CD4 Silencing in Thymocytes is Opposed by the Enforced Association of P300 HAT, HDAC1 or SUV39H1 with Runx Transcription Factors

Christyne Ayne Kane

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CD4 SILENCING IN THYMOCYTES IS OPPOSED BY THE ENFORCED ASSOCIATION OF P300 HAT, HDAC1 OR SUV39H1 WITH RUNX TRANSCRIPTION FACTORS

A Dissertation Presented

by

CHRISTYNE A. KANE

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Program in Animal Biotechnology and Biomedical Science
CD4 SILENCING IN THYMOCYTES IS OPPOSED BY THE ENFORCED ASSOCIATION OF P300 HAT, HDAC1 OR SUV39H1 WITH RUNX TRANSCRIPTION FACTORS

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by
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DEDICATION

To my family and friends
ACKNOWLEDGMENTS

Graduate school is a long journey which no student travels alone. As such I’d like to extend my deepest thanks to my advisor, Dr. Janice Telfer for her guidance and support through not only my graduate career, but for introducing me to the world of hands-on immunology as an undergraduate student. I would also like to extend my thanks to my committee members Dr. Cynthia Baldwin and Dr. Wilmore Webley for their patience and helpful suggestions concerning my experiments and data analysis.

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ABSTRACT

CD4 SILENCING IN THYMOCYTES IS OPPOSED BY THE ENFORCED ASSOCIATION OF P300 HAT, HDAC1 OR SUV39H1 WITH RUNX TRANSCRIPTION FACTORS

FEBRUARY 2014

CHRISTYNE A. KANE, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

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Directed by: Professor Janice C. Telfer

The transcription factors Runx1 and Runx3 are required for permanent silencing of CD4 in maturing CD8+ thymocytes. Runx binding to consensus sites within the CD4 silencer region is required for CD4 silencing post-positive selection. The Runx nuclear matrix targeting sequence (NMTS) is required for CD4 silencing and is implicated in binding to the histone acetyltransferase (HAT) p300, histone deacetylases (HDAC), and histone methyltransferase (HMT) SUV39H1 proteins. Epigenetic modifications of chromatin or post-translational modifications of Runx itself as a result of Runx association with these enzymes may be important for establishment of long-term CD4 silencing. In this study, we show that treatment of thymocytes with the HDAC inhibitor trichostatin A (TSA) and a resulting increase in histone acetylation in the CD4 silencer region results in an increase in CD4 silencing in a Runx-independent manner. We evaluated the role played by Runx lysines and their potential post-translational modification in CD4 silencing by mutational analysis of nine Runx lysine residues. Disruption of lysines within the Runt DNA binding domain known to reduce Runx DNA-binding activity resulted in CD4 derepression, indicating that Runx DNA-binding is
required for CD4 silencing. Mutation of other lysines not involved with DNA-binding and reported to be acetylated or methylated did not affect CD4 silencing by Runx. The transduction of thymocytes with the C-terminally truncated Runx1 or Runx1 lacking the NMTS fused with the p300 HAT domain, HDAC1, or SUV39H1 resulted in CD4 derepression, indicating that enforced association of these individual enzymes with the Runx DNA-binding domain promotes CD4 transactivation rather than CD4 silencing. Profiling chromatin marks present under c-terminally truncated Runx1d.190 treatment conditions revealed H3K9me3/H3K4me3 coenrichment in CD4 promoter and silencer regions suggesting the involvement of a dynamic instruction profile in the establishment of CD4 silencing.
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CHAPTER 1

BACKGROUND AND LITERATURE REVIEW

1.1 Thymocyte Lineage Commitment

1.1.1 Thymocyte Differentiation Surface Receptors

Thymocyte lineage commitment describes the differentiation of DP thymocytes into single surface receptor-expressing cells, expressing either CD4 or CD8 (Figure 1.1). This lineage commitment process begins with hematopoietic stem cell (HSC) derived early thymic progenitor (ETP) cells which lack lineage specific markers (Lin-) such as CD3, but express c-kit and Sca-1(1). As these ETPs begin to lose their multipotency, they become double negative CD4-CD8- (DN) cells (2). These DN cells are divided into four different stages as defined by their CD25, the alpha-chain of the IL-2 cytokine receptor, and CD44, an adhesion molecule involved in homing, expression (DN1: CD44+CD25-, DN2: CD44+CD25+, DN3: CD44-CD25+, DN4: CD44-CD25-) (Figure 1.1).

During the DN2 and DN3 stages, T cell receptor genes begin to generate diversity through V(D)J recombination of Tcra, Tcrg, and Tcra (3). Successful recombination of the Tcra by lymphoid-specific recombinase RAG (composed of RAG1 and RAG2) results in the formation of a pre-TCR from TCRβ and a pre-α (Figure 1.1). A γδ TCR is assembled of a successfully recombined Tcrg and Tcra. Once pre-TCR have been assembled, they must pass through β-selection; a process by which the cell must receive a signal through the pre-TCR to continue development. Cells which have not generated a functional pre-
TCR are eliminated by apoptosis (2, 3). Thymocytes which have passed β-selection undergo a downregulation of recombinase expression, a robust proliferative burst, and upregulate expression of both CD4 and CD8 to become double positive cells (DP, CD4+CD8+). This population is the largest represented in the thymus. These DP cells re-express RAG for the recombination of Tcra, resulting in surface expression of αβ TCR.

Once these cells express a functional αβ TCR, they undergo positive selection to test the strength of the αβ TCR reactivity with self peptide-MHC complexes (4). Cells which do not interact with the self peptide-MHC complexes undergo death-by-neglect. Thymocytes which have functional αβ TCR and interact with self peptide-MHC complexes are rescued from programmed cell death. In the event that thymocytes express a αβ TCR which reacts too strongly with self peptide-MHC complexes, these cells upregulate BIM (BCL-2-interacting mediator of cell death) leading to an apoptotic death, a process termed negative selection (5). Cells which have upregulated a successful TCR now differentiate into CD4 or CD8 single positive (SP) thymocytes, T helper or cytotoxic T cells respectively. Several models have been proposed and challenged concerning influences of CD4 vs. CD8 lineage choice, however a great deal has yet to be explained in order to fully understand this process (6).

1.1.2 Transcription Factors in Lineage Commitment

Several transcription factors have been described as important for the emergence of ETP. The Notch signaling pathway was first demonstrated as critical for the generation of ETP as inhibition of Notch signaling causes loss of ETP (7). T-cell factor
1 (TCF-1) is induced by Notch signaling and is important for inducing T-cell specific transcription factors GATA-binding protein 3 (GATA3) and B-cell lymphoma/leukemia 11b (Bcl11b)(8). Gata3 is important for ETP development as well as ETP to transition through to the DN2 stage(9). Basic helix-loop-helix transcription factor E2A is critical at multiple points in early T-cell development, beginning with the generation of adequate ETP numbers(10). E2A is also important for cells to transition from DN1-DN2 as accumulation of DN1 cells is reported in E2A-deficient mice(11). Runx transcription factors are also essential for early thymic development, as disruption of binding partner CBFB resulted in a decrease of ETP as well as inefficient ETP-DN3 transitions(12). Additionally, Runx1 is required for an efficient DN2-DN3 transition(13, 14).

Once cells reach the DN2 stage, there are fewer developmental options for them as they are mostly committed to the T-cell lineage. Bcl11b has been shown to be required for the commitment to the T-cell lineage within the DN2 stage(15-18). E2A plays additional critical roles during later stages of thymocyte development such as activating VDJ recombination at the DN2-DN3 stages(19, 20) and regulating the expansion of thymocytes which have passed β-selection(21, 22). Notch also is required at later stages of T-cell development as Notch1 inactivation results in accumulation of cells in the DN3 stage with defects in VDJ rearrangement(23, 24). Gata3 and Runx1 also play roles in the later thymocytes development as Gata3 is important for TCRβ expression and Runx1 is required for proliferative expansion of cells which have passed β-selection(25, 26).
The events transitioning DP cells toward CD4 or CD8 lineage commitment are less well understood than those in the DN stages. However, it is known that this process involves the upregulation of transcription factors functioning as master regulators specific for CD4 (THPOK) or CD8 (Runx) lineages (27). It is unknown what brings about the choice of transcription factor upregulation, but the kinetics of the TCR signal is a common working model of lineage commitment determination (2). Under this kinetic signaling model, TCR signaling represses CD8 expression, resulting in a CD4+CD8int phenotype. Persistent TCR signaling in MHC class II-restricted thymocytes results in CD4 lineage commitment. However, TCR signaling in MHC class I-restricted thymocytes is impaired by the downregulation of CD8. This shorter signal allows for ‘coreceptor reversal’, the downregulation of CD4 expression and upregulation of CD8 surface expression. It is not yet known how the kinetics of TCR signaling effects Runx3 and ThPOK expression.

