hSET1 HISTONE METHYLTRANSFERASE ACTIVITY IS REQUIRED FOR TAL1-MEDIATED ACTIVATION OF TAL1 TARGET GENES DURING HEMATOPOIESIS

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2011

To Baron K. Faille, who has given me the opportunity to finish this

ACKNOWLEDGMENTS

I acknowledge my family and friends for their continued encouragement in me. I also acknowledge my adviser for giving me the opportunity to study in his laboratory.

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ADP	Adenosine diphosphate, a phosphate donor.
AML	Acute myeloid leukemia
ASH2L	hSET1 complex component, important in the structure and catalytic activity of the hSET1 and MLL histone methyltransferase complexes.
Cdk6	Cycling dependent kinase 6, a cell cycle checkpoint protein
CD34	Cluster of differentiation marker for early hematopoietic stem cells
ChIP-seq	Chromatin immunoprecipitation. Chromatin is isolated from nuclear or whole cell extract, before immunoprecipitation (see below.) followed by addition of Solexa adaptors and massive parallel sequencing.
DMSO	Dimethyl sulfoxide, used to differentiate MEL cells
DTT	Dithiothreitol, a small-molecule redox reagent known as Cleland's reagent, $C_4H_{10}O_2S_2$;its oxidized form is a disulfide-bonded 6-membered ring
EDTA	Ethylenediaminetetraacetic acid, is a polyamino carboxylic acid and a colourless, water-soluble solid. It is widely used to dissolve scale and in buffers, as a hexadentate ("six-toothed") ligand and chelating agent, i.e. its ability to "sequester" metal ions such as Ca^{2+} and Fe^{3+} and diminish their reactivity.
EPO	Erythropoietin, required for differentiating CD34+ cells into CD36+ hematopoitic stem cells to erythroid progenitors.
GATA1	Transcription factor involved in erythroid differentiation which binds to GATA motifs in DNA
GST	Gluathione-S-tranferase, which has an affinity for biotin.
H2A	Histone protein 2A
H2B	Histone protein 2B
H3	Histone protein 3
H3K4	Lysine at the 4^{th} position of the N-terminal tail of H3
H3K4-me3	Trimethylated H3K4

H4	Histone protein 4
HDAC	Histone deacetylases
HSC	Hematopoitic stem cell, the precuror to all of the blood cell types in the body.
HMT	Histone methyltransferase
^{3}H-SAM	Tritiated S-Adenosyl Methionine, the donor of hydrogen atoms in methylation reactions, both in vivo and in vitro
hSET1	Human histone methyltransferase, member of the complex which methylates histone H3 on lysine 4; required in transcriptional activation of gene promoters
IP	Immunoprecipitation, using antibodies to pull down proteins of interest, the digesting the proteins and purifying the DNA which can then be polymerase chain reaction (PCR) amplified using primers specific to a gene of interest.
LCR	The locus control region involved in regulating globin genes, including β-globin.
Ldb1	A transcription factor also misregulated in T-ALL. Associated with TAL1 to aid in differentiation of erythroid cells. Involved in P4.2 activation. Also known as NLI.
LMO2	A transcription factor also misregulated in T-ALL. Associated with TAL1 to aid in differentiation of erythroid cells.Involved in P4.2 activation.
LSD1	Lysine specific demethylase 1 also known as KDM1
MEL	Mouse erythroleukemia cells derived from spleens of mice susceptible to the Friend virus.
MLL	Mixed lineage leukemia protein, a histone methyltransferase from the hSET1 family in complexes MLL1-4.
P4.2	Red cell membrane protein, band 4.2
PMSF	phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride, a serine protease inhibitor commonly used in the preparation of cell lysates.
RbBP5	hSET1 complex member, important to the structure and function of the hSET1 and MLL complexes.

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis, a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight.) SDS gel electrophoresis of samples have identical charge per unit mass due to binding of 1.4g SDS per g of protein, which results in fractionation by size alone towards a cathode.
TAL1	T-cell acute lymphoblastic leukemia protein number 1
T-ALL	T-cell acute lymphoblastic leukemia
Tris-HCl	Tris has a pKA of 8.06. Tris-HCl, the acid salt, when titrated to pH = pKa with the corresponding counterion (OH- for tris-HCl) it is an effective buffer similar to biological condition. It is widely used as a component of buffer solutions, especially for solutions of nucleic acids.
WDR5	hSET1 complex component important in the structure and function of hSET1 and MLL histone methyltransferase complexes.
WDR82	hSET1 complex component unique to this complex, and also important in trimethylation of H3K4.

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

hSET1 HISTONE METHYLTRANSFERASE ACTIVITY IS REQUIRED FOR TAL1-MEDIATED ACTIVATION OF TAL1 TARGET GENES DURING HEMATOPOIESIS

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May 2011

Chair: Suming Huang
Major: Medical Sciences--Biochemistry

T-cell acute lymphocytic leukemia-1 (TAL1) also known as stem cell leukemia protein (SCL) is frequently over-expressed in T-cell acute lymphoblastic leukemia (T-ALL). TAL1 is an important transcription factor that functions at two crucial stages of hematopoiesis. First, it is important in the expansion of early hematopoietic stem cells. Second, TAL1 is important for terminal erythroid differentiation. Deregulation of these processes is commonly involved in leukemic transformation [1]. TAL1 is a member of the basic helix-loop-helix (bHLH) family of proteins. TAL1 forms heterodimers with other bHLH proteins, known as E2A proteins. TAL1 confers tissue-specific transcription and regulates target genes involved in early hematopoietic stem cell differentiation and erythroid differentiation [2]. TAL1 can activate or repress transcription of target genes by recruiting transcriptional coactivators or corepressors, respectively. The results herein show that TAL1 is associated with the histone H3K4 methyltransferase hSET1 complex. More specifically, these results show that TAL1 directly associates with the ASH2L subunit of the hSET1 complex and TAL1 associates with endogenous hSET1 complex in T-ALL Jurkat cells. In addition, hSET1 is required for TAL1-mediated activation of the P4.2 target gene by changing the histone methylation status from a repressed status to

active status (H3K4 trimethylation). Furthermore, knockdown of hSET1 decreases the TAL1 mediated activation of the P4.2 promoter activity. The regulation of P4.2 in erythroid differentiation reveals a common epigenetic mechanism in which histone H3K4 methyltransferase hSET1 is involved in the TAL1-mediated transcriptional activation during hematopoiesis and possible leukemogenesis. This information will eventually help to design new therapeutic approaches to treat leukemia. Moreover, the study will shed light on the control of transcription factors on hematopoiesis.

CHAPTER 1
INTRODUCTION

Hematopoiesis

Hematopoiesis is characterized by the development of distinct cell types through the regulated differentiation and proliferation of pluripotent hematopoietic stem cells (HSC) and oligo-potent progenitors [3]. The two common oligopotent hematopoietic progenitor cell lineages, are lymphoid and erythroid/myeloid. The lymphoid progenitor cells have the ability to differentiate into all of the white blood cell types that exist in the body, including B-cells and T-cells. The erythroid/myeloid progenitor cells have the ability to differentiate into erythrocytes as well as megakaryocytic and granulocytic cells (Figure 1-1). Transcription factors specific to each cell type as well as cytokines play key roles in the process [4]. Gene-targeting studies in mice have identified a series of regulators that function non-redundantly at various stages of hematopoietic cell differentiation and in specific blood cell lineages [5]. Thus a carefully orchestrated binding of transcription factors is key in proper development of hematopoietic cells and the deregulation of this process leads to leukemogenesis [6].

Leukemia and Leukemogenesis (Regulation)

The study of transcriptional regulation of hematopoiesis provides crucial insight into how aberrant regulation of gene expression leads to leukemogenesis. Many genes that encode crucial hematopoietic transcription factors are also deregulated in human leukemias. Fusions as well as deletions of these transcription factors resulted from chromosome translocation events are frequently involved in leukemogenesis [3]. For example, a fusion of SET domain containing MLL protein, which is also a histone methyltransferase, and of the same family as the SET1 protein, is involved in acute

myeloid and lymphoblastic leukemia [7]. Further, Ldb1 and LMO2, which are hematopoietic transcription factors and also specifically involved in activation of the erythroid target gene P4.2 are also involved in translocations leading to leukemogenesis as is TAL1 [1,8,9].

Introduction to TAL1

TAL1 was originally identified due to a (1:14) translocation in human T-cell acute lymphoblastic leukemia (T-ALL) patients, which involves deletion of a 90 bp regulatory segment placing the TAL1 gene under the control of a T-cell receptor promoter which is active in T-cell development. This leads to the ectopic expression of TAL1 in T-cells. In fact, TAL1 is ectopically expressed in 60% of all T-cell acute leukemias [3]. It is a member of the basic helix-loop-helix (bHLH) family of transcription factors and is required for the development of all hematopoietic cell lineages [10,11]. TAL1 forms heterodimers with the products of the ubiquitously expressed bHLH genes, E2A or HEB, and binds a hexanucleotide sequence known as an E-box, CANNTG, to regulate transcription [12]. (Figure 1-2). The DNA binding function of TAL1 is dispensable in the early expansion of hematopoietic cells yet crucial in terminal erythroid differentiation [13]. In addition to direct effects as a transcription factor, TAL1 also exerts influence on target genes by recruiting histone methyltransferases, histone acetyltransferases, histone demethylases, histone deacetylases and transcription factors which change the conformation of chromatin to allow transcription of the target genes at the appropriate stages of development for specific mature hematopoietic cell types.

