of chromatin remodeling (see Chapter 3) is insufficient to fulfill this role *in vivo.* Indeed, expression of Δ221EKLF in a whole animal model results in a block of terminal erythroid differentiation similar that observed in complete loss of EKLF. However, proliferation of erythroid progenitors is unaffected by expression of Δ221EKLF *in vivo*. Furthermore, expression of Δ221EKLF fails to rescue the expression of ankyrin, band3, Epb4.9, BKLF, AHSP, and all seven genes involved in heme biosynthesis to wild-type levels. It is worth noting that none of the heme biosynthetic genes are known direct targets of EKLF. It would be interesting to speculate on the chromatin structure at these gene promoters; however, studies to examine the DNase I sensitivity at the gene promoters are ongoing in

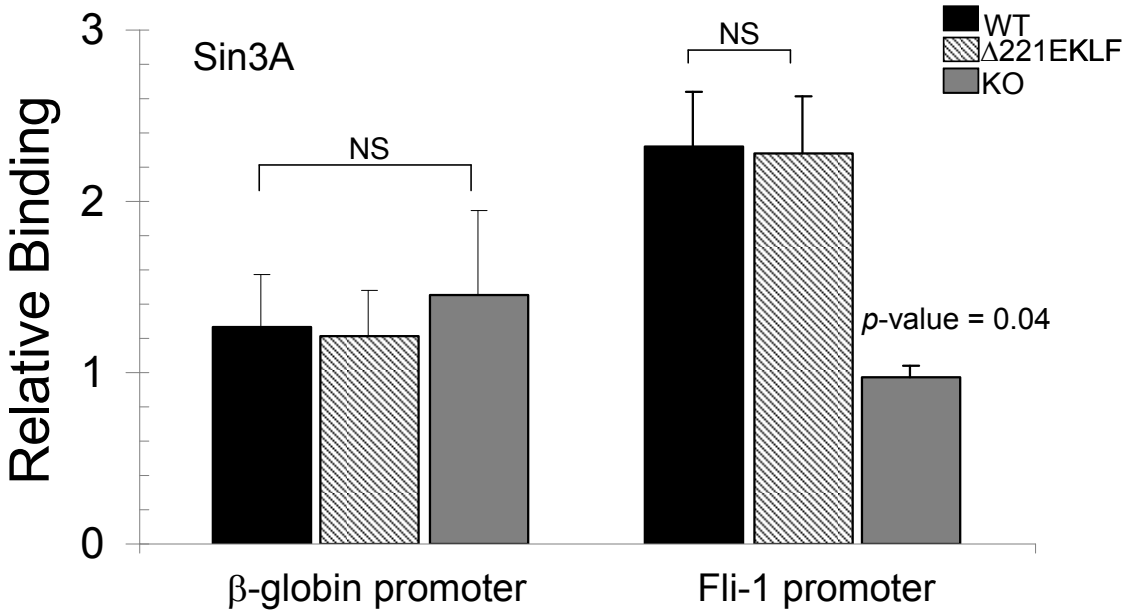

Figure 4.7. Recruitment of a Sin3A-containing repressor complex. ChIP analysis was performed in fetal liver erythroblasts from E13.5 wild-type, Δ221EKLF, and EKLF-null embryos. Occupancy of Sin3A was determined at Fli-1 promoter contrasted with occupancy at β-globin promoter. Data shown as averages from at least three independent experiments performed in duplicate (mean ± SEM). *p*-value shown for significant difference. NS = not significant.

our laboratory and should provide significant insights into the role of Δ221EKLF at non-β-globin erythroid-specific gene loci. While it is interesting that Δ221EKLF is insufficient to rescue non-β-globin-like erythroid gene expression examined in the present study, the question remains if there are genes that require only the chromatin remodeling domain of EKLF for high-level expression *in vivo*. Gene expression profiling studies utilizing the Δ221EKLF mice are ongoing in our laboratory to address this hypothesis.

Δ221EKLF regulates Fli-1 gene and inhibits expansion of megakaryocytic progenitors *in vivo*

The most compelling conclusion of the studies presented in this chapter is that expression of Δ221EKLF is sufficient to repress megakaryocytic differentiation *in vivo.* This result is particularly interesting given that studies in the previous chapter have shown that Δ221EKLF mutant protein is expressed at a lower level than wild-type EKLF protein. Despite this caveat, my studies show that Δ221EKLF protein is expressed at a sufficient level to repress megakaryopoiesis *in vivo.* This conclusion is supported by results from colony-forming assays demonstrating equivalent numbers of megakaryocytic progenitors in fetal liver from Δ221EKLF and wild-type animals (Figure 4.4). Furthermore, chromatin immunoprecipitation assays show that expression of Δ221EKLF increases Δ221EKLF binding to the Fli-1 promoter and decreases PolII occupancy and acetylation of histone H3 at the Fli-1 promoter (Figure 4.6A-C). Given these results, I would have expected Fli-1 mRNA levels to be reduced in fetal liver erythroblasts from E14.5 wild-type and Δ221EKLF embryos as compared to EKLF-null erythroblasts; however, real time RT-PCR analysis showed no significant difference in Fli-1 mRNA levels (Figure 4.5B). Expression arrays between EKLF wild-type and EKLF-null fetal liver cells demonstrate only a 2.5-fold up-regulation of Fli-1 in the absence of EKLF (Frontelo *et al.*, 2007). By contrast, it is possible that the lineage fate decision has already been determined prior to E14.5 of murine development. Thus, Fli-1 mRNA level has been measured in cells that have already committed to the erythroid lineage in Δ221EKLF homozygous and wild-type animals. In a similar manner, cells lacking EKLF are driven towards the megakaryocytic lineage. As a result, the level of Fli-1 at this stage of murine development may not play a role in the lineage fate decision. To reconcile this discrepancy, I have also analyzed the levels of Fli-1 mRNA in cells derived from yolk sac of E9.5 of wild-type, Δ221EKLF, and EKLF-null embryos (Figure 4.5C). During this stage of murine development, the yolk sac is the source of all hematopoietic progenitor cells (Palis *et al.*, 1999; Lux *et al.*, 2008). Moreover, hematopoietic progenitor cell types, including erythrocyte and megakaryocyte progenitors, can be cultured from E9.5 yolk sac cells (Rampon & Huber, 2003). Indeed, I found that Fli-1 mRNA was decreased 2-fold in erythroid cells derived from E9.5 Δ221EKLF and wild-type yolk sac. Thus, these results are consistent with the idea that the level of Fli-1 is important during early murine development in order to influence lineage fate decisions.