1.2 Runx Transcription Factors

1.2.1 Runx Family of Transcription Factors

The Runt-related transcription factor family is made up of three proteins which share structural and functional similarity with the *Drosophila* protein, Runt(28). These Runt-related transcription factors were independently cloned in many laboratories, resulting in many different aliases for each protein(29-34). The Runt-related transcription factors were originally described as DNA-binding proteins which bind CBFβ (PEP2B) in order to form the heterodimeric core-binding factor(CBF) or polyomavirus
enhancer-binding protein 2 (PEBP2) transcription complex (35, 36). In 1999, the 
Nomenclature Committee of the Human Genome Organization (HUGO) adopted the 
name 'Runx', referring to genes encoding runt-related proteins (37)(Table1.1).

Runx transcription factors Runx1 (AML1, CBFα-2, PEBP2α-B), Runx2 (AML3, CBFα- 
1, PEBP2α-A), and Runx3 (AML2, CBFα-3, PEBP2α-C) have a highly conserved, 128 amino 
acid Runt homology domain (RHD), named for its homology to the Drosophila runt 
gene (28, 35, 38, 39). This characterizing Runt DNA-binding domain is highly conserved 
among humans, mice, Xenopus, chicken, C. elegans, puffer fish, sea urchins, and 
Drosophila (37, 38, 40) (Figure 1.2). The Runt binding domain preferentially binds to the 
common DNA motif TGPyGGTPy (Py is Pyrimidine) (41, 42). This DNA binding domain 
also mediates heterodimerization with CBFβ, which does not directly bind DNA, but 
rather increases DNA binding efficiency with Runx proteins (32, 38, 43).

As Runx proteins are transcription factors, they possess two distinct and 
conserved trafficking signals which are required for subnuclear transport. The first, and 
highly conserved, domain of these proteins is the nuclear matrix targeting sequence 
(NMTS), located in the C-terminus of the protein (44, 45). This 31 amino acid motif is 
necessary and sufficient to direct Runx transcription factors to nuclear matrix associated 
sites where gene transcription takes place (46-49). The second signal required of Runx 
proteins in order for subnuclear targeting is a nuclear localization signal (NLS) 
(PRRHRQKL), located downstream of the RHD, supports nuclear import of the 
proteins (44, 50, 51).
Additionally, almost every single Runx protein contains a highly conserved C-terminal VWPRY sequence which is required for the proteins to associate with the WD domain of Groucho/TLE corepressors (36, 52-55). This complex functions to repress the transcription of proteins when targeted to their promoter regions. Runx family member functions are regulated by this complex (53-56). However, not all domains of these proteins are highly conserved; a repression domain immediately C-terminal to the RHD (amino acids 208-237) has been shown to bind the corepressor Sin3a in vivo (35, 52). Methylation of Runx1 at specific arginine residues R206 and R210 by arginine methyltransferase PMRT1 regulates this Sin3a binding (57). Additionally, some domains, such as the QA-rich regions within Runx2, are rather divergent from the sequences of other two proteins (58).

The Runx transcription factors contain many sites for interaction with various cofactors in order to regulate gene expression. While this list is ever-expanding and those listed are representative, coregulatory proteins and factors include CBFB (43), ETS family members (59-62), C/EMBP (63), Groucho/TLE (36, 52-55), HES-1 (64, 65), ALY (66), GATA-1 (67, 68), and SMADs (69-73). While many factors (SOX9, STAT5, and PAX5 (74)) bind the RHD and may compete with each other for binding; other factors interact with C-terminal protein regions. These context-dependent protein interactions can also vary greatly, resulting in repression, such as with the case of Groucho/TLE interaction or antagonize this interaction resulting in activation, as in the case of HES binding the VWPRY motif (64).
Runx family members may have very highly conserved regions; however their individual functional requirements differ greatly, with minimal overlapping function. Runx1 has a primary role in hematopoiesis, developing cell types including definitive erythroid cells, granulocytes, macrophages, and lymphoid cells (75, 76). Additionally, Runx 1 is required for CD8 T-cell development(77). Runx 2 proteins plays a primary role in the differentiation of osteoblasts, cell migration, and vascular invasion of bone(78). The final mammalian family member, Runx 3, is required for CD8 T-cell development (77, 79).

Runx 1 and Runx 3 are primarily expressed in the thymus, though Runx2 may also be detected (80). Also, while all three family members may be detected in developing bone, Runx2 is primarily expressed (80-82). Within the thymus, Runx1 is primarily expressed in the cortex, while Runx3 is largely expressed in the medulla (Figure 1.1). These overlapping expression patterns of Runx family members may serve as functional substitutes in specific contexts (80). Runx transcription factors function to activate or repress transcription of target genes in a context-dependent and cell type-specific fashion indicating their diverse roles in cellular processes. These context-dependent or cell-type specific fashions may involve the recruitment or cooperativity of other proteins or complexes.

Runx transcription factors have different isoforms, determined by alternate promoter transcription and alternative splicing, which play an important role in translational regulation of Runx expression(40, 83). The N-terminal isoforms can be transcribed either from the distal or proximal promoters, resulting in a nineteen or 5
amino acid N-terminal difference, respectively. The distal promoter of Runx1, resulting in a protein with the N-terminal sequence MASDSIFESFPSYPQCFMR, is predominately active in the T cell lineages and in hematopoietic cells (84). The proximal promoter of Runx1, resulting in a protein with the N-terminal sequence MRIPV, is primarily active in myeloid cell lineages. There are also many C-terminal and truncated isoforms of Runx determined by alternative splicing of the C-terminus. While at least three of these c-terminal isoforms are expressed in the thymus, analysis of the contribution of these isoforms to CD4 silencing revealed that the C-terminus downstream of the runt domain is required for CD4 silencing (85). Additionally, Runx family proteins may be translated from an internal transcriptional initiation site, however distinct biological functions of these isoforms are unknown(86).

1.2.2 Roles in T cell immunity

Runx1 and Runx3 transcription factors in T lymphocytes have many diverse roles in T cell immunity (27). In some T cell subsets, such as CD4-CD8- (DN), Runx1 and Runx3 exhibit redundant activities; while in other subsets, such as T helper subsets (Th1, Th2), these transcription factors have distinct functions. These function discrepancies may be due to the differing expression patterns of Runx1 and Runx3 (80). In wild type thymi, Runx3 is expressed in CD4-CD8+ (CD8SP) cells while Runx1 can be detected in DN, CD4+CD8+ (DP), CD8SP, and CD4SP cells (87). These subset expression differences largely translate to specific CD4 silencing roles. Runx1 is predominantly expressed in the cortex, while Runx3 is expressed in the medulla, downstream of B-selection (80).
Despite these expression differences, Runx1 and Runx3 are both required to establish CD4 silencing resulting in lineage commitment to CD8SP’s (77, 88). Runx1 has been shown to bind Positive transcription elongation factor b (P-TEF-b), inhibiting the elongation of CD4 by RNA Polymerase II (RNAPII), possibly contributing to CD4 silencing (89). Additionally, Runx1 activates CD8 as the DN population progresses to the DP thymocytes (87).

Runx proteins have many highly conserved domains, some of which may play a role in CD4 silencing. Truncation of Runx at amino acid 361 (Figure 1.2), removing the Groucho/TLE association motif does not affect CD4 silencing, indicating that this motif does not play a primary role (85). However, the deletion of amino acids 263-360 substantially derepresses CD4 silencing (85). The Runx Nuclear Matrix Targeting Sequence (NMTS) is found within these residues, suggesting that the NMTS is required for efficient CD4 silencing. Further specific deletions of the NMTS alone confirm this suggestion. Deletion of the Sin3a corepressor association domain (Figure 1.2) in Runx1 does not alter the state of CD4 expression, indicating that this repression-associated domain is not required for the establishment of CD4 silencing (85).