TAL1 Is A Critical Regulator of Hematopoiesis and Leukemogenesis

During the process of hematopoiesis, genes critical for differentiation of early hematopoietic stem cells into specific lineages of oligopotent progenitors, then finally

into mature blood cells are actively transcribed. At the same time, transcription of cell proliferation genes are turned off, in part by epigenetic mechanisms [1]. Deletion of Tal1 in mice leads to embryonic lethality 8.5 days post conception (E8.5) due to complete loss of yolk sac hematopoietic cells [14]. Further, Tal1-null embryonic stem (ES) cells are unable to generate both primitive and definitive erythroid cells in vitro and do not contribute to hematopoiesis in vivo in chimeric mice suggesting a key role of TAL1 in erythropoiesis [15]. In addition, studies indicated that TAL1 is also required for proper B- and T-lineage development [15,16] (Figure 1-1).

During hematopoiesis, TAL1 can function as repressor or activator of transcription depending on the sequence context and differentiation stage of the cells. TAL1 recruits mSin3a- and histone deacetylase 1/2 (HDAC1/2)-associated corepressor complexes, which mediate its transcription repressive activity in certain stages of erythroid differentiation. While TAL1 activity is required throughout erythroid differentiation, the TAL1 complexes containing HDACs and deacetylase activity are markedly decreased during differentiation [17]. Consistent with the role of histone methylation in TAL1-mediated transcriptional repression, our lab has recently discovered that TAL1 interacts with the histone lysine specific demethylase 1 (LSD1 or KDM1) in T-ALL Jurkat and erythroleukemia cells [18]. LSD1 removes methyl groups from histone H3 lysine 4 (H3K4) residues. This H3K4 trimethyl modification is found in transcriptionally active or competent chromatin [19]. In proliferating erythroleukemia cells, TAL1 represses target gene expression in part by recruiting LSD1 to demethylate H3K4 dimethylation marks at promoter regions [18]. However, despite the role of TAL1 in transcriptional repression, it has also been shown that TAL1 interacts with co-activating histone modifying enzymes

such as p300 and the p300/CBP associated factor (PCAF) in hematopoietic cells as well as in T-cell leukemia [17,20]. These data also support that despite its known status as a transcriptional repressor, TAL1 can also positively regulate transcription.

Chromatin Structure and Function

Within eukaryotic nuclei, DNA wraps around histone octamers, consisting of two copies each of H2A, H2B, H3 and H4, to form repetitive units known as nucleosomes. The N-terminal tails of core histones protrude out from the nucleosome and are dynamically regulated by covalent post-translational modifications. Enzymatic modifications of the histone N-terminal tails have been implicated in the modulation of chromatin structure and gene expression. Like DNA methylation, histone modification patterns, associated with specific gene loci, are often maintained during replication and transmitted to daughter cells [21]. Histone modifications include phosphorylation, acetylation, methylation, ubiquitination, sumolation, adenosine di-phosphate (ADP)-ribosylation and proline isomerization [22]. Among these modifications, histone acetylation and methylation occurring at lysine (K) residues on histone H3 and H4 tails have been recognized as an important epigenetic mechanism with links to transcriptional activation and repression [22,23]. These epigenetic modifications are critical for maintaining normal gene expression patterns in developmental processes such as hematopoiesis. Further, hematopoietic cell differentiation is often perturbed in malignancies such as leukemia [4,24,25].

Throughout hematopoietic lineage development, histone acetylation and methylation patterns play critical roles in converting cell fate decisions into epigenetic information that determine the gene expression patterns into specific mature blood cell types [16]. This process is initiated by the binding of sequence-specific transcription

factors to the regulatory elements of target genes in hematopoietic cells. These transcription factors then recruit coregulatory enzymes, many of which possess intrinsic histone modification activities to augment or inhibit target gene transcription [1,17,20,26,27]. For example, erythroid-specific transcription factors, such as TAL1, GATA1, EKLF and NF-E2 [1,28,29] form complexes and differentially recruit histone acetyltransferases (HATs) which correlate with actively transcribed genes [20,30] or histone deacetylases (HDACs) which repress transcription [2,17] to modulate expression of erythroid specific target genes (Table 1-1.) Aberrant regulation of hematopoietic specific transcription factors and coregulators often leads to the development of specific forms of leukemias [2,16,24,29,31-33]. Changes in epigenetic marks due to misregulation of histone modifying enzymes may alter the function of TAL1, resulting in aberrant transcription during hematopoietic development which results in malignant hematopoiesis.

hSET1 Is A Histone Modifying Enzyme Involved in Transcriptional Activation.

The human SET1 complex (hSET1) is a SET domain containing H3K4 methyltransferase whose activity is correlated with 'open' chromatin structure and active transcription [34]. The hSET1 complex contains SET1, in which the SET domain confers H3K4 specific histone methyltransferase (HMT) activity [35] (Table 1-2, Figure 1-3). H3K4me2/3 is essential for recruitment of RNA polymerase II (RNAPII) and transcription machinery to the promoter of the target gene [22,35]. There are several SET domain containing protein complexes (hSET1, and MLL1-4) and they are highly conserved from yeast to man (Table 1-2). Though yeast has only one SET1 complex, man has several homologs [34,36]. All of the SET family histone methyltransferase complexes contain ASH2L, WDR5, RbBP5 and HCFC1 (Table1-2.) Though both MLL1 and hSET1 have H3K4 specific HMT activity, the hSET1 complex has a broader activity than the MLL

complexes, which have more specific target genes [34]. Further, the hSET1 complex is known to bind to the serine-5 phosphorylated, large subunit of RNAPII, Rtf, in yeast. This is at the C-terminal domain of the elongating polymerase. When serine 5 of Rtf is phosphorylated, transcription is active; [37] this provides an additional link between hSET1 and activation of genes.

The proteins within the SET1 complex have the following functions: ASH2L is required for molecular regulation of H3K4 trimethylation, but not di- or mono-methylation in yeast [36]. Therefore, it is likely that knockdown of this component will only affect trimethylation levels. However, the data herein show that TAL1 binds directly to ASH2L. Thus knockdown of ASH2L may affect transcriptional activation of the TAL1 target gene due to failure to recruit RNAPII. The hSET1 complex components WDR5 and WDR82 are also both important for the stability of the SET1 complex [38]. Further, WDR82 may be unique to the hSET1 complex as is hSET1 itself, and thus knockdown of this component and hSET1 will only affect hSET1 mediated H3K4 methylation at promoters and hSET1 target gene expression [38]. It is likely that TAL1 recruits hSET1 H3K4 methyltransferase to regulate target gene expression. One example suggesting this is the following: in addition to TAL1 binding at the P4.2 promoter, an increase in H3K4 dimethylation and trimethylation at the P4.2 promoter is also observed during DMSO induced differentiation of MEL cells. Since H3K4 trimethylation is associated with active genes, this could be the signal to activate target genes at the correct developmental stage in the cell.

Protein 4.2 (P4.2): Red Blood Cell Membrane Protein

Along with β-globin, the expression of protein 4.2 (P4.2) is a mark of erythroid differentiation, used in previous studies because P4.2 mRNA is expressed in early

erythroblasts. P4.2 is an important component of mature erythrocyte membrane skeletal networks which regulates the stability and flexibility of the mature red blood cells [39]. Deficiency of P4.2 leads to hemolytic anemia with various penetrance levels, especially prevalent in Japanese populations [40]. There are 7 isoforms of P4.2, the largest of which encodes a 72 kDA membrane protein. The expression of this gene has been studied utilizing erythroblasts from bone marrow and erythroid cells cultured by the two-phase liquid culture method from burst-forming unit erythroid (BFU-E) cells in peripheral blood in mice [41].

Transcription of the mouse P4.2 gene initiates at multiple sites, with the major initiation site mapped at 174 nucleotides upstream of the ATG start codon. The mouse P4.2 promoter is TATA-less and contains multiple potential binding sites for erythroid transcription factors GATA-1, NF-E2, EKLF, and tal-1/SCL. Transient transfection experiments demonstrated that a 1.7-kb mouse P4.2 promoter fused with the luciferase coding regions was induced in DMSO-treated MEL cells. TAL1 is known to associate with many binding partners including E2A, GATA1 LMO2, LDB1, and ETO2, all of which are involved in modulating hematopoietic cell growth and differentiation and expression of P4.2 [8,42,43].

Deletion analysis showed that the GATA-binding site at position -29 to -24 (upstream, relative to the promoter) is required for p4.2 expression after induction in differentiated MEL cells [39]. Further studies show that GATA1 bound to the dual GATA sites in the promoter form a bridge with TAL1/E-protein heterodimers linked by LMO2 and Ldb1 to initiate transcription of the p4.2 gene [43]. GATA1, Ldb1, LMO2 and TAL1 are among transcription factors whose function is critical in hematopoiesis [1,8,43].

Additionally, in the case of P4.2 the ETO2 protein binds to the dual GATA sites to prevent premature activation of the gene by this TAL1/Ldb1/LMO2 bridge [42].

Previous research regarding the P4.2 promoter shows that LSD1 is recruited to restrict the ability of progenitor cells response to cellular differentiation programs [8,12,32,42-44]. Also, after differentiation, LSD1 is switched to repress TAL1 target genes that promote cellular proliferation such as P4.2. In supporting this view, LSD1 disappeared from the p4.2 promoter which became H3K4-hypermethylated and histone-hyperacetylated during differentiation [6,18].