Δ221EKLF uniquely inhibits Fli-1 by recruitment of a Sin3A-containing corepressor complex

Previous studies have shown that EKLF can function as a transcriptional repressor through its zinc finger DNA binding domain, which can recruit Sin3A and HDAC1 (Chen & Bieker, 1996; Chen & Bieker, 2004) and more recently through sumoylation of lysine 74, which can then recruit the Mi-2β component of the NuRD repressor complex (Siatecka *et al.*, 2007). Interestingly, in contrast to wild-type EKLF, expression of a K74R EKLF mutant that cannot be sumoylated cannot repress megakaryopoiesis in transgenic mice (Siatecka *et al.*, 2007). Several new results obtained from my studies directly challenge the latter observation. First, I show that expression of Δ221EKLF is sufficient to repress megakaryocytic differentiation *in vivo* in a similar manner to wild-type EKLF (Figure 4.4). Thus, this indicates that sumoylation of lysine 74 is not required for EKLF-mediated repression of megakaryopoiesis *in vivo* (Siatecka *et al.*, 2007). Furthermore, my studies utilized a model in which the Δ221EKLF protein is expressed from the endogenous EKLF promoter under the control of *cis* regulatory elements. In contrast, studies by Siatecka *et al.* (2007) were performed in a transgenic murine model in which the mutant protein was over-expressed under the control of the PF4-gene promoter (Siatecka *et al.*, 2007), which may not recapitulate the events *in vivo* precisely. The most obvious pitfall is that proteins expressed from heterologous promoters may not be expressed in a tissue- and developmentally-specific manner as the endogenous protein. My studies also found that Sin3A occupancy is increased at the Fli-1 promoter in Δ221EKLF and wild-type erythroblasts (Figure 4.7). These observations are in contrast to studies demonstrating that the NuRD repressor complex is involved in EKLF-mediated repression of megakaryopoiesis (Siatecka *et al.*, 2007). Sin3A is not part of the NuRD complex (Downes *et al.*, 2000). Taken together, my results provide strong support for the Δ221EKLF domain in EKLF-mediated repression of megakaryocytic differentiation through recruitment of a Sin3A-containing repressor complex.

Three potential models of EKLF-mediated repression of megakaryopoiesis can be derived from review of the recent literature (see Introduction above; Figure 4.1). Although support exists for each model, the precise mechanism by which EKLF represses megakaryocytic differentiation remains to be fully explored. Based on my current studies, I propose a modified model in which EKLF represses megakaryopoiesis, at least in part, by inhibition of Fli-1 through recruitment of a Sin3A-containing repressor complex (Figure 4.8). The Δ221EKLF domain alone is sufficient for this recruitment. At this time, studies in our laboratory are ongoing to address the other two potential models in the context of Δ221EKLF. We have planned experiments to explore expression of megakaryocytic genes (i.e. GpIX) *in vivo* and occupancy of Δ221EKLF protein and additional factor binding at these gene promoters. Furthermore, we propose studies to confirm EKLF and Fli-1 interaction *in vivo* by purifying Δ221EKLF protein complexes. Regardless of the exact mechanism of EKLF-mediated repression of megakaryopoiesis, the Δ221EKLF mice have proven to be an indispensable model in which to explore this question *in vivo*.