1.3 ThPOK

Another prominent piece of thymocyte lineage commitment is the zinc-finger protein Th-POK (T-helper-inducing POZ/Krüppel-like factor, also known as Zbtb7b and Ckrox) which specifies CD4 lineage choice (90). ThPOK also functions in part to repress CD8 lineage specific genes, such as Runx3 (91, 92). Runx may also act to regulate ThPOK
in some capacity, indicating cross-regulatory relationship between these lineage commitment master regulators (91).

ThPOK expression is limited to CD4 lineage cells, is promoted by positive selection signals, and is regulated by an upstream regulatory element having both positive and negative function (93). The ThPOK silencing element or distal regulatory element (DRE) is bound by Runx proteins; a required, but not sufficient event in the silencing of ThPOK in DP thymocytes (91). This silencer is active in pre-selection DP thymocytes and cytotoxic lineage cells (91). Deletion of the silencer using a knock-in ThPOK reporter allows ThPOK expression in pre-selection DP thymocytes as well as cytotoxic lineage cells (94). ThP0k may also antagonize the activity of DRE as well as the CD4 silencer element (95). In the absence of Runx1, ThPOK mRNA is upregulated, supporting a role for Runx1 in the regulation of ThPOK before positive selection. A role for Runx3 in the regulation of ThPOK is suggested by increased ThPOK expression upon the inactivation of Runx1 and Runx3 compared to Runx1 inactivation alone (94).

1.4 The CD4 Locus

The CD4 gene is regulated in a development stage-specific manner during thymocyte maturation (Figure 1.3). CD4 gene transcription takes place during the DP and CD4SP stages, but not during DN or CD8SP stages. This transcription is driven by a promoter region, two enhancer regions, and a locus control region (96). As these component regions are thought to be constitutively active, the variations in CD4
expression observed through different development stages are controlled by a silencer element, the CD4 silencer (96).

The CD4 silencer, a cis negative regulatory element within the CD4 gene, is required for the establishment of CD4 repression in maturing CD8 T cells (97, 98). Within the first intron of the CD4 gene, Runx may bind to at least two of five consensus Runx binding sites (99-102) within the 434bp CD4 silencer (Figure 1.3) (103-105). Runx1 and Runx3 transcription factors are required for the establishment of CD4 silencing in all peripheral CD8 T cells (77, 88), while Runx2 is able to silence CD4 at reduced efficiency. Once CD4 expression is silenced in T cells, this silencing is maintained through multiple cell generations, and is not able to be reversed through deletion of the CD4 silencer in mature CD8 T cells (88, 98). In PMA/ionomycin stimulated cells, a process mimicking positively selecting signals, Runx1 and Runx3 are able to bind the CD4 silencer (Figure 1.4A). Runx binding the CD4 silencer is detectable at 20hrs post-treatment and increases in intensity over a 108hr timecourse (Figure 1.4B). Runx3 bound to the CD4 silencer is greater than the bound Runx1, supporting the prominent role of Runx3 in CD4 silencing. Taken together, these features suggest that CD4 silencing is controlled by an epigenetic mechanism(102).
1.5 The "Histone Code"

1.5.1 Histone Assembly

In the nucleus, chromatin is packaged into nucleosomes, \(~146\text{bp}\) of DNA wrapped in 1.65 turns around histone octamers separated by \(~50\text{bp}\) of free DNA (Figure 1.5) (106, 107). Each nucleosome is composed of two copies of each of four histone proteins, H2A, H2B, H3, and H4 (107, 108). The assembly process begins with the dimerization of H2A with H2B. Two of these dimers can then join together with a H3 H4 tetramer to form the histone octamer (106). These octamers may then associate with and condense DNA. Histone 1 (H1) is a linker histone that binds the DNA separating histones. A critical piece of this nucleosome assembly event is that residues within the unstructured histone tails as well as residues within the histone globular domains act as regulators of the chromatin condensation process. They are exposed to chromatin modifying proteins and complexes outside of the nucleosome/DNA assembly, so that they can be post translationally modified. The order and position of these chromatin modifications provide important signals for many cellular processes (109).

Post-translational modifications of histones are known as the “Histone Code”. These modifications act as guides for many biological processes such as transcriptional regulation, DNA replication, alternative splicing, DNA repair, and chromosome condensation (106). Examples of these post-translational modifications include acetylation, deacetylation, methylation, ubiquitination, phosphorylation, and sumoylation.
1.5.2 Histone Modifications

Acetylation is commonly associated with the decondensation of chromatin, resulting in euchromatin facilitating transcriptional activation (106, 109-112). The source of the acetyl groups transferred to the lysines of the protein is Acetyl-Coenzyme A. Conversely to acetylation, deacetylation is commonly associated with the condensation of chromatin to heterochromatin, resulting in transcriptional repression (106, 109-111). This removal of acetyl groups from the proteins transfers the acetyl groups back to Coenzyme A.

Methylation can be associated with either transcriptional activation or repression based on the site of methylation. For example, methylation of histone residues H3K4 or H3K36 are associated with transcriptional activation while methylation of residues H3K9 and H3K27 are associated with transcriptional repression (106, 113). Unlike acetylation, which only attaches one acetyl group to a lysine, lysines may be methylated with one, two, or three methyl groups. This quantity of methylation marks can also determine transcriptional activation or repression (111).

Ubiquitination of histone residues is involved in many nuclear processes, including transcriptional activation, silencing, and DNA repair (114). Ubiquitination of proteins described the conjugation of the 76 amino acid protein ubiquitin to protein substrates. This process involves three separate enzymatic activities (E1-E3) (114). Monoubiquitination of histone residues H2AK119 and H2BK120 are the most common and well-described examples of ubiquitination of histones (106, 114, 115). Monoubiquitination is functionally distinct from polyubiquitination which targets
proteins for degradation by the 26S proteasome (114). Ubiquitination of these histone proteins may be reversed through removal of the ubiquitin by ubiquitin-specific proteases (114).

Little is known about phosphorylation as a histone modification other than that it requires the activation of upstream signaling pathways and kinases (116). Given the few known marks of histone phosphorylation, it is not surprising that direct protein phosphorylation by specific kinases has a greater effect on gene expression that the phosphorylation of histones. However, phosphorylation of residue H3S10 or H2B residues have been shown to enhance HAT activity and transcriptional activation respectively (116). Additionally, the nonspecific phosphorylation of H3 residues has been observed to facilitate DNA damage repair.

Heterochromatin protein 1 (HP1) is a highly conserved adapter protein functioning in heterochromatin condensation and transcriptional repression(113). While these proteins do not modify chromatin themselves, specific chromatin modifications are involved in recruiting HP1 to certain sites of the genome. Specifically, methylation of H3 lysine 9 (H3K9me) is responsible for this recruitment (117). Additionally, it has been demonstrated that this modification in the presence of H3S10ph is sufficient to eject the HP1 proteins from the heterochromatin (117). This serves as a dynamic example of the ever-changing properties of chromatin which contribute to transcriptional regulation.
1.6 Chromatin Modifying Enzymes

Epigenetic modification investigation of CD4 locus regulatory component regions, the promoter, enhancer, and silencer, have revealed hyper acetylation of H3K9 at all three regulatory regions in DP and CD4SP developmental stages (118). However, in DN and CD8SP cells, H3K9 hypoacetylation is observed and H3K9me3 is enriched at the CD4 promoter region (118). Observation of protein recruitment to these regulatory regions revealed increased association of p300 histone acetyl transferase with all three of these regions in DP cells (118). Additionally, Runx1 is associated with the CD4 silencer in DN and DP populations, overlapping the association pattern of p300 with the CD4 silencer as well as enhanced H3K9 acetylation in DP populations.