Rationale for This Study

We undertook the biochemical purification of TAL1 associated nuclear protein complexes from T-ALL leukemia cells and showed that TAL1 copurifies with the hSET1complex suggesting that hSET1 may confer positive effects on TAL1-mediated transcription. My hypothesis is that recruitment of hSET1 plays an important role in activation of the TAL1 target gene P4.2 in erythropoiesis. Colocalization of TAL1 and increased H3K4 methylation at the p4.2 promoter during transcriptional activation of p4.2 gene and the hSET1 is present in the TAL1 associated protein complex support the hypothesis that recruitment of hSET1 by TAL1 and subsequent H3K4 trimethylation of target gene promoters plays an important function on TAL1 mediated transcriptional regulation and hematopoiesis. Thus in addition to associating with the LSD1 complex, TAL1 also associates with the hSET1 complex, perhaps acting as a molecular switch to activate hematopoietic specific gene expression patterns during hematopoiesis.

Table 1-1. Histone modifying enzymes required for erythropoiesis

TFs	Coactivators	Corepressors	References
TAL1/SCL	P300, PCAF, hSET1*	mSin3A, HDACs, Brg-1, LSD1	Huang, at al. 2000; Huang, et al. 1999; Huang & Brandt, 2000a; Schuh et al., 2005; Goardon et al., 2006; Hu et al., 2009
GATA-1	CBP	NURD, Mi-2, HDACs	Rodriguez et al., 2005; Hung et al., 1999; Hong et al., 2005; Blobel et al., 1998
EKLF	CBP/p300, Brg-1	mSin3A, HDACs	Chen & Bieker, 2001; Zhang et al., 2001; Armstrong et al., 1998
NF-E2	CBP/p300		Huang, et al. 1999

*added because of this study

Table 1-2. Conserved SET domain containing complexes ySET1, hSET1 and MLL1 complexes share components. WDR82 is unique to SET1. ASH2L is required for H3K4 trimethylation activity.

ySET1	hSET1	MLL1
SET1	SET1	MLL
CPS60	ASH2L	ASH2L
CPS50	RbBP5	RbBP5
CPS40	CXXC1	-
CPS35	*WDR82*	-
CPS30	WDR5	WDR5
CPS25	hDPY30	hDPY30
-	HCF1	HCF1/2
-	-	*Menin*

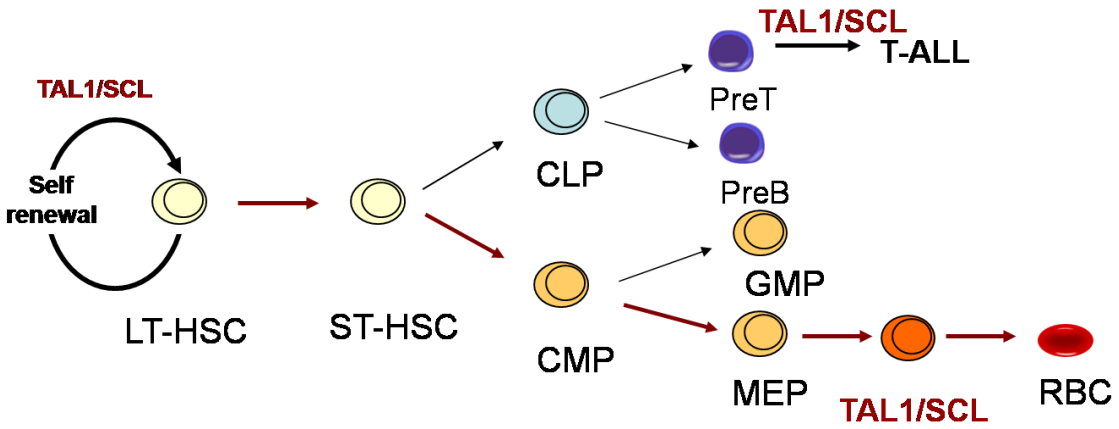

Figure 1-1. TAL1 is critical for differentiation of hematopoietic cells. TAL1 is important for early HSC/HPC differentiation and later in erythroid differentiation (black arrows.) In Jurkat cells, TAL1 is ectopically expressed, preventing differentiation into lymphoid lineage cell types.

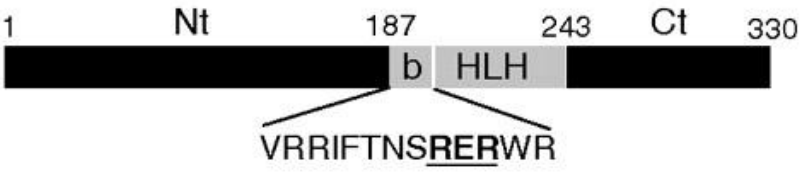

Figure 1-2. The structure of the TAL1 protein: Shown is the N terminus (Nt) the basic DNA binding region (b), the helix loop helix region (HLH) (the bHLH domain is made up of aa 187-243) and the C terminus (Ct.) The region within aa 143-185 is required to bind protein partners such as LSD1 [13].

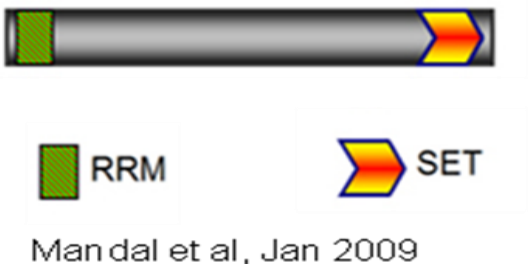

Figure 1-3. SET1 protein domains [45]. SET1a forms a HMT complex that associates with TAL1. The SET domain is the catalytic unit of the protein. There is also an RNA Recognition motif (RRM.)

CHAPTER 2
MATERIALS AND METHODS

Cell Culture

Murine erythroleukemia (MEL) cells were grown at 37°C with 5% CO^2, and maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S.) Induction of erythroid differentiation was performed by incubating cells in medium containing 1.8% dimethylsulfoxide (DMSO) for 24 hours.

Mass Spectrometry and Immunoprecipitation

The TAL1 associated complexes were purified from nuclear extract (NE) of the Jurkat T-ALL cell line transduced with flag-tagged TAL1 (Flag-TAL1) and identified by mass spectrometry at the Taplin facility in Harvard medical school. To prepare the nuclear extract, cells were lysed using the protocol developed by Nakatani and colleagues [46]. To confirm whether TAL1 associates with hSET1 in T-ALL, TAL1 associated complexes were immunoprecipitated from Jurkat nuclear extract using α-flag antibody (Sigma F3290) and then purified using an α-TAL1 antibody (sc-12984.) The resulting complex was resolved by SDS-PAGE and analyzed by western blotting using an α-SET1 antibody (Bethyl labs A300-289A, formerly BL1192) targeting the catalytic component of the hSET1 complex.

GST Pull-Down Assay

Both *in vitro* transcribed and translated ^{35}S-Met labeled hSET1 complex components as well as bacterially expressed GST-TAL1 were incubated in binding buffer (50 mM Tris HCl [pH 8.0]; 2 mM EDTA; 150 mM NaCl; 0.1% NP-40; 20 mM $ZnCl_2$; 10 mM $MgCl_2$; 1 mM DTT, 1 mM PMSF) containing 0.1 mg/mL bovine serum

albumin (BSA) for 1 hour at room temperature and then 1 hour at 4°C. This incubation was followed by washing with the same binding buffer to remove all protein not bound to GST or GST-TAL1.

H3K4-Specific Methylation Assay

Nuclear extracts were generated from both induced and uninduced MEL cells overexpressing FH-TAL1, before and after 24 h of DMSO induced differentiation (1.8% DMSO for 24 h) in the standard procedure used in our lab developed by Nakatani and colleagues [46]. These nuclear extracts were then immunoprecipitated using α-Flag antibody; the resulting complex was then purified with α-TAL1 antibody. This complex was then incubated with ^{3}H-Ado-Met and commercially available H3 peptides, or fully H3K4 trimethylated peptides in a buffer favorable to histone methylation (20 mM Tris-HCl, PH 8.0; 4 mM EDTA; 1mM PMSF, 500 uM DTT) for 1 h at 30°C. The products of this reaction were then run on a 10% SDS-PAGE gel. The presence of a band on an autoradiograph of this gel (incubated for 24 h at -80°C) indicates the specificity of the methylation activity of the TAL1 associated hSET1 complex for K4 residues in the histone H3 tails.

Chromatin Immunoprecipitation Sequencing (ChIP-seq)

ChIP-seq data was provided by our collaborators in the laboratory of Dr. Keji Zhao. Nucleosomes were isolated from CD34+ cells and cells differentiated to CD36+ with erythropoietin (Epo.) The DNA was purified and Solexa adaptors added before massively parallel sequencing of the P4.2 promoter region.

P4.2 Promoter Driven Luciferase Reporter Assay

To test if hSET1 is important for TAL1 transactivation, an assay containing a P4.2 driven luciferase reporter gene was used. This pREP-4.2-luc contains a P4.2 promoter,

upstream of the luciferase reporter gene. The pREP reporter plasmid is unique in that it can be transfected into mammalian cells and will replicate as an episome, in which the DNA and histones form the chromatin structure as it does within chromosomes.

Cloning of the pREP4-P4.2-luc Plasmid

The cloning of this plasmid involved taking a pREP4 plasmid, in which there is a multi-cloning site. The particular one used in this study already contained an insert with a long terminal repeat (LTR) sequence, which contains triple E-box motifs that can potentially also bind to TAL1. The P4.2 promoter, which contains dual GATA motifs and dual E-boxes, was cut out of a pCDNA3.1 vector backbone and inserted into this pREP4 episomal plasmid (Figure 2-1) An episomal plasmid replicates as a miniature chromosome within the cell in culture and forms chromatin as a normal chromosome. The P4.2 promoter was inserted with a single restriction site XhoI, into the pREP4 plasmid backbone and confirmed to be the correct orientation by restriction digest with XhoI and NotI, which is w/in the promoter clone inside the pREP4 backbone (data not shown.)