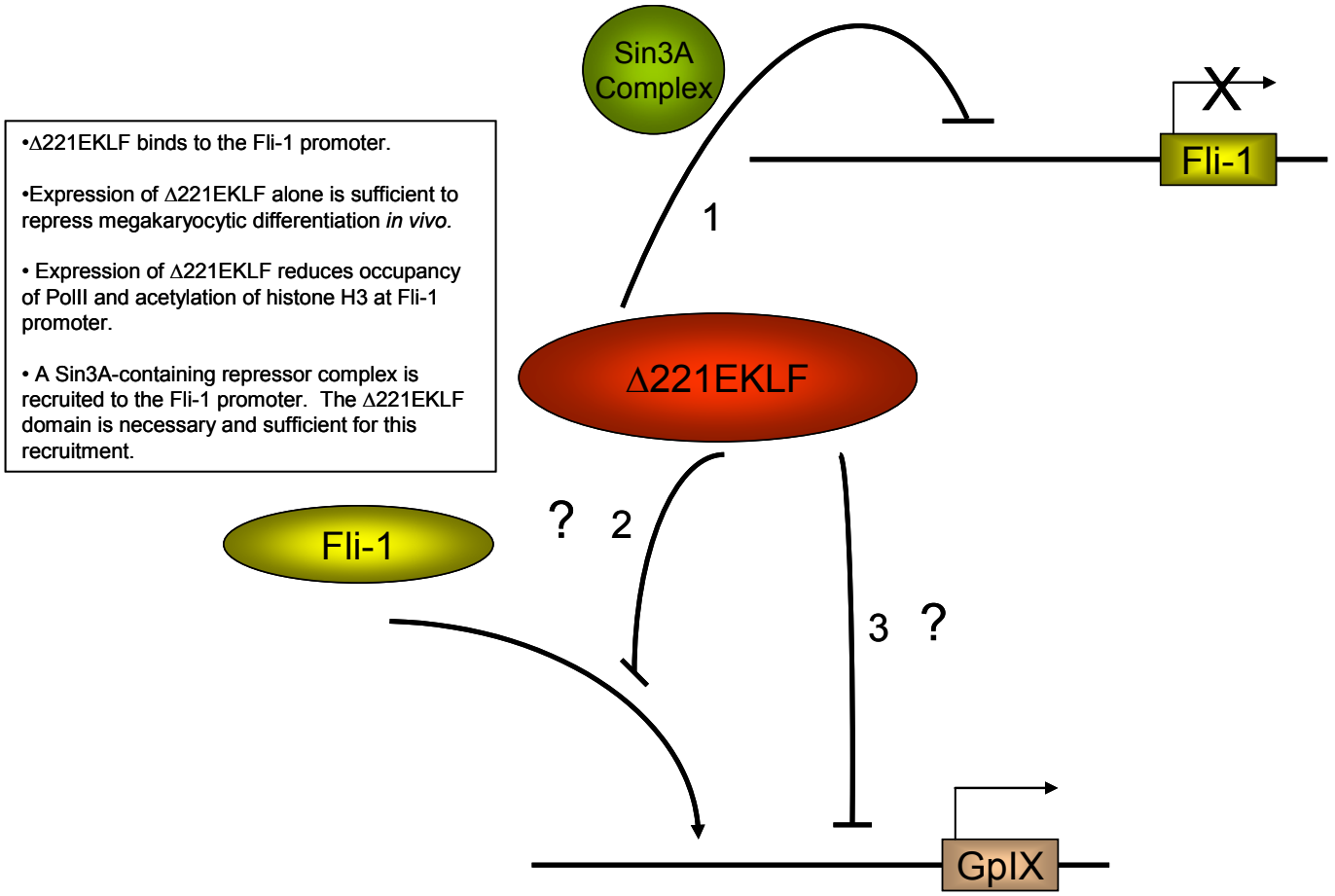

Figure 4.8. Modified potential models of Δ221EKLF-mediated repression of megakaryopoiesis. Shown here is a simplified diagram depicting three possible mechanisms for EKLF-mediated repression of megakaryopoiesis. Results obtained from the current studies in my dissertation support the model in which EKLF inhibits Fli-1 gene transcription through recruitment of a Sin3A-containing repressor complex. The Δ221EKLF domain alone is sufficient for this repression *in vivo*. It is unclear at this juncture how Δ221EKLF affects the other two potential models of EKLF-mediated repression of megakaryopoiesis. Studies are ongoing to address these questions.

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

The studies presented in this dissertation contribute to our understanding of the role of the zinc finger transcription factor EKLF or erythroid Krüppel-like factor in erythroid and megakaryocytic differentiation. I have generated mice expressing an amino truncation mutant of EKLF (Δ221EKLF). This domain of EKLF has been shown to retain full and appropriate chromatin remodeling activity of wild-type EKLF *in vivo.* However, expression of this domain is insufficient to rescue high-level β-globin gene transcription *in vivo*. In contrast, expression of Δ221EKLF is sufficient to inhibit expansion of megakaryocytic progenitors observed with a complete loss of EKLF. Thus, the studies described in the preceding chapters provided novel and insightful knowledge on the role of EKLF-mediated activation and repression at erythroid and megakaryocytic gene loci.

Δ221EKLF mice are an excellent model to study separable chromatin remodeling and ß-globin gene transcription *in vivo*

I have generated mice expressing a unique domain of EKLF that uncouples chromatin remodeling and transactivation of β -globin transcription in an intact animal model. Utilizing these animals, I have shown in Chapter 3 that expression of Δ221EKLF cannot rescue the EKLF-null phenotype. Similar to EKLF-null mice, the Δ221EKLF mice succumb *in utero* at E15.5 of gestation, partially as a result of severe anemia. This observation is supported by low levels of β-globin transcripts in Δ221EKLF erythroblasts. In contrast to lack of EKLF, expression of Δ221EKLF restores DNase I HS at the β-globin promoter and induces H3 acetylation at the β-globin promoter. Thus, chromatin remodeling and activation at the β-globin promoter has been uncoupled *in vivo*. Furthermore, the Δ221EKLF mice provide a unique opportunity in which to dissect the determinants required for chromatin remodeling and histone modification from those essential for β-globin gene transcription *in vivo.*

EKLF has been shown to associate with a SWI/SNF chromatin remodeling complex. EKLF interaction with Brg1, the ATP-catalytic core of the E-RC1, results in chromatin remodeling of a chromatinized β-globin template (Armstrong *et al.*, 1998; Kadam *et al.*, 2000). Additionally, Δ221EKLF and Brg1 interaction has been confirmed by *in vitro* studies in our laboratory (Brown *et al.*, 2002). However, evidence for EKLF interaction with Brg1 at the β-globin *in vivo* is still lacking. Thus, the Δ221EKLF mice are the ideal reagents in which to explore this question *in vivo*. I propose to immunoprecipitate EKLF and Brg1 in fetal liver erythroblasts to confirm this interaction *in vivo*.