1.6.1 p300 Histone Acetyl Transferase (HAT)

Histone acetyl transferases, such as p300, are known to modify histones resulting in a transcriptionally active chromatin conformation or directly modify protein residues of chromatin-associated proteins, basal transcriptional machinery components, and transcriptional activator proteins. There are two types of HATs, type A-HATs which are involved in chromatin assembly and gene transcription and type B-HATs which are cytoplasmic proteins which acetylate histones prior to incorporation into new chromatin (110). HATs are further categorized into families based on sequence similarities (111).

p300 is a member of the nuclear p300-CBP coactivator family, comprised of only two closely related members, p300 and CBP. These proteins contain multiple conserved functional domains including a bromodomain, a glutamine (Q)-rich region, and three
cysteine-hisidine (CH)-rich domains (Figure 1.6A) (119). The bromodomain plays a role in the association of factors with chromatin as well as protein-protein interactions. Acetylated histones or non-histone proteins are targeted by these bromodomains (120) (Figure 1.6A). The Q-rich and CH-rich regions are in the C-terminus of the protein and enable p300 to act as an adaptor or scaffold molecule for the assembly of relevant protein complexes, such as with p300 association with PCAF through the CH3 region (119, 121). Additionally, p300 possesses a large acetyl transferase domain (amino acids 934-1652 (HAT)), conferring acetyl transferase activity to the protein (111).

p300 has been shown to interact with Runx1, stimulating Runx1-dependent transcription (122, 123), Runx2, and Runx3 (124). The nuclear matrix targeting sequence of Runx (amino acids 318-358 (NMTS)) as well as the c-terminus of p300 (amino acids 963-2369), which includes the histone acetyl transferase domain (HAT) (Figure 1.6A), are required for this interaction (122, 124). Runx1 and Runx3 are directly acetylated by p300 at lysine residues K24, K43, K144, K182, and K188 (124, 125). p300 also associates with the CD4 promoter, enhancer, and silencer elements in DP cells, overlapping the association pattern of Runx1 with the CD4 silencer (118).

### 1.6.2 Histone Deacetylases (HDACs)

In contrast to p300’s activity, histone deacetylases (HDACs) function to deacetylate histones or non-histone proteins; commonly associated with an inactive, or neutral, transcriptional state. Considering their broad functions, these enzymes may also be referred to as “lysine deacetylases” (126). Classical HDACs may be classified into
three different groups based on their homology to yeast proteins, class I HDACs (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 7, 9), and class II (HDAC6 and HDAC10) (108). Class I HDACs are mostly localized in the nucleus whereas class II HDACs are shuttled between the nucleus and the cytoplasm (108, 126, 127). HDAC1 and HDAC2 lack a nuclear export signal (NES) and are consequently exclusively localized in the nucleus. This lack of NES is attributed with the very small size of the class I HDACs, ~500bp (Figure 1.6B) (108). The domain possessing deacetylase activity, or the catalytic domain, is comprised of ~390 amino acids and is highly conserved among classical HDACs (Figure 1.6B) (127). This domain operates via a charge relay system requiring a Zn$^{2+}$ ion to bind the zinc binding site within the active site pocket (127). HDAC inhibitors (HDACi) function by displacing the requisite Zn$^{2+}$ ion from the pocket, leaving the charge relay system inoperable.

Trichostatin A (TSA), a fermentation product of *Streptomyces*, was the first natural hydroxamate discovered to inhibit HDACs (128). One of the most potent HDACi’s, TSA is commonly used at nanomolar concentrations *in vitro* to inhibit HDACs1-9, causing the accumulation of acetylated histones and non-histone proteins (Figure 1.5.) (129). The IC$_{50}$ value of TSA against HDAC1 in human Jurkat T cells is 70 nM (88). TSA is thought to chelate the acetylase-required Zn$^{2+}$ ion in the active site pocket of HDAC through its hydroxamic group (127).

While it is well-described that HDACs modify histone lysines, many non-histone proteins may also be modified by HDACs. Histone deacetylase 6 (HDAC6) has been reported to interact with Runx2, resulting in the repression of Runx2-dependent transcription (130). Runx1 associates strongly with HDACs 1, 3, and 9, while its
interaction with HDACs 2, 5, and 6 is milder, and interaction with HDACs 4, 7, and 8 is minimal (131, 132). Runx3 interacts with HDACs 1, 2, and 5 (124, 133). Additionally, HDACs 5 and 6 share a similar punctuate nuclear distribution to Runx1 transcription factors (48, 130, 134).

1.6.3 Histone Methyltransferase

HDAC-mediated deacetylation of lysines exposes these lysines to other modifications, such as methylation. SUV39H1 is a member of the suppressor of variegation 3-9 homolog family containing a chromodomain and a conserved c-terminal SET domain flanked by two cysteine-rich domains (Figure 1.6C) (135). The chromodomain, 60 amino acids in length, serves a location of interaction and directs the protein to euchromatic or heterochromatic chromatin (136). The 130 amino acid SET domain confers enzymatic activity, though all three of these domains are required for histone methyltransferase activity (Figure 1.6C) (135). Additionally, the catalytic SET domain is negatively regulated by the acetylation of lysine residue 266 (137). Deacetylation of this residue by HDAC SIRT1 is sufficient to restore methyltransferase activity (137). The preferential methylation target of SUV39H1 is H3K9, resulting in tri-methylation of this residue.

Runx1 and Runx3 associate with a histone methyltransferase, SUV39H1, through multiple interaction sites including the runt domain, the nuclear matrix targeting sequence, and amino acid residues 379-430 (138, 139). Interestingly, mutation of Runx1 residues 379-430 abolishes HDAC1 and HDAC3 association (138). Runx can also be
associated with indirect HMT activity through binding of an HDAC to heterochromatin
protein 1 (HP1) which binds to SUV39H1 (140). This association may result in
methylation of histone H3 at lysine 9, a known substrate of SUV39H1 (141), and lead to
repression of the associated DNA.

1.7 Cell-penetrating Peptides

1.7.1 The HIV-1 TAT Cell-penetrating peptide

Cell penetrating peptides (CPPs) are a diverse class of peptides ranging in size
from 5-30 amino acids which have a unique ability to cross the cell membrane. CPPs are
also able to shuttle a variety of cargoes such as plasmid DNA, siRNA, small molecules,
proteins and peptides, drugs, and nanoparticulate pharmaceutical carriers across the
cell membrane in vitro as well as in vivo (142, 143). While CPPs are utilized for a wide
variety of applications, they are unable to discriminate between cell types, rendering
the inappropriate for some in vivo applications. Despite tremendous variety among
their amino acid composition and structure, CPPs may be identified into three major
classes: cationic, amphipathic, and hydrophobic (142). Cationic classified peptides
primarily have a net positive charge while amphipathic CPPs are the largest class of
CPPs, comprised of cationic and anionic peptides. Hydrophobic peptides are the
smallest classification, only identifying 15% of CPPs (142).

A cationic cell penetrating peptide, the transactivator of transcription (TAT)
protein of HIV-1 was first described in 1988 (144, 145). While the full TAT protein is 86
amino acids long, it contains a highly basic region composed of two lysines and six
arginines (arg-lys-lys-arg-gln-arg-arg-arg) (144). In 1997, a minimal sequence which enabled cell entry including a nuclear localization signal (Ruben) was described which included this basic region (146)(Figure 1.7). This sequence is critical as CPPs rely on these positively charged sequences in order to interact with the cell membrane. Specifically, the guanidine head group of arginine may form hydrogen bonds with negatively charged phosphates and sulfates on the cell surface membrane. While lysine does not possess a guanidine head group, this residue contributes its positive charge to the peptide sequence (143). This amino acid characteristic indicates that lysine alone is less effective at cell membrane penetration.

1.7.2 Mechanism of Cellular Transduction

In order to gain entry to a cell, CPPs utilize two distinct mechanisms of intracellular uptake: Direct translocation and/or Endocytotic pathways ((143)). Direct transduction through the lipid bilayer is mediated by both electrostatic interactions as well as hydrogen bonds formed between arginine residues and negatively charged phosphates and sulfates on the cell membrane surface (Figure 1.7A).