P4.2 Reporter Assay

Since the P4.2 linked luciferase promoter used in this study expresses luciferase upon activation the first step was to determine if P4.2 promoter driven luciferase was expressed in mouse erythroleukemia (MEL) cells upon DMSO induction. First, the MEL cells were co-transfected with pREP-4.2-luc and a plasmid encoding Tal1 cDNA (pCDNA-TAL1) using the Lipofectamine™ 200 kit performed in 24 well plates. 24 hours later, the cells were induced to differentiate by adding 1.5% DMSO and additional Optimem media for a total of 1mL per transfection in a 24-well plate. The resulting luciferase expression was measured at 72 hours after DMSO induction by correlating

bioluminescence produced with expression of the protein using a luciferase reporter kit (Promega.) Therefore, this assay measured TAL-mediated activation of the P4.2 driven luciferase reporter gene as a function of the amount of pCNDA3.1-Tal1 co-transfected into the promoter during erythroid differentiation. The resulting luciferase expression was measured using 100 uL per well in 96-well plates with a spectrophotometer.

siRNA Knockdown of hSET1 in the P4.2 Reporter Assay

Further, siRNA targeting hSET1 was co-transfected in an otherwise identical experiment, to determine the effect of hSET1 presence on TAL1 mediated activation. The hSET1 knock down (kd) studies were done using 100 ng of TAL1 and 10 ng of si-hSET1 co-transfected into MEL cells in the presence of the pREP4-P4.2-luc reporter. Lipofectamine™ was also used in this transfection, and the siRNA was added at the same time as the pCDNA3.1-Tal1 and pREP4-P4.2-luc plasmids, 24 hours before DMSO and additional Optimem media was added.

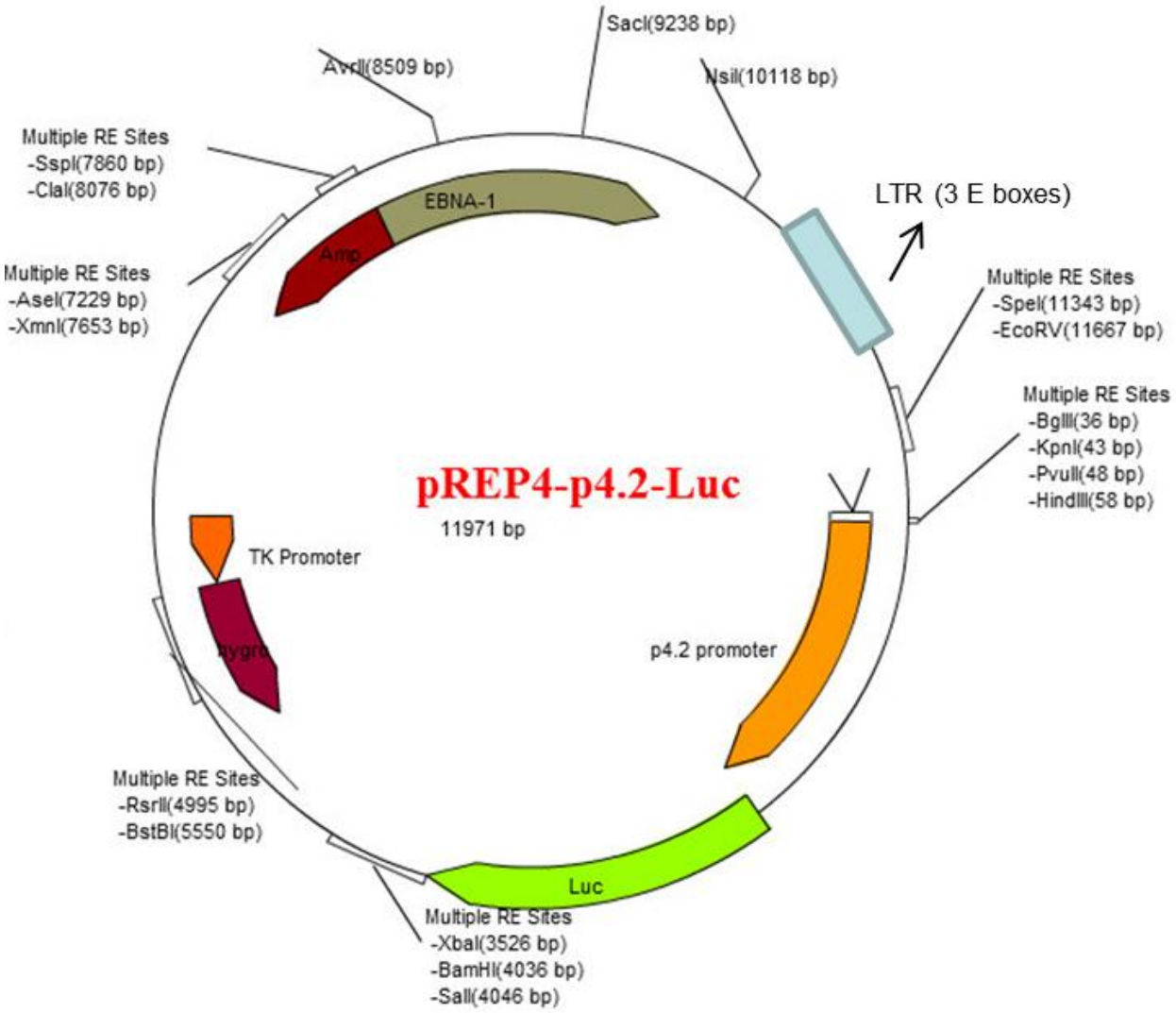

Figure 2-1. Structure of the pREP-P4.2-luc plasmid: Contains the P4.2 promoter driving the luciferase gene, which contains dual E-boxes and dual GATA sites. Further, long terminal repeats from the HIV virus which contain triple E-box motifs are also contained in this plasmid.

CHAPTER 3
THE ASSOCIATION OF TAL1 WITH hSET1

In this project, I focused on investigating how the opposite histone modifying enzymes, LSD1 and hSET1 complexes, which have been identified as TAL1-associated cofactors regulate the function of TAL1 in hematopoiesis and leukemogenesis. The specific aims of this research proposal are: 1.) Analyze the role of histone methyltransferase hSET1 complex in TAL1-mediated transcriptional regulation during erythroid differentiation; 2). Understand the molecular basis of histone modifying enzymes in TAL1-induced normal. The first aim was accomplished by characterizing the association of the TAL1-associated hSET1 complex with the TAL1 protein, and the H3K4-specific methylation of the complex. The second aim examined the effect of hSET1 onTAL1 mediated transcriptional regulation in hematopoiesis as well as the hSET1 mediated epigenetic modification, H3K4 trimethylation, which coincides with activation of the P4.2 gene, a marker for erythropoiesis.

The TAL1 Complex Associates with the hSET1 Complex

Previous studies in our lab have indicated that TAL1 activation or repression is determined by combinatorial association with coactivators versus corepressors. Therefore, it was important to first purify TAL1 associated complexes. The polypeptide signatures found in this analysis are listed in the end of this section (Figure 3-1). These data provide evidence that the hSET1 HMT complex associates with the TAL1 complex in a T-ALL leukemia cell line. Data gathered from mass spectrometry analysis shows that TAL1 containing complexes in Jurkat cells include several transcriptional corepressor and coactivator proteins (Figure 3-1). They are: 1) one complex containing corepresssors such as LSD1 and Co-REST and HDAC1, and 2) another complex

containing coactivators such as hSET1, and components of the hSET1 complex (ASH2L, WDR5) suggesting that TAL1 can activate or repress target genes by interacting with coregulatory enzymes and functions like a molecular switch.

The TAL1 complex is associated with hSET1 in the Jurkat T-ALL cell line. Jurkat leukemia cells are T-cell precursors in which TAL1 is ectopically expressed, a cell culture model for T-ALL leukemia. In this T-ALL cell line, TAL1 associates with several components of the hSET1 complex, including: hSET1, ASH2L, HCF1, and RBBP5 (Figure 3-1). To confirm whether TAL1 associates with hSET1 in T-ALL, TAL1 associated complexes were immunoprecipitated from Jurkat nuclear extract using α-TAL1 antibody (Santa Cruz Biotechnology, sc-12984) and western blotted (WB) with α-hSET1 antibody (Bethyl Labs A300-289A.) These data confirm that endogenous TAL1 interacts with hSET1 (Figure 3-2). These results support the idea that hSET1 associates with TAL1 in normal hematopoiesis and in leukemogenesis.

TAL1 Directly Interacts with the ASH2L Component.

In addition to determining the association of hSET1 and TAL1, I determined which component of the hSET1 complex directly associates with TAL1 using *an in vitro* GST-pulldown assay (Figure 3-3 A). These data show that TAL1 interacts with ASH2L, a component required for trimethylation of H3K4 [36]. Further, the results show that there is no direct interaction of TAL1 with the other hSET1 complex components tested, including RbBP5, WDR5 or the SET1 catalytic unit itself (Figure 3-3 B).

The TAL1 Complex Displays H3K4 Specific Histone Methyltransferase (HMT) Activity

To determine whether the TAL1 complex exhibits HMT activity throughout differentiation, nuclear extracts from FH-TAL1 over-expressed MEL cells, before and

after DMSO induced differentiation were prepared. The TAL1 complex was purified using an α-Flag immunoaffinity column, then the resulting purified complex was then used in an in vitro methylation assay. These results indicate that the TAL1 complex not only has HMT activity in general but also that this HMT activity is H3K4 specific. This is because the fully trimethylated H3K4 histones were not methylated by the complex (Figure 3-4, lanes 2 and 4) whereas H3 alone was methylated (lanes 1 and 3).