What is the role of Δ221EKLF in erythroid-specific transcription factories?

Work from Fraser's group has focused on understanding how genes migrate to specialized compartments in the nucleus for transcription called transcription factories (Osborne *et al.,* 2004). These transcription factories are discrete foci containing nascent RNA production and high concentrations of RNA PolII. Using a combination of techniques including three-dimensional (3D) fluorescence in situ hybridization (FISH), immunofluorescence, and chromosome conformation capture (3C) assays, their laboratory has provided support for the existence of such discrete factories. In these studies, they have shown that the β-globin gene is transcribed in the same factory as AHSP, a gene that is similarly expressed in the erythroid cell. These genes are separated by more than 50 mega bases on murine chromosome 7. Furthermore, they have shown that genes present on different chromosomes such as the β- and α-globin genes, also co-localize in the same transcription factory but at a lower frequency than genes linked in *cis* (Osborne *et al.*, 2004).

The idea of a discrete foci dedicated to transcription is plausible given that the number of factories visible in the nucleus is less than the numbers of expressed genes (Obsorne *et al.*, 2004). However, it is highly tempting to speculate on the factor(s) that recruit the genes to the specialized factories. It would be interesting to determine if EKLF is co-localized with erythroid-specific genes such as AHSP. Our laboratory currently has an on-going collaboration with the Fraser group to address this key question. Furthermore, studies should be extended to include the role of Δ221EKLF in these transcription factories.

Is Δ221EKLF sufficient for formation of the Active Chromatin Hub (ACH)?

High-level expression of the β-globin genes requires the upstream enhancer LCR. The precise mechanism of how the LCR functions to enhance transcription remains unsettled; however, it is relatively agreed upon that it involves communication between the LCR and the promoter. Indeed, establishment of the chromosome conformation capture or 3C technique has greatly advanced our knowledge of this communication. Using the 3C technique, the Grosveld and Fraser groups have determined that the LCR moves in close proximity to actively transcribed genes, forming a structure called the active chromatin hub (ACH). The ACH is a spatial configuration of the locus in which the LCR loops toward the active β-globin genes (Tolhuis *et al.*, 2002). Moreover, EKLF, along with GATA-1 but not NF-E2, has been shown to be required for ACH formation (Palstra *et al.*, 2003; Vakoc *et al.*, 2005; Kooren *et al.*, 2007). Thus in the absence of EKLF, a substructure is formed consisting of the regions outside of the locus and part of the LCR but does not contain the genes (Palstra *et al.*, 2003). A recent study from Bresnick's group has demonstrated that the chromatin remodeling factor, Brg1, is required for loop formation between the LCR and the β-globin promoter (Kim *et al.*, 2009). Despite these advances in our knowledge on LCR/β-globin promoter interactions, there are still many unanswered questions. Is chromatin remodeling mandatory for LCR/β-promoter interaction via looping? Results from mutant BRG1 mice imply this is a

requirement (Kim *et al.*, 2009). If yes, I hypothesize that Δ221EKLF is sufficient to form the ACH given this domain retains full chromatin remodeling activity. Is active gene transcription an absolute requirement for ACH formation? If yes, I hypothesize that Δ221EKLF would be insufficient to establish the ACH given that Δ221EKLF cannot activate β-globin gene transcription. Whatever the results, it would be interesting to know the determinants of EKLF required for ACH formation *in vivo*.

Does Δ221EKLF regulate other megakaryocytic genes *in vivo?*

In Chapter 4, I reported on new and interesting results demonstrating that expression of Δ221EKLF alone is sufficient to block expansion of megakaryocytic progenitors observed with complete loss of EKLF. This inhibition of megakaryopoiesis occurs, at least in part, by repression of Fli-1, a key regulator of megakaryocytic differentiation. Results from my ChIP analysis of the Fli-1 promoter suggest that Δ221EKLF directly regulate Fli-1 expression *in vivo* through a mechanism involving recruitment of a Sin3A-containing repressor complex to the Fli-1 promoter. These observations are contrasted with previous studies in cell lines that demonstrated sumoylation of EKLF at lysine 74 inhibits megakaryopoiesis through recruitment of a Mi-2β-containing complex (Siatecka *et al.*, 2007). It is of interest to note that Mi-2β is not part of any Sin3A-containing corepressor complex (Downes *et al.*, 2000). Thus, my results are consistent with the idea that sumoylation of EKLF is not the only mechanism for inhibition of megakaryopoiesis *in vivo*, as Δ221EKLF domain lacks the lysine 74 region.

I presented three potential, but not mutually exclusive, models by which Δ221EKLF could inhibit megakaryocytic differentiation. Although my studies have provided support for the model in which Δ221EKLF inhibits megakaryopoiesis by directly repressing Fli-1 expression levels *in vivo,* it would be interesting to explore the other potential models. It is possible EKLF, or Δ221EKLF, could directly regulate other megakaryocytic genes, such as glycoprotein (GP) IX. I propose studies in which the expression of GpIX is determined in Δ221EKLF erythroblasts compared to wild-type and EKLF-null erythroblasts. I would expect the expression level of GpIX to be reduced in Δ221EKLF and wild-type erythroblasts as compared to EKLF-null erythroblasts. It is possible we may not see a significant difference in expression of GpIX in wild-type, Δ221EKLF, and EKLF-null cells. Explanations for this observation are i) GpIX is not a direct target of EKLF-mediated repression or ii) GpIX expressions needs to be determined in the context of megakaryocytic progenitors only. Additionally, using ChIP analysis, we should examine the binding of Δ221EKLF at GpIX promoter. If Δ221EKLF regulates GpIX, we expect to see Δ221EKLF protein enrichment at the gene promoter. These future studies would provide significant information on the role of Δ221EKLF in the regulation of megakaryocytic genes.