While energy-dependent macropinocytosis is a primary endocytotic method of CPP intracellular entry, other pinocytosis processes such as clatherin-mediated endocytosis, caveolae/lipid raft-mediated endocytosis, and clatherin/caveolae-independent endocytosis may facilitate entry. TAT peptide, in addition to direct transduction, has been shown to utilize macropinocytosis, clatherin-mediated endocytosis, and caveolae/lipid raft-mediated endocytosis simultaneously (147) (Figure
1.7B). It is important to note that the preferred method of intracellular entry is dependent upon the cargo of the CPP. TAT peptides carrying large cargo may enter the cell by macropinocytosis (143) though utilize lipid raft-mediated endocytosis when conjugated to a protein (148) or clatherin-dependent endocytosis when conjugated to a fluorophore (149). Once inside the cell, conjugated cargo may bind to DNA targets in order to regulate transcription of these target genes (Figure 1.7C). Despite the diverse cellular entry mechanisms used, CPPs serve as a very efficient non-viral strategy to deliver various cargoes to intracellular compartments.
### Table 1 Nomenclature for Runx Family Transcription Factors

<table>
<thead>
<tr>
<th>Human Genome Organization (HUGO)*</th>
<th>Acute Myeloid Leukemia</th>
<th>Core binding factor</th>
<th>Polyoma enhancer binding protein 2</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runx1</td>
<td>AML-1</td>
<td>CBFα -2</td>
<td>PEBP2α-B</td>
<td></td>
</tr>
<tr>
<td>Runx2</td>
<td>AML-2</td>
<td>CBFα -1</td>
<td>PEBP2α-A</td>
<td>OSF-2</td>
</tr>
<tr>
<td>Runx3</td>
<td>AML-3</td>
<td>CBFα -3</td>
<td>PEBP2α-C</td>
<td>NMP-2</td>
</tr>
</tbody>
</table>

*This manuscript utilizes the HUGO nomenclature.*
Figure 1.1. Diagram of thymocyte development.

Early thymic precursor (ETC) cells enter the thymus and proceed to differentiate into thymocytes. These cells are termed double negative (DN) thymocytes and can be divided into four developmental stages as determined by their surface expression of CD25 and CD44. Between DN2 and DN3 stages, the cells begin to undergo VDJ rearrangement, resulting in a pre-TCRa/B. Thymocytes undergo B-selection, resulting in a robust proliferative burst and upregulation of CD4 and CD8, becoming double positive (DP) cells. These cells are able to test the strength of their TCR interaction with MHC class I or MHC class II molecules. Cells that react with their respective molecules are positively selected, while cells which do not interact undergo death-by-neglect. Cells which interact too strongly are negatively selected for, resulting in apoptosis. Due to the upregulation of various transcription factors (Runx, ThPOK, Gata3) cells differentiate into CD4 or CD8 single positive (SP) cells. The thymus location where each of the different development stages takes place is indicated.
Figure 1.2. Runx family proteins.

Runx transcription factors Runx1, Runx2, and Runx3 are characterized by the highly conserved runt DNA-binding domain. Another highly conserved domain of these proteins is the nuclear matrix targeting sequence (NMTS). The QA-rich regions in Runx2 are rather divergent from the other two proteins. Two different splice isoforms determined by the transcription from either the proximal or distal promoters is also found in Runx proteins. Use of the distal promoter of Runx1 results in a protein with the N-terminal sequence MASDSIFESFPSYPQCFMR, while use of the proximal promoter of Runx1 results in a protein with the N-terminal sequence MRIPV. The C-terminus of the protein also includes a conserved sequence for Groucho/TLE association.
Figure 1.3. The regulatory elements of the CD4 locus.

CD4 transcription is regulated through two enhancer elements, a promoter element, and a silencer element. The 5' enhancer element and the promoter elements are thought to be constitutively active, leaving the silencer element responsible for variations in gene expression. The 3' end of the CD4 locus contains a locus control region (LCR) and a T cell specific enhancer region (TE). Within the CD4 silencer element, Runx may bind up to five Runx consensus sites.
Figure 1.4. Association of Runx proteins with the CD4 silencer in PMA/ionomycin-stimulated thymocytes.

This treatment simulates positively selecting signals, leading to the upregulation of endogenous Runx3 expression. **A.** Runx proteins bound to the CD4 silencer and that this event was detectable between 20 and 48hrs. **B.** The intensity of this observed interaction increased over the time course, up to 108hrs. Low amounts of Runx1 were observed bound to the CD4 silencer, supporting a dominant role for Runx3 in the establishment of CD4 silencing.
Figure 1.5. Chromatin structure and modifications.

DNA is condensed into heterochromatin through association with nucleosomes. These nucleosomes are composed of two H2A/H2B dimers and one H3/H4 tetramer. Post-translational modifications of the unstructured N-terminal histone tails or the globular histone domains are able to regulate transcription of the genome. These post-translational modifications include acetylation, deacetylation, and methylation. Acetylation is catalyzed by histone acetyltransferases (HATs) adding an acetyl group to the lysines of these histone tails or non-histone proteins. Acetylated histone tails decondense chromatin, facilitating to the recruitment and assembly of transcriptional machinery to the genome. Histone deacetylases (HDACs) function to remove this acetyl group through a charge relay system requiring a Zn\textsuperscript{2+} ion in the binding pocket. Histone deacetylase inhibitors (HDACi), such as Trichostatin A (TSA), remove the required Zn\textsuperscript{2+} ion from the HDAC, resulting in preserved acetylation of the chromatin. Histone methyltransferases (HMT) function to methylate histone tails, resulting in heterochromatin formation and consequently, transcriptional repression. Specific methylation marks of histone tails may result in transcriptional activation.
A. The p300 family of proteins contains multiple conserved functional domains including a bromodomain which plays a role in the association of factors with chromatin as well as protein-protein interactions, and a glutamine (Q)-rich region in the C-terminus of the protein which enables p300 to act as an adaptor or scaffold molecule for the assembly of relevant protein complexes. p300 also possesses an acetyl transferase domain (amino acids 934-1652 (HAT)), conferring acetyl transferase activity to the protein. B. Class I HDACs, including HDAC1, are small histone deacetylases (~500 amino acids) which are localized in the nucleus due to their lack of a nuclear export signal (NES). The catalytic domain possessing deacetylase activity is comprised of ~390 amino acids and is highly conserved among classical HDACs. This domain operates via a charge relay system requiring a Zn2+ ion to bind the zinc binding site within the active site pocket. C. SUV39H1 is a SET family histone methyltransferase containing a conserved SET domain flanked by two cysteine-rich domains. All three of these domains are required for histone methyltransferase activity.
Figure 1.7. Mechanism of Cellular Transduction by Cell-Penetrating Peptides

Runx1 truncated at amino acid 190 (Rx1d.190) was fused to Cell Penetrating TAT peptide.  
A. The negative charge of the cell membrane attracts the positively charged cell penetrating peptide (CPP).  
B. CPPs containing cargo may enter the cell by direct translocation through the plasma membrane and/or by macropinocytosis, clathrin-mediated endocytosis, caveolae/lipid raft-mediated endocytosis, and clathrin/caveolae-independent endocytosis.  
C. In the event that the CPP contains a NLS, it can translocate to the nucleus where it may bind DNA, and in the case of Runx proteins, regulate gene transcription.
CHAPTER 2

CD4 SILENCING IN THYMOCYTES IS OPPOSED BY THE ENFORCED ASSOCIATION OF P300 HAT, HDAC1 OR SUV39H1 WITH RUNX TRANSCRIPTION FACTORS

The transcription factors Runx1 and Runx3 are required for permanent silencing of CD4 in maturing CD8+ thymocytes. Runx binding to consensus sites within the CD4 silencer region is required for CD4 silencing post-positive selection. The Runx nuclear matrix targeting sequence (NMTS) is required for CD4 silencing and is implicated in binding to the histone acetyltransferase (HAT) p300, histone deacetylases (HDAC), and histone methyltransferase (HMT) SUV39H1 proteins. Epigenetic modifications of chromatin or post-translational modifications of Runx itself as a result of Runx association with these enzymes may be important for establishment of long-term CD4 silencing. In this study, we show that treatment of thymocytes with the HDAC inhibitor trichostatin A (TSA) and a resulting increase in histone acetylation in the CD4 silencer region results in an increase in CD4 silencing in a Runx-independent manner. We evaluated the role played by Runx lysines and their potential post-translational modification in CD4 silencing by mutational analysis of nine Runx lysine residues. Disruption of lysines within the Runt DNA binding domain known to reduce Runx DNA-binding activity resulted in CD4 derepression, indicating that Runx DNA-binding is required for CD4 silencing. Mutation of other lysines not involved with DNA-binding and reported to be acetylated or methylated did not affect CD4 silencing by Runx. The transduction of thymocytes with the C-terminally truncated Runx1 or Runx1 lacking the
NMTS fused with the p300 HAT domain, HDAC1, or SUV39H1 resulted in CD4 derepression, indicating that enforced association of these individual enzymes with the Runx DNA-binding domain promotes CD4 transactivation rather than CD4 silencing. Profiling chromatin marks present under c-terminally truncated Runx1d.190 treatment conditions revealed H3K9me3/H3K4me3 coenrichment in CD4 promoter and silencer regions suggesting the involvement of a dynamic instruction profile in the establishment of CD4 silencing.