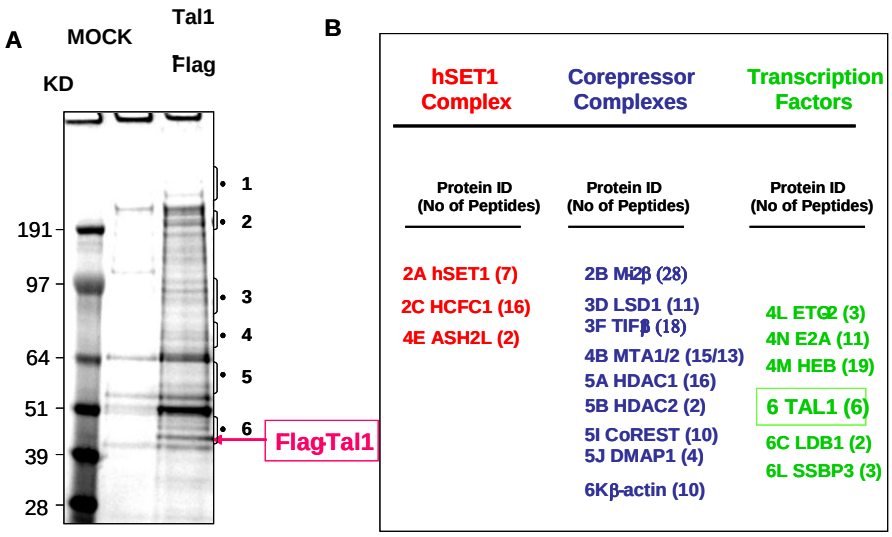

Figure 3-1. Identification of the TAL1 interacting proteins, including hSET1 components as well as corepressors. A partial list of polypeptides identified by mass spectrometry above is grouped by biological function. TAL1 associating polypeptides were purified from the nuclear extract of 1×10^9 Flag tagged-TAL1 transduced and mock transduced Jurkat cells by using an α-Flag antibody conjugated column, eluting polypeptides containing the Flag peptide. The resulting flag-TAL1 complexes were resolved by SDS-PAGE and the proteins visualized with Coomassie blue staining and analyzed by mass spectrometry.

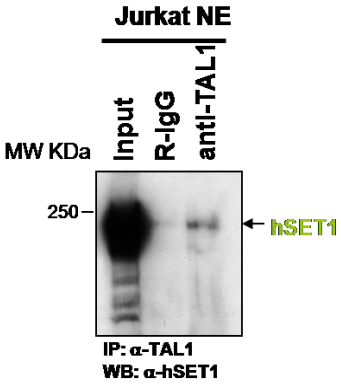

Figure 3-2. TAL1 recruits endogenous hSET1 and the H3K4 HMT in Jurkat cells. The Jurkat nuclear extracts were immunoprecipitated with TAL1 antibody. The immuno-complexes were resolved by SDS-PAGE, and analyzed with western-blot using hSET1 antibody.

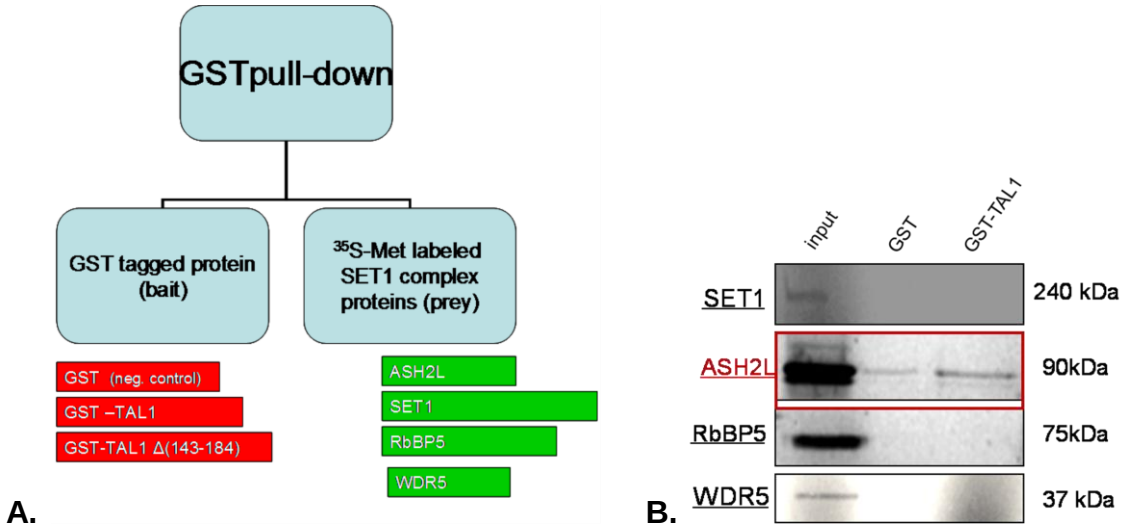

Figure 3-3. GST-pulldown assays. A) Experimental scheme of GST-pulldown assays. B) Radioactively labeled protein input (positive control) GST (negative control) and GST-TAL1 (full length.)

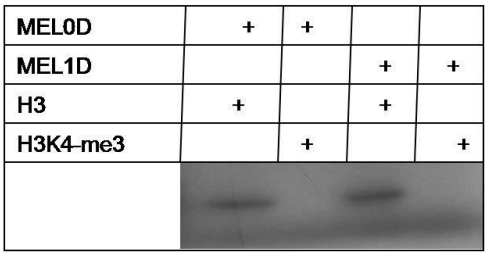

Figure 3-4. HMT assay. The FH-TAL1 MEL nuclear extract before and after treatment w/ 1.8% DMSO for 1 day was immunoprecipitated using α-Flag antibody; the resulting complex was then purified with α-flag immunoaffinity column. This complex was then incubated with 3H-Ado-Met and commercially available H3 peptides. In lanes 2 and 4, completely tri-methylated H3K4-me3 was also used to determine the specificity of the methylation activity of the TAL1 containing complex. The presence of a band on the autoradiograph of the gel indicates methylation.

CHAPTER 4
THE EFFECT OF hSET1 ON TAL1 TARGET GENES

After confirming the association of the two complexes, the next logical step was to confirm that these complexes exert a discernable effect of the transcription of TAL1 target genes, such as P4.2 in erythroid development. To ascertain this, two different approaches were undertaken. First of all, chromatin immunoprecipitation is the standard biochemical method for determining colocalization of complexes at a promoter as well as any changes in chromatin structure discernable via histone modifications. Our collaborator Keji Zhao contributed the ChIP-seq studies of the colocalization of TAL1 and H3K4 trimethylation at the P4.2 promoter. Therefore, another approach using a reporter assay was also employed to determine the direct effect on the protein expression of the P4.2 gene itself.

TAL1 Binding Is Associated with Increased H3K4me3 in the P4.2 Promoter.

If TAL1 associates with SET1, it is likely that hSET1 HMT activity plays a role in activation of TAL1 target genes during normal hematopoiesis. P4.2 is an erythroid cell membrane protein and a marker for red blood cell development1. ChIP-seq data (Figures 4-1 and 4-2) from our collaboration with the laboratory of Dr. Keji Zhao suggest a correlation between TAL1 occupancy of the P4.2 promoter and increased H3K4me3 in ES cells (Figure 4-1). Further, in CD34+ hematopoietic stem cells (HSC) where the P4.2 gene is silent, there is no H3K4 methylation detected at the P4.2 promoter (Figure 4-2). After differentiation from CD34+ HSCs into the CD36+ lineage, H3K4me3 levels increase (data not shown).

The Effect of hSET1 on TAL1 Mediated Transcription Using a P4.2 Promoter Driven Reporter Assay.

After determining the association of the TAL1 and hSET1 complexes, and the presence of histone methyltransferase activity of the TAL1 associated hSET1 complex, a more direct *in vivo* approach was used to determine the effect of hSET1 on TAL1 mediated target gene P4.2 during erythroid differentiation (Figure 4-3) The *in vitro* model system used does lead to P4.2 promoter driven luciferase expression upon DMSO induction (Figure 4-4) Unexpectedly, the level of pCDNA3.1-TAL1 required for maximum activation of the reporter construct varied between endogenous levels and an addition of 100 ng of pCDNA-TAL1. For example, co-transfection with pREP4-P4.2-luc with higher concentrations of pCDNA3.1-Tal1 (150ng to 200 ng) and thus overexpression of TAL1, may result in repression of P4.2 (data not shown). This is likely due to varying transfection efficiency. This is not actually surprising since very little TAL1 binds DNA and is required in activation of the P4.2 gene. In any case, TAL1 is shown to be required for P4.2 expression in previous studies [8,39].

To address the effect of hSET1 on TAL1 transactivation, DMSO treated MEL cells were then co-transfected with siRNA targeting hSET1 to disrupt the function of hSET1 on TAL1-mediated activation of P4.2. It was expected that cotransfection of siRNA targeting the hSET1 complex would strongly decrease the TAL1 transactivation of the P4.2 driven luciferase reporter (Figure 4-5). 100ng was the optimum concentration of co-transfected pCDNA-TAL1 for activation of the reporter gene in siRNA cotransfection studies. Repression of the gene activation did occur upon transient hSET1 knockdown. Unexpectedly, derepression also occurred in cells transfected with higher then endogenous levels of pCDNA-TAL1 co-transfected with si-hSET1 (Figure 4-5) In

addition to the transfection efficiency issues it would make sense that knocking down hSET1 may not be completely abolishing hSET1 histone methyltransferase activity and overexpression of TAL1 via cotransfection with higher concentration of pCDNA-TAL1 may compensate for this. Nonetheless, these results do indicate that hSET1 is involved in TAL1-mediated P4.2 activation in differentiating erythrocytes, but that there is a specific level required.