Results from *in vitro* and cellular studies provide evidence for EKLF and Fli-1 interactions, and this protein-protein interaction may play a role in repression of

megakaryopoiesis. However, evidence for this interaction *in vivo* is still lacking. It is possible EKLF inhibits megakaryopoiesis by preventing Fli-1 protein binding to its target genes through protein-protein interaction. Studies in MEL cell lines have shown that Fli-1 occupancy at megakaryocytic gene promoters is increased with EKLF knock-down. These studies, however, did not confirm EKLF-Fli-1 interaction as the mechanism for this observation. A confirmatory experiment would be to immunoprecipitate Δ221EKLF and Fli-1 proteins *in vivo.*

In conclusion, the results in this study provide novel and interesting insights in to the *in vivo* role of the chromatin remodeling domain of EKLF in definitive hematopoietic cells. The results are consistent with the idea that expression of a truncated Δ221EKLF protein is sufficient to remodel the local chromatin architecture at the β-globin promoter; however, expression of the Δ221EKLF protein alone is not sufficient to transactivate β-globin gene transcription *in vivo.* Furthermore, the current study supports a role for Δ221EKLF in repression of the megakaryocytic progenitor expansion observed with complete loss of EKLF. Future experiments should be directed at exploring the role of Δ221EKLF in β-globin LCR/promoter interaction and transcription of erythroid- and megakaryocytic-specific genes.

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APPENDIX: SUPPORTING FIGURES AND TABLES

Table A.1. Putative EKLF-dependent genes.

Gene	Gene symbol	Signal log ratio (null/wt)
SRY-box containing gene 2	Sox2	6.1
acetyl-Coenzyme A synthetase 2	Acas2l	4.2
calcium channel, voltage-dependent	Cacna1b	4.0
benzodiazapine receptor associated protein 1	Bzrap1	2.3
inhibitor of DNA binding 4	Id4	1.7
potassium voltage-gated channel	Kcnq5	1.7
erythroid differentiation regulator 1	Erdr1	1.4
eukaryotic translation initiation factor 2	Eif2s3y	1.3
insulin-like growth factor binding protein 1	Igfbp1	1.3
talin 1	Tln1	1.3
erythropoietin receptor	Epor	-1
ankyrin repeat and BTB (POZ)	Abtb1	-1.1
eosinophil-associated, ribonuclease	Ear1	-1.1
myeloblastosis oncogene-like 2	Mybl2	-1.1
transportin 2 (importin 3, karyopherin beta 2b)	Tnpo2	-1.1
B-cell CLL/lymphoma 11A (zinc finger protein)	Bcl11a	-1.2
B-cell translocation gene 2, anti-proliferative	Btg2	-1.2
calmodulin regulated spectrin-associated protein	Camsap1l1	-1.2
cyclin-dependent kinase inhibitor 2D	Cdkn2d	-1.2
lamin A	Lmna	-1.2

Table A.1 (continued).

Gene	Gene symbol	Signal log ratio (null/wt)
aminolevulinic acid synthase 2, erythroid	Alas2	-1.3
biliverdin reductase B	Blvrb	-1.3
STEAP family member 3	Steap3	-1.3
synuclein, alpha	Snca	-1.5
ankyrin repeat domain 9	Ankrd9	-1.7
erythrocyte protein band 4.2	Epb4.2	-1.8
aminolevulinate, delta-, dehydratase	Epb4.2	-1.8
aminolevulinate, delta-, dehydratase	Alad	-1.9
eukaryotic elongation factor-2 kinase	Eef2k	-1.9
E2F transcription factor 2	E2f2	-2.4
erythrocyte protein band 4.1	Epb4.1	-2.4
Kruppel-like factor 1 (erythroid)	Klf1	-2.5
ankyrin 1, erythroid	Ank1	-2.6
LIM domain only 2	Lmo2	-2.5
Kruppel-like factor 3 (basic)	Klf3	-2.8
transferrin receptor	Tfr	-2.8
SRY-box containing gene 6	Sox6	-3.4
Duffy blood group	Dfy	-3.8
erythrocyte protein band 4.9	Epb4.9	-5.1
erythroid associated factor	Eraf; AHSP	-7.2