2.1 Introduction

Runx transcription factors Runx1, Runx2, and Runx3 are highly conserved mammalian runt family members. They are characterized by the runt DNA-binding domain which is highly conserved among humans, mice, chicken, Xenopus, chicken, C. elegans, sea urchins, and Drosophila (37, 38, 40) (Figure 1.2). Runx1 and Runx3 are primarily expressed in thymus while Runx2 is expressed largely in bone (80-82). Runx transcription factors may either activate or repress transcription of target genes in a context-dependent and cell type-specific fashion indicating their diverse roles in cellular processes.

While CD4 versus CD8 lineage commitment has been extensively studied, it is still unknown how the change in gene expression programming is introduced. The CD4 silencer is required for the establishment of CD4 repression in maturing CD8 T cells (97, 98). Within the first intron of the CD4 gene, Runx may bind to at least two of five consensus Runx binding sites (99-101) within the 434bp CD4 silencer (103-105). Runx1
and Runx3 transcription factors are required for the establishment of CD4 silencing in all peripheral CD8 T cells (77, 88), while Runx2 is able to silence CD4 at reduced efficiency. Once CD4 is silenced in T cells, this silencing is maintained through multiple cell generations, and is not able to be reversed through deletion of the CD4 silencer in mature CD8 T cells (88, 98). These features suggest that CD4 silencing is controlled by an epigenetic mechanism.

Histone acetyl transferases, such as p300, are known to modify histones resulting in a transcriptionally active chromatin conformation or directly modify protein residues. p300 has been shown to interact with Runx1, stimulating Runx1-dependent transcription (122, 123), Runx2, and Runx3 (124). The nuclear matrix targeting sequence of Runx (amino acids 318-358) as well as the C-terminus of p300 (amino acids 963-2369), with includes the histone acetyl transferase domain (HAT), are required for this interaction (122, 124). Runx1 and Runx3 are directly acetylated by p300 at lysine residues K24, K43, K144, K182, and K188 (124, 125).

In contrast to p300’s activity, histone deacetylase 6 (HDAC6) has been reported to interact with Runx2, resulting in the repression of Runx2-dependent transcription (130). Runx1 associates strongly with HDACs 1, 3, and 9, while its interaction with HDACs 2, 5, and 6 is milder, and interaction with HDACs 4, 7, and 8 is minimal (131). Runx3 interacts with HDACs 1, 2, and 5 (124). Additionally, HDACs 5 and 6 share a similar punctuate nuclear distribution to Runx1 transcription factors (48, 130, 134).

HDAC-mediated deacetylation of lysines exposes these lysines to other modifications, such as methylation. Runx1 and Runx3 associate with a histone
methyltransferase, SUV39H1, through multiple interaction sites including the runt
domain, the nuclear matrix targeting sequence, and amino acid residues 379-430 (138,
139). Interestingly, mutation of Runx1 residues 379-430 abolishes HDAC1 and HDAC3
association (138). Runx can also be associated with indirect HMT activity through
binding of an HDAC to heterochromatin protein 1 (HP1) which binds to SUV39H1 (140).
This association may result in methylation of histone H3 at lysine 9, a known substrate
of SUV39H1 (141), and lead to repression of the associated DNA.

Here we show that preservation of acetylation in positively signaled thymocytes
expressing endogenous Runx3 leads to CD4 repression in a dose-dependent manner.
Specific lysines within the runt DNA-binding domain are shown to be required for the
establishment of CD4 silencing in developing thymocytes. Preservation of acetylation in
cells expressing exogenous Runx1 or Runx1 lysine point mutants enhances the
repression of CD4. Additionally, the enforced association of chromatin modifying
enzymes p300 HAT, HDAC1, or HMT SUV39H1 with truncated Runx1 constructs opposes
Runx-mediated CD4 silencing in developing thymocytes. Profiling of the chromatin
marks present under c-terminally truncated Runx1d.190 treatment conditions revealed
H3K9me3/H3K4me3 coenrichment in CD4 promoter and silencer regions suggesting the
involvement of a dynamic instruction profile in the establishment of CD4 silencing.
2.2 Results

2.1.1 Inhibition of HDAC increases the kinetics of Runx3-mediated CD4 silencing in positively signaled thymocytes.

Runx3, whose expression is induced by positively selecting signals, binds the CD4 silencer. Runx1 and Runx3 have been shown to associate with the histone deacetylase HDAC1 (124, 131). Potential roles for histone deacetylase activity include transcriptional repression as a result of removing acetylation as well as priming lysines for methylation and subsequent binding of an HP-1 homolog (150). We investigated whether HDAC recruitment by Runx3 plays a role in the establishment of CD4 silencing. Thymocytes were stimulated with phorbol myristal acetate (PMA) and ionomycin (IM) to simulate positively selecting signals (151-154), leading to the upregulation of Runx3 expression. These cells were then treated with 3.3-33 nM (1-10 ng/mL) HDAC inhibitor trichostatin A (TSA) which inhibits the deacetylase activity of HDACs 1-9 (129). The IC50 value of TSA against HDAC1 in human Jurkat T cells is 70 nM, but increased thymocyte death was observed at doses above 2 nM or 7 ng/mL ([8], data not shown). Despite the lower doses of TSA necessitated by the propensity of the thymocytes to die at higher doses, the intensity of CD4 immunofluorescent staining was decreased with increasing doses of TSA (Figure 2.1). The most robust interval increases in CD4 silencing occurred at 3ng/ml and 6ng/ml (Figure 2.1B). CD8 surface expression was normal (data not shown), indicating that HDAC inhibition did not generally increase surface receptor endocytosis. The level of CD4 down-regulation seen with 6ng/ml TSA is comparable to non-positively signaled thymocytes transduced with Runx1 or Runx3 at 60 hours post-transduction.
(85). TGFβ1 treatment of PMA/IM stimulated thymocytes increases the p300 HAT-mediated acetylation of Runx3 (124). TGFβ1 treatment of PMA/IM stimulated thymocytes also increased CD4 silencing (data not shown). These results suggest that the preservation of acetylation on Runx3, histones, or other proteins from endogenous deacetylases increases the kinetics of Runx3-mediated CD4 silencing.

2.1.2 Specific lysine residues in the runt DNA-binding domain are required for Runx1-mediated CD4 silencing.

The increased kinetics of Runx3-mediated CD4 silencing upon HDAC inhibition or TGFβ1 suggests a role for lysine acetylation in the establishment of CD4 silencing. Runx1 truncation of the C-terminus at amino acid 190 (Rx1d.190) leads to a complete loss of silencing activity, indicating a requirement for the C-terminus in order to establish CD4 silencing (85). This developed the hypothesis that the Runx C-terminus may bind chromatin modifying enzymes such as acetylases or deacetylases which could acetylate or deacetylate Runx lysines, resulting in CD4 silencing. There are nine highly conserved lysine residues in Runx transcription factors, 8 of which are conserved between human/mouse Runx1, 2, and 3, and skate Runx3, one (K43) which is conserved between human/mouse Runx 2, and 3, and skate Runx3, and five of which are located within the runt DNA-binding domain (amino acids 50-177).