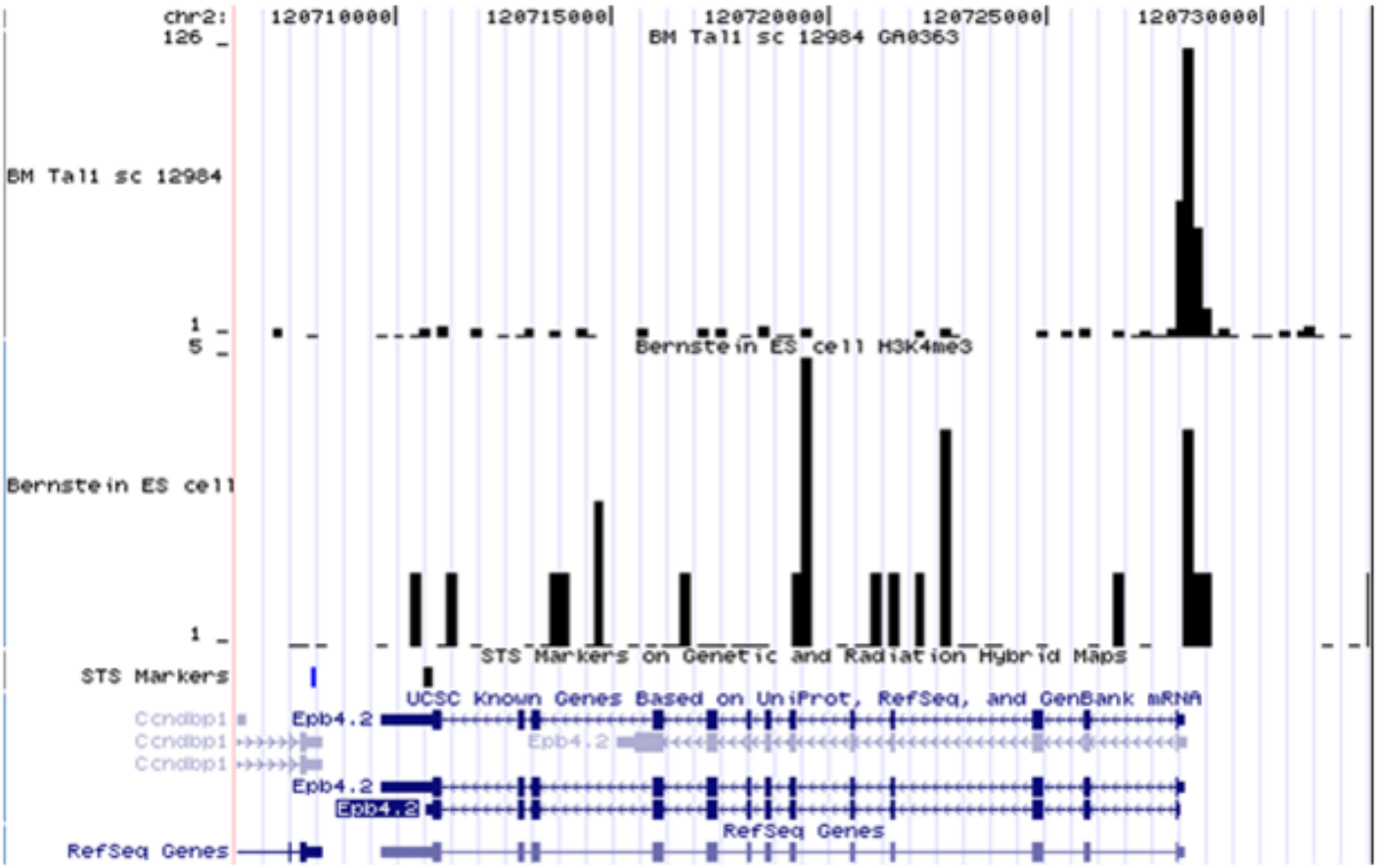

Figure 4-1. A correlation between TAL1 localization at the P4.2 promoter and H3K4 methylation (ChIP-seq data on Bernstein ES cells over the P4.2 promoter provided by Dr. Keji Zhao, NIH.)

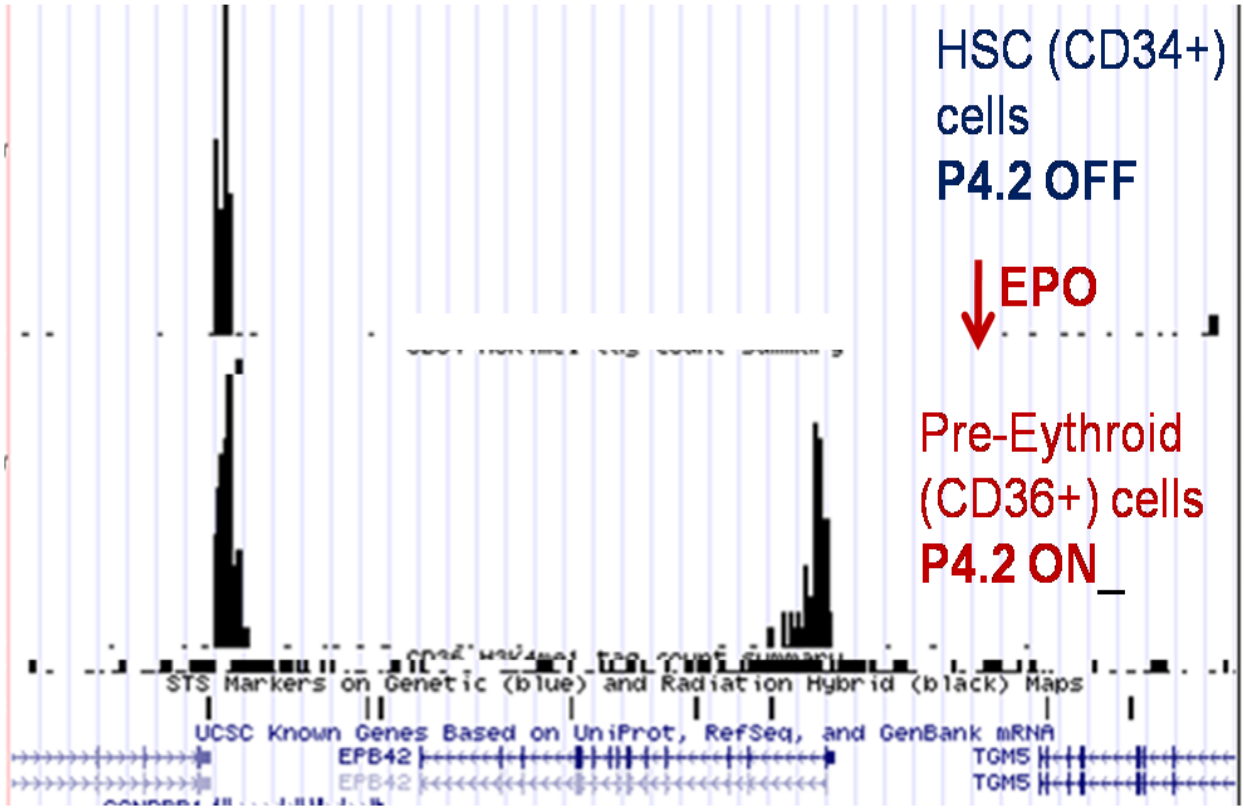

Figure 4-2. TAL1 and H3K4me3 are correlated with expression of P4.2 upon differentiation. ChIP-seq data indicating a correlation between H3K4 methylation at the promoter of P4.2 in CD34+ cells upon differentiation with erythropoietin (EPO). (data provided by Dr. Keji Zhao, NIH.)