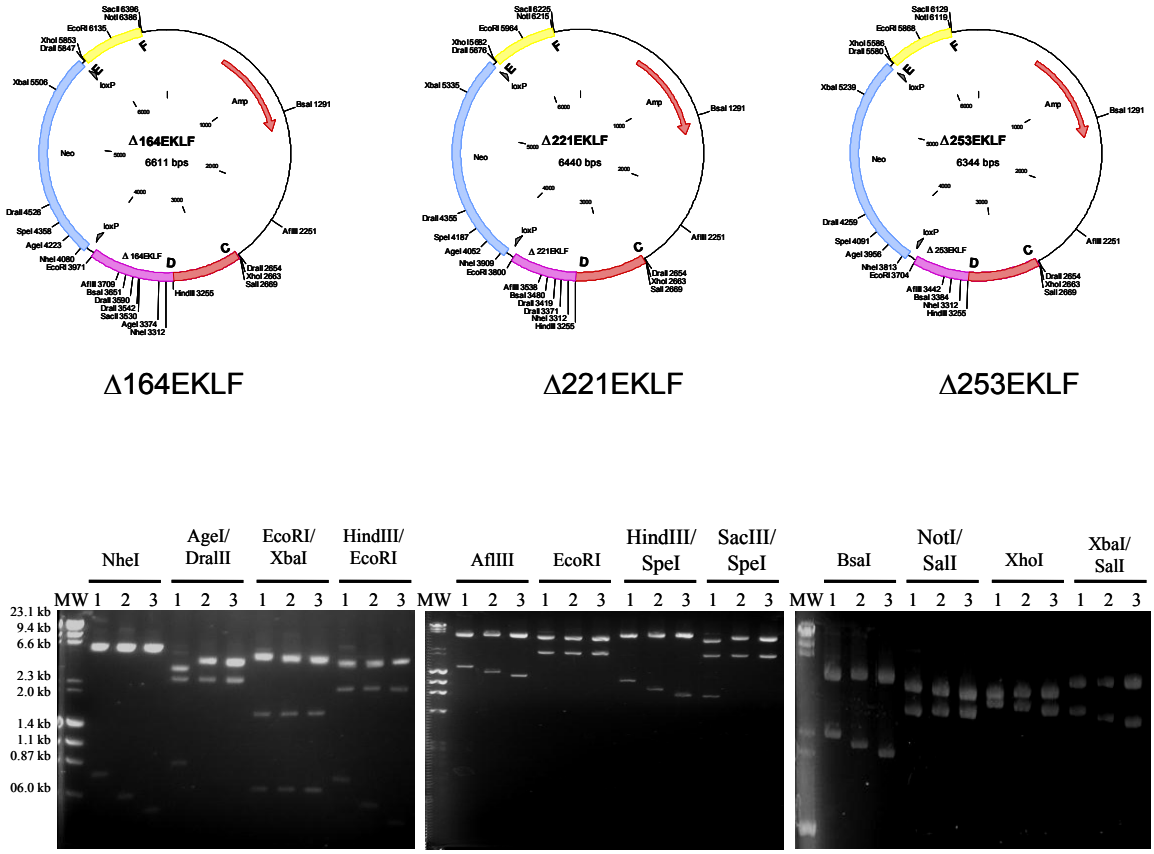

Figure A.1. Confirmation of the mini-targeting vectors. The top panel shows the plasmid maps of the mini targeting vectors, HA-Δ164EKLF, HA-Δ221EKLF, and HA-Δ253EKLF, respectively. The bottom panel is representative agarose gels after digestion of the mini targeting vectors with the restriction enzymes. Lane 1 = HA-Δ164EKLF; lane 2 = HA-Δ221EKLF; lane 3 = HA-Δ153EKLF. The predicted fragments are given in Appendix Table A.2.

Table A.2. Predicted fragment size with restriction enzyme digestion.

Enzyme	Δ164 (1)	Δ221 (2)	Δ253 (3)
NheI	5.8 0.8	5.8 0.6	5.8 0.5
AgeI/DraIII	3.2 2.6 0.9	3.8 2.6	3.7 2.6
EcoRI/XbaI	4.4 1.5 0.6	4.3 1.5 0.6	4.2 1.5 0.6
HindIII/EcoRI	3.7 2.2 0.7	3.7 2.2 0.6	3.7 2.2 0.5
AflIII	5.2 1.5	5.2 1.3	5.2 1.2
EcoRI	4.4 2.2	4.3 2.2	4.2 2.2
HindIII/SpeI	5.5 1.1	5.5 0.9	5.5 0.8
SacIII/SpeI	3.7 2.0 0.8	4.4 2.0	4.3 2.0
BsaI	4.3 2.3	4.3 2.2	4.3 2.1
NotI/SalI	3.7 2.9	3.5 2.9	3.4 2.9
XhoI	3.4 3.2	3.4 3.0	3.4 2.8
XbaI/SalI	3.8 2.8	3.8 2.7	3.8 2.6

A. 5' Southern blot probe

CTAGCTAAAGCCTTTCTTATTAATGCCAAAAGAACTGAAGCAGGAAATCA
CGGTGGTTACACAGCCCAGCACCTAAGCCCAATTTACAGGACAAGGCTCA
TTTTTATTATTAGAGCTCCTTTGGTAACAGAGAAGGCTGCTCACAGCCAGC
ATGCTCAAGGTTGTATAAAGCACAACAATGTTATTTGTCTTCAGAAAAAC
ATCTGAGGTGTGGGAACATCATTTACCCTGACTATGCT

B. 3' Southern blot probe

AATTCGCCCTTAAGGGCCAGAACTTGGAGCGCCTTAGAAGTCACGTGACA
GCCAATGCCCAAGGGAGAGGCGTGTCCACGGATGAAGCCTATCCGGAGC
TGAGTCGGCCAGAAGCGGGAGGACGGGGCGGGGAGGACATTGGAGGAG
GCGGGGTCCGCTTGTGGGACAACTTCATAGGAAGCCCCGCCTCCGTATTG
GGCCTGGCGGGCTTCGTCTCCACCCTCGCGAAGCCGGGACACAGATCTCT
TCGGTTTTTAGGGGAAGTTCATCTGAATCTCGTGACCCGGCTGTCCCCGGG
TCTTTTAGAGTCCATTGCTGTCGCCTCATGGCAACACTGAGATCGCTGCTG
CTGGCTGCGCTGCTGTGGGTCCCTGCCGAAGCCCTGAGCTGCTATGGGGA
CTCCGGGCAGCCTGTGGATTGGTGAGTAAGTAGTCGCGGGACTGTCCCCC
GCACACTGCCTGGGGACCGGCGCGGGAATCCAAAAAACCTCAGATTCCTT
CTCTCCCCCAACCTCATGTCTTCACGGACCTCCAGGTTCGTGGT

Figure A.2. Sequences of Southern blot probes used in screening targeted ES cells and genotyping of animals. (A) The 239 bp 5' probe was used with *EcoRV* digest to identify an 8.7 kb wt band and 6.8 kb KI band. (B) The 543 bp 3' probe was used with *NheI* digest to identify an 11.1 kb wt band and 9.1 kb KI band.