To investigate the role of Runx lysines in the establishment of CD4 silencing, we individually mutated each of the nine Runx1 lysines to alanines. Of these residues, K24 and K43 have been reported to be acetylated by p300, an event which augments the
DNA binding and transactivation activity of Runx (125). Residues K24 and K43 are in close proximity and have been shown to have overlapping function (125). A double mutant (K43AK24A) was made to address this possibility of redundant function and investigate whether p300 HAT-mediated acetylation of these residues augments DNA binding in the context of the CD4 silencer. Residues 144, 182, and 188 have been reported to be acetylated by p300 in response to treatment with TGFβ1, resulting in protection from ubiquitin-mediated degradation (124). The mutation of residues K83 and K167 has been shown to impair DNA binding through the disruption of salt bridges or hydrogen bonds mediated by the side chains (155-157). Additionally, mutation of residue K144 is known to disrupt direct DNA contact (155). Remaining residues K90 and K125 were investigated due to their proximity to these other crucial residues as they may provide redundant or overlapping function.

In order to confirm that each of the Runx1 lysine point mutants were expressed at robust and similar levels compared to Runx1 full length proteins, HEK 293T cells were transfected with the point mutant constructs. The cells were cultured for 36hrs before evaluation of intracellular HA Tag and GFP levels with flow cytometry. Protein levels of each Runx lysine mutant were consistent with Runx1 full length protein expression (Figure 2.2). Additionally, residues K182 and K188 whose acetylation was reported to protect the protein from ubiquitination showed substantial amounts of protein, indicating that mutation of these residues was sufficient to protect the protein from ubiquitination. As GFP expression levels are in direct accordance with levels of HA Tag
staining, we can also conclude that GFP fluorescence is an adequate indicator of Runx protein levels.

Bulk thymocytes were harvested from BCL-2+ transgenic mice (158) and purified to a CD4-CD8-(DN) population using magnetic bead negative selection (Figure 2.3). This DN population is critical to the efficiency of the retroviral strategy as these cells are undergoing beta-selection and thus are robustly proliferating and able to uptake the virus upon cellular membrane breakdown and reformation. Cells retained by the magnetic beads, CD4+ and CD8+ populations are stained and evaluated with flow cytometry to determine the efficiency of sorting.

Purified DN thymocytes were infected with murine Runx1 bicistronic retroviral constructs bearing point mutations in each of nine lysine residues, also expressing a GFP marker. After retroviral transduction, the thymocytes were cultured to allow for maturation into the double-positive (DP) stage as well as expression of Runx1 and GFP (Figure 2.3). Immunofluorescent staining was used to monitor GFP, CD4, CD8, and TCR expression at 16, 36, and 60 hour timepoints. Cell populations were first gated for viability, followed by GFP expression, and CD8 expression (Figure 2.4). This gating scheme results in cells which are CD4+CD8+ (DP) and cells that have downregulated CD4 expression to varying extents.

Once infected, there is a delay in expression of the Runx1 proteins, allowing CD4 to be expressed during earlier time points such as 16 hours, and silenced as Runx1 protein expression increases at 36 and 60 hour time points (Figure 2.4 (85)). No CD4 de-repression was observed with the mutation of residues K24, K43, or with the double
mutant K43K24 (Figure 2.5). This indicates that the p300 HAT-mediated acetylation of these specific residues important for increased DNA binding affinity does not play a role in the establishment of CD4 silencing. The disruption of specific residues K83, K144, and K167 was able to completely de-repress Runx1-mediated CD4 silencing (Figure 2.6), indicating a requirement for DNA binding by Runx proteins.

Mutagenesis of remaining lysines outside the runt domain whose acetylation is protective against ubiquitin-mediated degradation of the protein (K182 & K188) (Figure 2.7) did not disrupt Runx1-mediated CD4 silencing. This data indicates that these residues as well as their acetylation-mediated ubiquitination protection is not critical to the establishment of CD4 silencing. As mutation of residue K144 disrupts DNA binding resulting in CD4 derepression, we are unable to access whether p300 acetylation of this residue and subsequent protection from ubiquitination play a role in the establishment of CD4 silencing. Additionally, residues which do not have a significant reported involvement with Runx protein function (K90, & K125) did not disrupt Runx1-mediated CD4 silencing. These results indicate that the direct acetylation of specific Runx lysines does not play a role in establishing CD4 silencing. However, these observations also indicate that Runx must bind to the CD4 silencer through the Runt DNA-binding domain in order to establish CD4 silencing.

2.1.3 HDAC inhibition promotes CD4 silencing and opposes derepression of CD4 by a truncated Runx1 mutant.
As we found that inhibition of HDAC in the presence of endogenous Runx increased the kinetics of CD4 silencing, we questioned what effect HDAC inhibition would have in the presence of exogenous Runx1. We have shown that enforced expression of Runx1 is able to silence CD4 expression and that Runx1 truncation of the c-terminus at amino acid 190 (Rx1d.190) leads to a complete loss of silencing activity (85). Enforced expression of Runx1 with mutations in lysines not required for DNA-binding still resulted in CD4 silencing, but perhaps any phenotype would be more pronounced in an environment of higher acetylation. Preserving acetylation with the HDAC inhibitor TSA may augment Runx binding to the CD4 silencer, resulting in an enhanced silencing phenotype.

We tested the requirement of exogenous Runx in the promotion of CD4 silencing by TSA. CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with murine Runx1 constructs as indicated and treated with TSA at 16 hours post-infection. In cells treated only with the carrier DMSO, Runx1 and lysine point mutants were able to silence CD4 while the C-terminal truncated Rx1d.190 de-repressed CD4 expression (Figure 2.8). Exogenous Runx was not required for CD4 silencing, as CD4 was partially silenced by 3ng/mL TSA in thymocytes transduced with control empty vector (Figure 2.8). Runx1-transduced cells treated with 3ng/mL TSA showed a similar decrease in the intensity of CD4 staining compared to Runx1-transduced cells not exposed to TSA (Figure 2.8). TSA treatment of cells infected with lysine point mutants K24A, K43A, or K43AK24A did not diminish Runx1-mediated CD4 silencing, as would be expected if acetylation of these residues was critical to establishing CD4 silencing (Figure 2.8). This suggests that p300
HAT acetylation of these residues and the resultant augmentation of DNA binding affinity does not play a role in the Runx-mediated CD4 silencing process (125).

Surprisingly, the inhibition of HDAC activity through TSA treatment resulted in the silencing of CD4 in thymocytes transduced with the control empty vector. Inhibition of HDAC activity through TSA treatment also overcame Rx1.d.190 CD4 de-repression, resulting in intermediate silencing of CD4 comparable to that seen in TSA-treated cells transduced with the control empty vector. Since this effect is seen in the absence of positively-selecting signals and the resulting upregulation of Runx3, and it is dominant over Rx1.d.190, it is likely that HDAC inhibition is promoting CD4 silencing through a Runx-independent pathway.

2.2 Runx proteins and chromatin modifying enzymes

2.2.1 Enzymatic activity of Runx1 fusion partners p300 HAT domain, HDAC1, and HMT SUV39H1.

Truncation of the Runx C-terminus abrogates CD4 silencing, indicating a requirement for the C-terminus. Deletion of the nuclear matrix targeting sequence (amino acids 318-358) diminishes CD4 silencing, indicating that this sequence is also important for the establishment of CD4 silencing (85). p300 HAT has been reported to associate with the NMTS of Runx1 (122). Human Runx1 (AML1) has been shown to interact with HDACs 1-3, 5, 6, and 9 (131), while Runx2 is able to associate with HDAC6 through its NMTS (130). Additionally, a histone methyltransferase, SUV39H1, has been reported to bind to Runx1, resulting in methylation of Runx1 and an abrogation of DNA
binding (139). It is noteworthy that HMT SUV39H1’s target residue, H3K9, is thought to be required for HP-1 recruitment, resulting in gene silencing (159-161). Association of any or all of these chromatin modifying enzymes with the Runx C-terminus could acetylate, deacetylate, and/or methylate nearby histones, resulting in CD4 silencing.