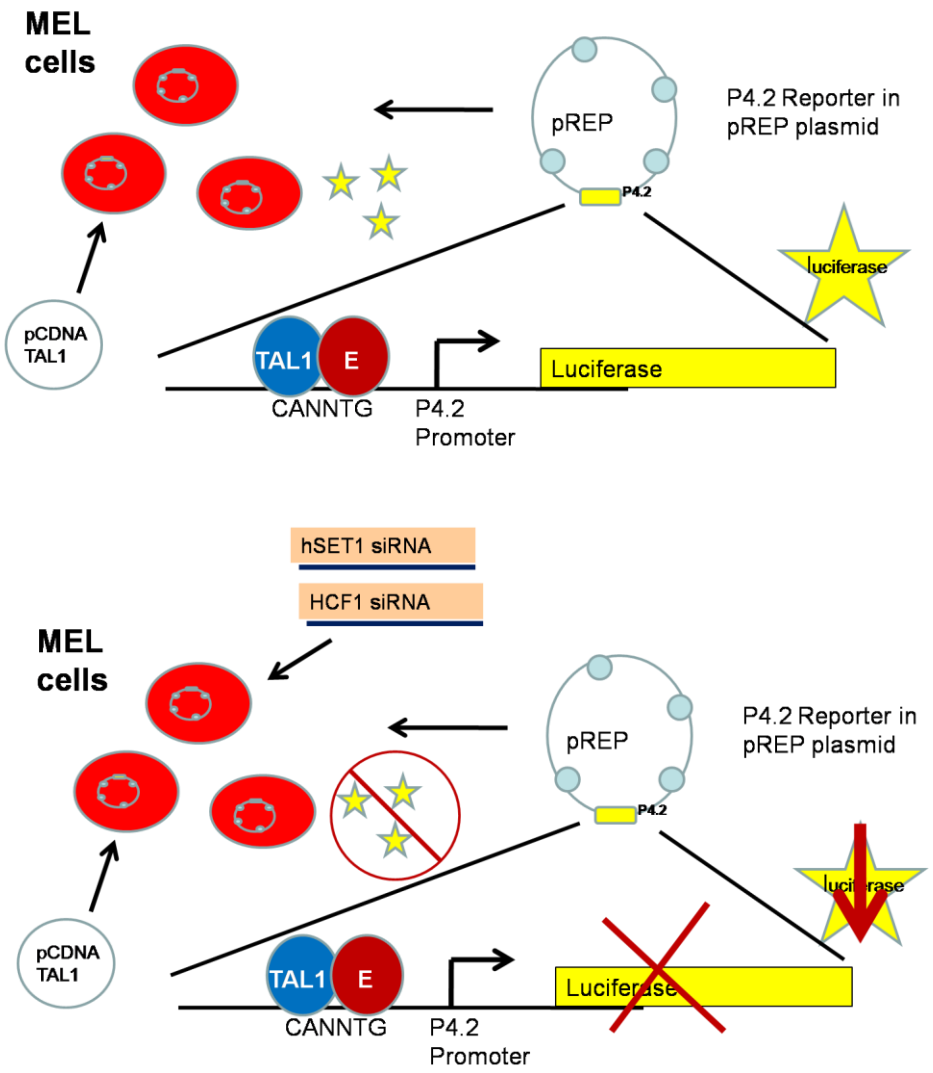

Figure 4-3. The experimental strategy for determining the effect of TAL1 as well as the effect of hSET1 knockdown on the P4.2 TAL1 target genes: A) MEL cells will be induced to differentiate after being co-transfected with pREP-4.2luc, pcDNA-*Ta1l.* B) Then the effect of cotransfecting α-hSET1, and α-HCF1 siRNA into these MEL cells, thus disrupting the hSET1 complex will be compared to the result with the wild type hSET1 complex.

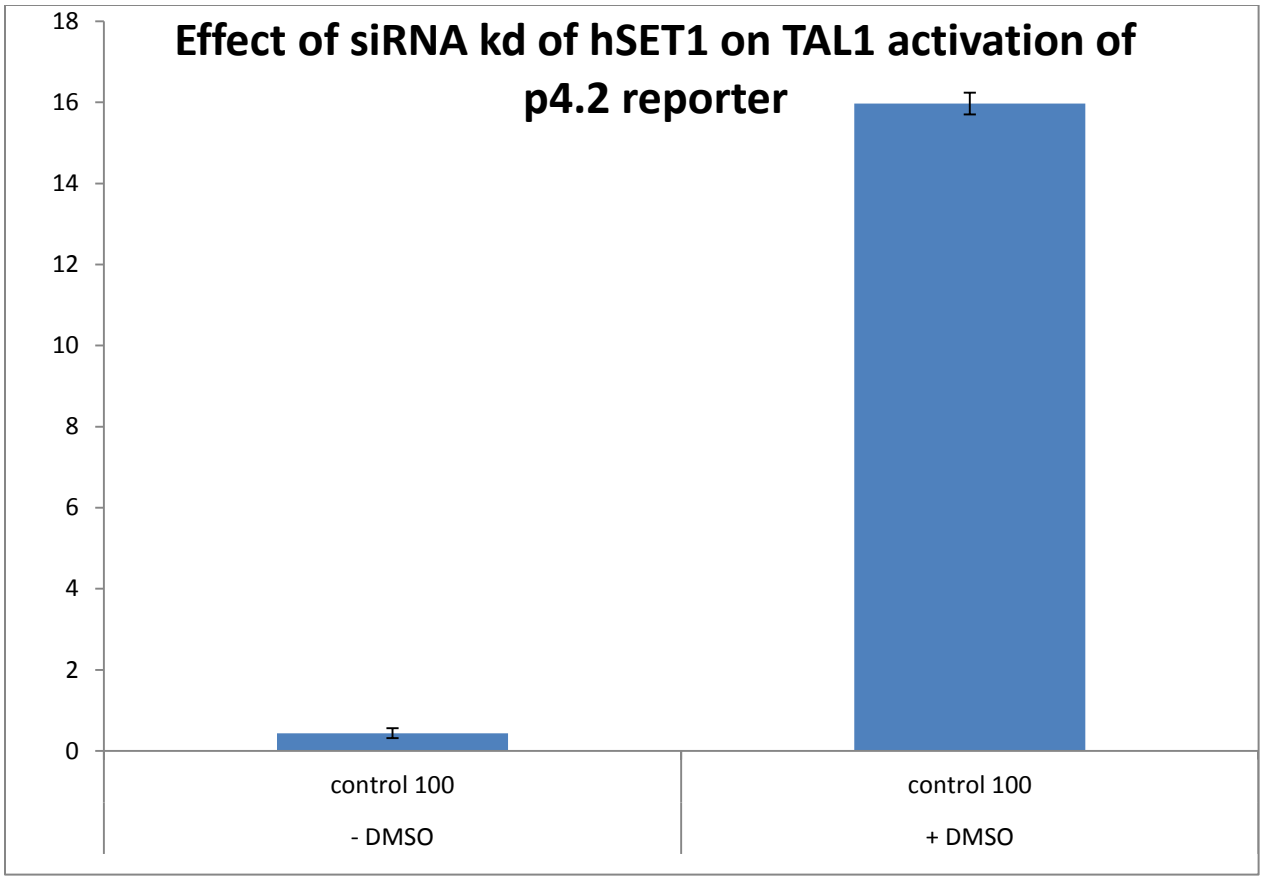

Figure 4-4. The effect of TAL1 concentration on TAL1 target genes. MEL cells were DMSO induced to differentiate after being co-transfected with pREP-4.2luc, pcDNA-*Ta1l,* and the respective luciferase activity was quantitated spectrophotometrically, indicating the activation level of the P4.2 promoter driving the luciferase construct.. T-tests show that this is a significant difference, $\alpha = 0.05$, n=6 (data not shown).

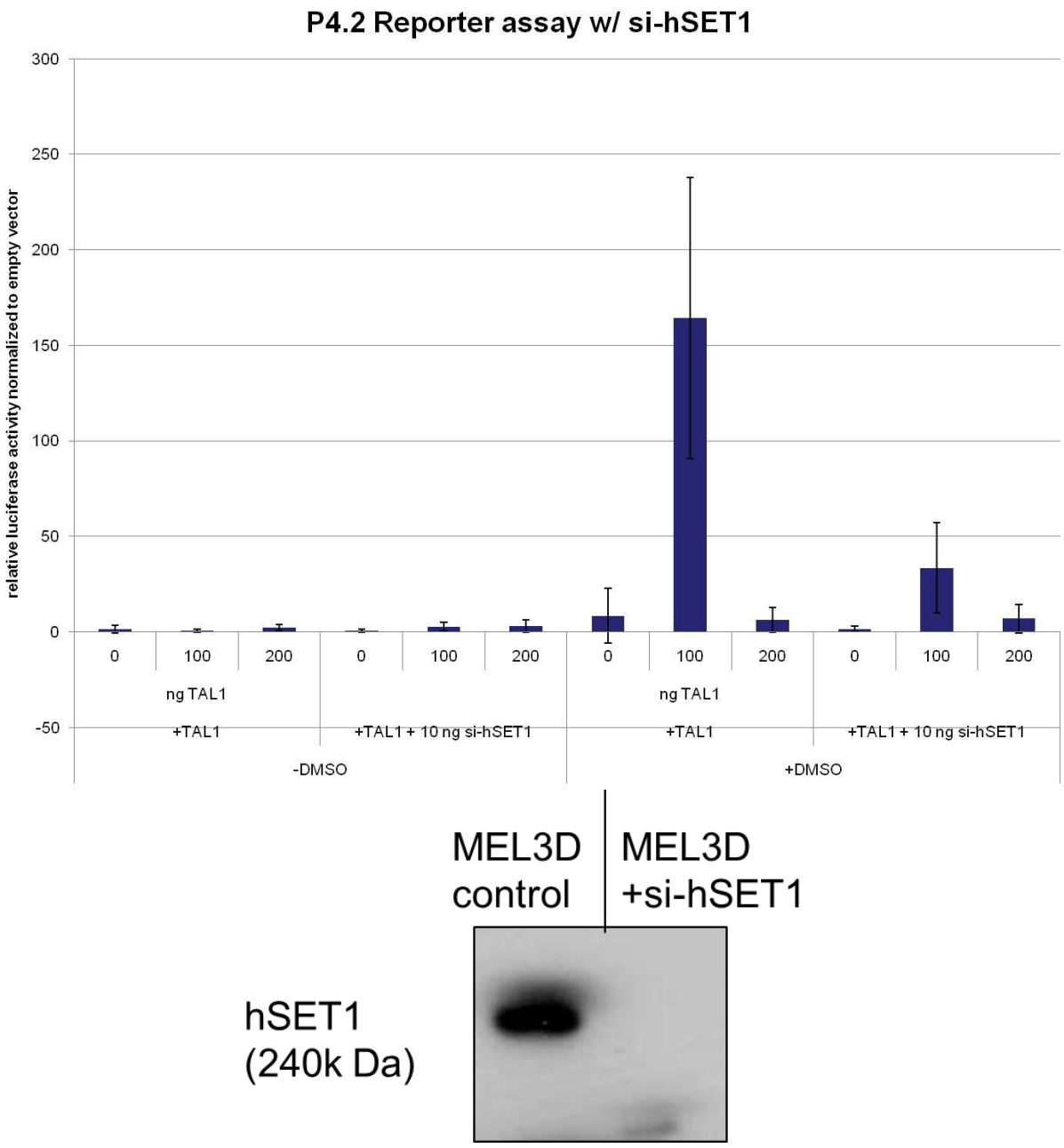

Figure 4-5. The effect of hSET1 knockdown on TAL1 target genes. MEL cells were DMSO induced to differentiate after being co-transfected with pREP-4.2luc, pcDNA-*Ta1l.* Then co-transfected with α-hSET1, and α-HCF1 siRNA into these MEL cells, thus disrupting the hSET1 complex will be compared to the result with the wild type hSET1 complex. T-tests show that this is a significant difference. α =0.05, n=2 (data not shown).