Table A.3. Buffer composition used in ChIP analysis.

Buffer	Composition
Cell Lysis	10mM Tris pH 8.0 10mM NaCl 0.2% NP40 Add Protease inhibitors (Roche) and PMSF before use.
Nuclei Lysis	50mM Tris pH 8.0 10mM EDTA 1% SDS Add Protease inhibitors (Roche) and PMSF before use.
IP Dilution Buffer	20mM Tris pH 8.0 2mM EDTA 150mM NaCl 1% Triton X-100 0.01% SDS Add Protease inhibitors (Roche) and PMSF before use.
IP Wash Buffer 1	20mM Tris pH 8.0 2mM EDTA 50mM NaCl 1% Triton X-100 0.1% SDS
IP Wash Buffer 2	10mM Tris pH 8.0 1mM EDTA 0.25M LiCl 1% NP40 1% Deoxycholic acid
Elution Buffer	100mM NaHCO3 1% SDS (use high quality SDS)

Table A.4. Primers used for ChIP analysis.

Region	Primer sequence	Reference
HS2	5'-AGTCAATTCTCTACTCCCCACCCT-3' 5'-ACTGCTGTGCTCAAGCCTGAT-3'	Kiekhaefer *et al.*, 2002
IVR	5'-GTATGCTCAATTCAAATGTACCTTATTTTAA-3' 5'-TTACCTCTTTATTTCACTTTTACACATAGCTAA-3'	Kiekhaefer *et al.*, 2002
βmaj pro	5'-CAGGGAGAAATATGCTTGTCATCA-3' 5'-GTGAGCAGATTGGCCCTTACC-3'	Kiekhaefer *et al.*, 2002
βmaj ex3	5'-GCCCTGGCTCACAAGTACCA-3' 5'-TTCACAGGCAAGAGCAGGAA-3'	Kiekhaefer *et al.*, 2002
AHSP	5'-CTAACTCCAGGGAAGCCTCACC-3' 5'-TTTGTGTGTCTTCTGCACTAAGCG-3'	Pilon *et al.*, 2006
Fli-1	5'-CGTGGACCCCGTCATTGTT-3' 5'-GCACTGCGCACACAGGATACT-3'	Frontelo *et al.*, 2007
RPII215	5'-GCGAATCTATAAAGGGCGTCACT-3' 5'-TCGGCGCTTCTGAGGAGA-3'	Kiekhaefer *et al.*, 2002
α-globin pro	5'-TGACCAAGGTAGGAGGATACTAACTTCT-3' 5'-TTGCCCGGACACACTTCTTAC-3'	Anquita *et al.,* 2004

Table A.5. Primers used for real time RT-PCR analysis.

Gene	Primer sequence	Reference
Alas 2	5'-CACCTATGCTTAAGGAGCCA-3' 5'-CAGAAGCACACAGGAAAGCA-3'	Drissen *et al.*, 2005
Alad	5'-CTTTGATCTCAGGACTGCTG-3' 5'-AACAGCTGCGGTGCAAAGTA-3'	Drissen *et al.*, 2005
Pbgd	5'-TACTTCTGGCTTCCAAGTGC-3' 5'-CAAGGTGAGGCATATCTTCC-3'	Drissen *et al.*, 2005
Urod	5'-ATCCCTGTGCCTTGTATGCA-3' 5'-AGGTTGGCAATTGAGCGTTG-3'	Drissen *et al.*, 2005
Cpox	5'-CAATTTGAAGCCAGTCCGTG-3' 5'-CTGGACTAGAACTCCCTTTG-3'	Drissen *et al.*, 2005
Ppox	5'-ATTCCAGCTTCAGAGCTCAG-3' 5'-TACTGCAGATTCACCACAGC-3'	Drissen *et al.*, 2005
Fech	5'-ACCAGTGACCACATTGAGAC-3' 5'-GGCCTTGGAGAACAATGGAT-3'	Drissen *et al.*, 2005
AHSP	5'-GGATCAGCAGGTCTTTGATG-3' 5'-AGAGTACTCAGCTCTTGCTG-3'	Drissen *et al.*, 2005

Table A.5 (continued).