CHAPTER 5
DISCUSSION

During hematopoiesis, TAL1 is essential for both early stem cell development and in erythropoiesis and its activity is subject to regulation. The specific function that TAL1 serves in regulating genes involved in differentiation towards either the erythroid or the megakaryocytic lineage depends on complex combinatorial effects of additional protein complexes in conjunction with TAL1/E-protein heterodimers. This suggests that TAL1 does in fact act as a molecular switch controlling cell fate by activating genes at the correct developmental stage. It makes sense that there is a histone methyltransferase associated with the coactivator complexes associated with TAL1 similar the association with LSD1 demethylation enzyme in TAL1 associated repressor complexes.

Further studies are necessary to elucidate how these proteins act in tandem to regulate genes involved in hematopoiesis. It is likely that TAL1 activates other erythroid-specific genes via the same mechanism. Further, given that hSET1 in conjunction with TAL1/E2A heterodimers is involved in activating TAL1 target genes in early hematopoietic expansion as well as in activating misregulated genes in T-ALL leukemogenesis, these studies will provide insight into the mechanisms by which the misregulation of this transcription factor contributes to leukemogenesis.

The Association of hSET1 and TAL1

The data presented in this thesis support the proposed role of hSET1 in the TAL1-mediated transcriptional activation of erythroid genes in erythroid progenitor cells and is accompanied by temporal changes in promoter chromatin status throughout hematopoietic cell development. The biological action of hSET1 in hematopoiesis was previously unknown. Further, these results show that in fact there is endogenous

association between TAL1 and hSET1 in hematopoietic cells, which colocalizes at the P4.2 promoter and has histone methyltransferase activity specific to histone H3K4 residue. Further these studies show that TAL1 associates with the hSET1 complex via the ASH2L subunit specifically. It remains to be shown which domain interacts with ASH2L however, and if this is the sole interaction between the two.

The Effect of hSET1 on TAL1 Target Genes

Further, the association of hSET1 and TAL1 can lead to activation with the correct concentration of transcription factors. However, the orchestration of this activation process is not simply linear. The concentration of TAL1 and how it interacts with other motifs nearby adds an additional layer of complexity to the puzzle of how TAL1 target genes and genes in general are activated at the proper developmental stage.

These studies contribute novel information on how this histone methyltransferase (HMT) complex regulates TAL1 transcriptional activity through alterations in 'the histone code' as a consequence of enzymatic activities, and the effect of these epigenetic modifications on hematopoietic differentiation. However, the data shown here has the limitation of only showing with certainty that TAL1 recruits hSET1 to the promoter and mediates H3K4 trimethylation and activation of the P4.2 gene in this *in vitro* model system.

One further limitation of this study is the fact that the pREP4-P4.2-luc plasmid also contains a segment with long terminal repeats (LTR) which contain E-boxes. These E-boxes which TAL1 heterodimers bind to may interfere with the GATA1 Ldb1, LMO2 bridge required for activating the gene. It is possible that if the threshold level of TAL1 is crossed, ectopic TAL1 blocks expression by sequestering transcription factors or by blocking binding of the GATA1, Lmo2, Ldb1, complexes that assist in bringing

enhancers to promoters via a looping mechanism as described in Wadman et al [43] Conversely, the E-boxes contained within the long terminal repeats (LTRs) that exist in the plasmid used in this study could also activate TAL1 in lieu of the enhancers or in addition to the enhancers that act *in vivo* within the P4.2 promoter.

Another direction for future studies on the effect of hSET1 on TAL1-mediated gene activation is determining that TAL1 and hSET1 colocalize at the promoter of target genes such as P4.2. The ChIP-seq data generously contributed by our collaborator Keji Zhao's data shown herein merely show that TAL1 and H3K4 trimethylation are co-localized. However, this H3K4 trimethylation is most likely due to hSET1 activity, because of the association of the complexes shown. Chromatin immunoprecipitation (ChIP) studies examining the binding of hSET1 at the P4.2 promoter as well as at the promoters of additional TAL1 target genes will more clearly show if there is indeed a pattern where hSET1 is the histone methyltransferase required for TAL1 activation of target genes.

Nonetheless, the data herein suggests that TAL1 does in fact act as a molecular switch controlling cell fate by activating genes at the correct developmental stage. It makes sense that there is a histone methyltransferase associated with the coactivator complexes associated with TAL1 similar the association with LSD1 demethylation enzyme in TAL1 associated repressor complexes. Previous research on the androgen receptor shows that other transcription factors also interact dynamically with both coactivators and corepressors to regulate cell fate decisions [18]. Therefore, dynamic changes of the TAL1 interaction with LSD1 and hSET1 dictate the TAL1 function. In fact, both coregulators are involved in regulated erythroid specific p4.2 gene. TAL1

recruits LSD1 to inhibit p4.2 transcription in undifferentiated stem or progenitor cells [18]. Upon induction of differentiation, disassociation of LSD1 and Recruitment of hSET1 complex (your data) appear to function as molecular switch to turn on the p4.2 expression. Since TAL1 only activates the p4.2 promoter in DMSO induced MEL cells and siRNA knockdown ofhSET1 abolishes the TAL1 mediated transcriptional activation in differentiated erythrocytes support the role of hSET1 in TAL1 mediated transcriptional activation during hematopoiesis.

Further studies are necessary to elucidate how these proteins act in tandem to regulate genes involved in hematopoiesis. It is likely that TAL1 activates other erythroid-specific genes via the same mechanism. Further, given that hSET1 in conjunction with TAL1/E2A heterodimers is involved in activating TAL1 target genes in early hematopoietic expansion as well as in activating misregulated genes in T-ALL leukemogenesis, these studies will provide insight into the mechanisms by which the misregulation of this transcription factor contributes to leukemogenesis.

How hSET1 Activates Transcription in Hematopoiesis.

These studies show that hSET1 is involved in TAL1 mediated transcriptional activation at the P4.2 promoter. The evidence for this is the fact that hSET1 is recruited to erythroid specific promoters by tissue specific transcription factors such as TAL1. Further, unlike the MLL catalytic subunit, hSET1 doesn't contain an intrinsic DNA binding domain; therefore it doesn't directly interact with DNA. The specificity of this binding is therefore conferred by the tissue specific transcription factor, TAL1. Furthermore, it is known that H3K4 mono-and di-methylation both mark the enhancers of active genes, while H3K4 trimethylation is enriched in the promoters of all active genes [22,47]. These data show that hSET1, a H3K4 methyltransferase, as well as H3K4

trimethylation are both highly enriched in active p4.2 promoter in mature erythrocytes. Further linking this process to transcription is the fact that H3K4 trimethylation catalyzed by hSET1, and hSET1 can directly interact with TAF7, an important component of TFIID/PolII complex [37].

Future Directions

First of all, the results of the GST-pull down experiments presented here have indicated that ASH2L directly interacts with TAL1. This suggests that ASH2L bridges the TAL1 and hSET1 interaction. ASH2L is important component of hSET1 complex. It is required for the integrity and enzymatic activity of whole histone methyltransferase complex. It will be interesting to map the domain that mediates TAL1 and ASH2L interaction in future experiments to determine the way in which hSET1 affects TAL1-mediated activation of target genes. Discovery of any differential regulation between different hematopoietic genes and leukemogenic genes is also key in determining the way that TAL1 mediated gene activation in tandem with hSET1.

As mentioned previously, the most important futures studies in this area should focus on confirming colocalization of hSET1 and TAL1 at target genes. The studies herein do suggest such an association due to the colocalization of H3K4 trimethylation and TAL1 at the P4.2 promoter. However, Chromatin immunoprecipitation of TAL1 and hSET1 binding at the P4.2 promoter and other TAL1 target genes in conditions that lead to activation of the gene should also be done to confirm this.

Another future direction of this work is to determine if hSET1 activates TAL1-mediated genes involved in leukemogenesis in the same way that it has been shown to activate P4.2, for TAL1 not only affects recruiting histone modifying enzymes and chromatin remodeling proteins, but may also activate genes affecting cell cycle arrest.

This is substantiated by the fact that TAL1 knockdown studies show an effect on cdk6 expression [5]. Further, the Runx transcription factor is also important in hematopoiesis of myeloid lineage and Runx genes are known downstream targets of TAL1 [48]. Finally complexes containing RBP2 associating w/ pRb has been shown to be involved in regulating cell cycle genes [45,49]. TAL1 has also been shown to interact with pRb in Mass spectrometry data from this lab (unpublished.) Discovery of the TAL1 target genes involved in cell cycle control as well as apoptosis may suggest potential new treatments for leukemia.

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BIOGRAPHICAL SKETCH

River Saenz (formerly River Ybarra) received a Bachelor of Science degree in 2007 from California State University in Sacramento with a major in molecular biology. During her undergraduate training, she prepared an honors thesis project entitled, “Identification of neuronal targets in Sydenham’s chorea by phage display.” She was also selected as a Louis Stokes Alliance for Minority Participation (LSAMP) Scholar as well as a McNair Scholar, two competitive programs designed to bring educationally and economically disadvantaged students from underrepresented groups into graduate programs in the sciences. In 2007, she was accepted to graduate school in the University of Florida’s Interdisciplinary Program in Biomedical Sciences (IDP) and joined the laboratory of Dr. Suming Huang. During her graduate studies, she received a research supplement from the National Heart, Lung and Blood Institute (NHLBI).