Gene	Primer sequence	Reference
Epb4.9	5'-TGCTCAAGACCCAAGGCTTA-3′ 5'-TCCTATCTGGTTTTGCCTGG-3′	Drissen *et al.*, 2005
GAPDH	5'-CCTGCCAAGTATGATGACAT-3′ 5'-GTCCTCAGTGTAGCCCAAG-3′	Drissen *et al.*, 2005
Ankyrin	5'-CTCCAGCCGGACCTGATAGAG-3′ 5′-GAACACGTGCGACCCTTCAGTAG-3′	Nilson *et al.*, 2006
Band 3	5′-CACAGTGCCTCTCCGTCGTCTCATC-3′ 5′-CCTTCCCCACCCACAGCCATAACAC-3′	Nilson *et al.*, 2006
BKLF	5'-GAAATGTCACCCCCTTTAATGAAC-3′ 5'-CACGATGACGGAAGGATGGT-3′	Funnell *et al.*, 2007
Fli-1	5'-CAACCAGCCAGTGAGAGTCA-3′ 5'-GCCCACCAGCTTGTTACATT-3′	
β-globin mRNA	5'-TTTAACGATGGCCTGAATCACTT-3′ 5'-CAGCACAATCAGATCATATTGC-3′	
β-globin 1^{o} transcripts	5'-CTTCTCTCTCTCCTCTCTCTTTCTCTAATC-3′ 5'-AATGAACTGAGGGAAAGGAAAGG-3′	

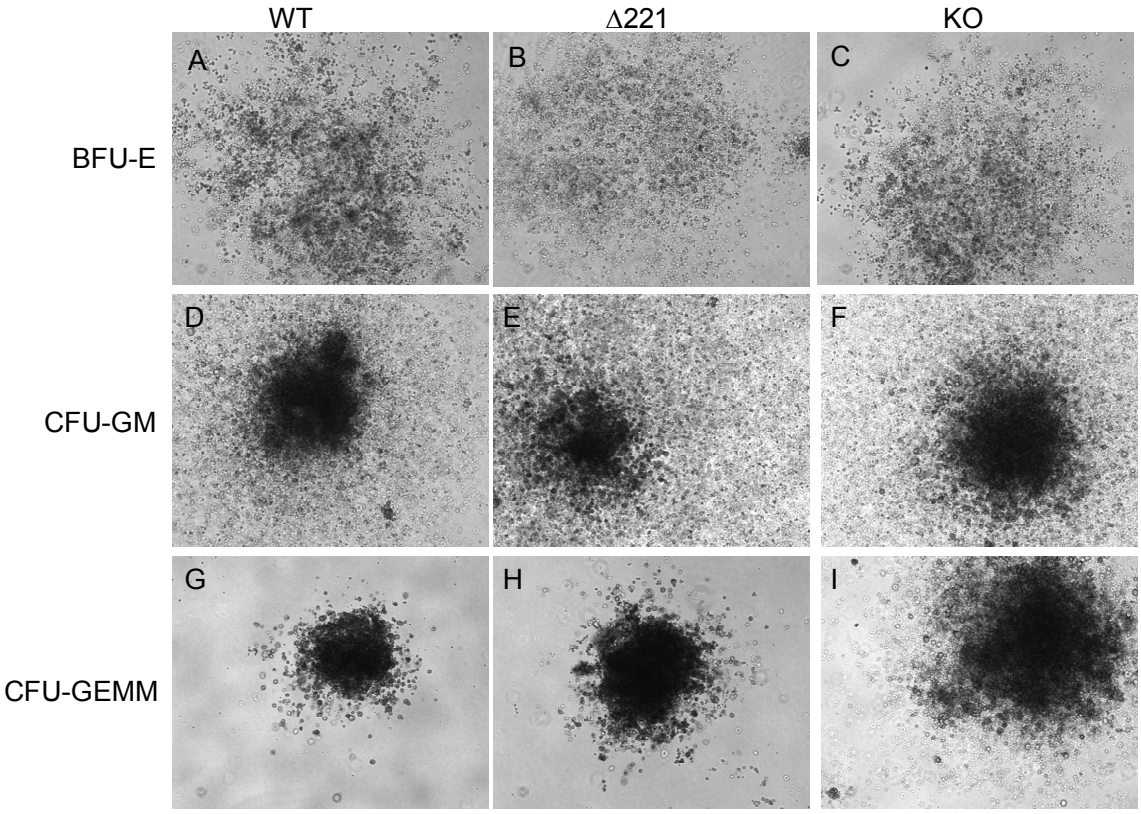

Figure A.3. Fetal liver hematopoietic progenitors. Fetal liver cells were plated in methocellulose medium and colonies were scored after 14 days of growth. Panels a-c: burst-forming unit-erythroid (BFU-E). Panels d-f: colony-forming unit-granulocyte-monocyte (CFU-GM). Panels g-i: colony-forming unit-granulocyte-erythroid-monocyte-megakaryocyte.

VITA

Valerie Malyvanh Jansen was born Phoukhaokham Malyvanh on September 18, 1977, in Vientiane, Laos. She immigrated with her parents to the United States at the age of 6 years and grew up in Nashville, Tennessee. In 1997, she graduated from John Overton Comprehensive High School and was accepted as a Presidential Scholar at Maryville College in Maryville, Tennessee. As an undergraduate student, she completed an internship at Oak Ridge National Laboratory in Oak Ridge, Tennessee, under the supervision of B.R. Evans, PhD, that resulted in a named U.S. patent. She graduated Magna Cum Laude with a BA in Chemistry in May 2001. In August 2001, she enrolled in medical school at the University of Tennessee Health Science Center in Memphis, Tennessee. In July 2003, she began her doctoral studies in the Department of Molecular Sciences, conducting her research at St. Jude Children's Research Hospital in the Division of Experimental Hematology under Dr. John M. Cunningham. In 2007, she moved with Dr. Cunningham's laboratory and completed her dissertation research at the University of Chicago, in Chicago, Illinois, in the Section of Pediatric Hematology/Oncology. Her PhD was granted in May 2009 from the University of Tennessee. She expects to graduate from Pritzker School of Medicine at the University of Chicago in June 2010.