To examine whether the Runx C-terminus functions to recruit any of these associated chromatin modifying enzymes to establish CD4 silencing, we fused either the p300 HAT domain (amino acids 934-1652) possessing acetyltransferase activity (162), HDAC1, or HMT SUV39H1 to Runx1d.190 (truncation at amino acid 190) and Runx1d.NMTS (deletion of amino acids 318-358). These constructs were cloned into the MSCV vector, adding an HA tag and a GFP marker (Figure 2.9).

To confirm that these fusions yielded functional protein, they were transfected into HEK 293T cells, fixed, and immunofluorescently stained and analyzed by flow cytometry at 36 hours post-transfection. Intracellular staining of fusion proteins using the HA tag indicated that all constructs were producing similar and adequate levels of the fusion proteins (Figure 2.9). While slightly diminished levels of protein were observed with Runx1 fusions to HMT SUV39H1, these levels are still substantially greater than controls. These results indicate that the proteins are being robustly expressed and are not subject to significant degradation within the cells.

p300 HAT is a well described histone acetyltransferase with many preferential histone acetylation targets, including histone H3 lysines 14 and 18 (163). While substrate specificity among HDACs varies, HDAC1 possesses an average range of specificities (164). To determine if the expressed fusion proteins are functional, we
transiently transfected 293T cells with the p300 HAT domain and HDAC1 fusion protein constructs and acid extracted the histones (165, 166) at 36 hours post-transfection to detect acetylated lysines with immunoblotting. We observed that histone H3 lysine acetylation was increased when the p300 HAT domain is fused to Rx1d.NMTS, indicating that the HAT domain of p300 is sufficient for acetyltransferase activity when fused to truncated Runx1 (Figure 2.10). Histone H3 lysine acetylation was also increased with expression of Rx1d.190. However, the fusion of the p300 HAT domain to Rx1d.190 further increased this acetylation intensity. Fusion of HDAC1 to Rx1d.NMTS did not diminish the global histone H3 lysine acetylation observed with expression of Rx1d.NMTS and only slightly diminished global histone H3 lysine acetylation when fused to Rx1d.190 compared to Rx1d.190 alone.

HMT SUV39H1 primarily catalyzes the methylation of histone H3 lysine 9 (141, 159-161). To determine the functionality of our HMT SUV39H1 fusion proteins, 293T cells were transfected as above with the HMT SUV39H1 fusion constructs, histones were acid extracted and immunoblotted for methylation of residue H3K9. Immunoblotting indicated that the fusion of HMT SUV39H1 to Rx1d.NMTS resulted in an increase in H3K9 methylation (Figure 2.10). Increased H3K9 methylation intensity was also observed with Runx1d.190 fusion to HMT SUV39H1. Notably, expression of HDAC1 fused to either Runx1d.NMTS or Rx1d.190 did not alter H3K9 methylation. As no increased H3K9 methylation was observed with the fusion of HDAC1 to truncated Runx1 constructs, HDAC1 when interacting with Runx is not likely playing a role in priming the H3K9 residues for methylation through deacetylation of this or surrounding residues.
These results indicate that all of the fusion proteins are stably expressed at adequate levels in the cells to carry out their specific chromatin modifying functions.

2.2.2 Runx1 association with specific chromatin modifying enzymes does not promote CD4 silencing.

Since the Runx C-terminus and NMTS are required for effective CD4 silencing and have been reported to associate with various chromatin modifying enzymes, including p300 HAT, HDAC1, and HMT SUV39H1, we examined whether the Runx C-terminus or NMTS played a role in the recruitment of these chromatin modifying enzymes to establish CD4 silencing. We transduced BCL2+ CD4-CD8-(DN) thymocytes with the Runx1 fusion protein constructs and examined the immunofluorescent staining of CD4 up to 60 hours post-transduction. Enforced expression of Runx1 resulted in CD4 silencing while silencing was diminished with the deletion of the NMTS and completely de-repressed with the truncation of the C-terminus, as previously described (85). Since HDAC inhibition resulted in enhanced CD4 silencing in the absence of induced endogenous or exogenously expressed Runx, acetylation is implicated in the establishment of CD4 silencing. Surprisingly, fusion of the p300 HAT domain to Runx1d.NMTS resulted in complete de-repression of CD4 silencing with CD4 levels similar to the truncation of the C-terminus (Figure 2.11). If the lack of association of p300 HAT with Runx is the reason that the Runx mutant deleted for the NMTS is less efficient at silencing CD4, then we would expect enhanced CD4 silencing with the expression of Rx1d.NMTS, but the opposite is the case. Similarly, the p300 HAT domain
fused to Runx1d.190 also resulted in complete de-repression of CD4 silencing, indicating that the enforced association of p300 HAT with the Runx DNA binding domain is not sufficient to overcome the CD4 de-repression seen with Rx1d.190 alone. Instead, as with Rx1d.NMTS, the level of CD4 expression is reproducibly increased with the expression of Rx1d.190-p300 HAT compared to the already high levels of CD4 induced by expression of Rx1.d190 alone.

To determine if HDAC1 plays a role in CD4 silencing when associated with the Runx1 C-terminus, we transduced the HDAC1 fusion proteins into BCL2+ CD4-CD8-(DN) thymocytes and tracked CD4 expression for up to 60 hours. The association of HDAC1 with Runx1d.NMTS resulted in an increase of CD4 expression levels as compared to thymocytes transduced with Rx1d.NMTS alone (Figure 2.12). Association of HDAC1 with Runx1d.190 does not appear to increase the de-repression of CD4 seen with Rx1.d190 alone. These results suggest that HDAC1 association with the Runx C-terminus is not sufficient for the establishment of CD4 silencing.

Runx3 can be associated with HMT activity directly (139) or through an association of a Runx3-associated type II HDAC with heterochromatin protein 1 (HP1) (140). This HDAC association deacetylates lysines, exposing them to methylation. While HDAC1 fusions to truncated Runx1 constructs didn’t result in CD4 silencing, the fusion could be blocking an essential interaction between HMT and the Runx C-terminus. To investigate whether HMT SUV39H1 plays a role in the establishment of CD4 silencing by associating with the Runx C-terminus, we transduced Runx-HMT SUV39H1 fusion proteins into BCL2+ CD4-CD8-(DN) thymocytes and observed CD4 expression levels for
up to 60 hours. Fusion of HMT SUV39H1 to Runx1d.NMTS resulted in complete de-repression of CD4, totally reversing the suboptimal expression of CD4 seen with expression of Rx1d.NMTS alone (Figure 2.13). Interestingly, the association of HMT SUV39H1 with Runx1d.190 further enhanced CD4 de-repression induced by expression of Rx1d.190 alone (Figure 2.13). These results indicate that the C-terminal association of Runx1 and HMT SUV39H1 is not sufficient to establish CD4 silencing. This phenotype of an increase in CD4 expression levels is similar to that seen with the fusion protein Rx1d.190-p300 HAT and supports the hypothesis that fusion of these enzymes to the Runx DNA binding domains does not result in null mutants, but instead promotes CD4 transactivation.

2.2.3 Chromatin Modifications in Rx1d.190-treated Thymocytes

In order to reliably access specific post-translational modifications associated with Runx protein-mediated CD4 silencing, protein transduction efficiency must be at a maximum. The ability to introduce protein into populations of cells which are not actively dividing such as CD4+CD8+ (DP) cells is also critical to achieve adequate cell numbers for downstream analysis. In order to efficiently deliver adequate amounts of Runx protein to cells which are not undergoing robust proliferation, Rx1d.190 fused to cell penetrating TAT peptide was used in place of the retroviral strategy. The relevant advantage of this strategy is that it boasts a near 100% transduction efficiency (P. T. Jacobs, personal communication). Bulk thymocytes were treated with TAT peptide, TAT-
Runx1.d190, or TAT-Rx1d.190K167A for 4 hours and qPCR examination confirms TAT-Rx1d.190's ability to derepress CD4 mRNA in thymocytes (Figure 2.14). Additionally, no upregulation of CD4 mRNA was observed with the mutation of lysine residue K167 which is required for DNA binding. Aside from functional confirmation of the TAT-protein transduction strategy, this indicates that c-terminally truncated Runx1d.190 protein must bind DNA in order to derepress CD4 silencing.

To elucidate the mechanism by which Runx proteins c-terminally truncated at amino acid 190 derepress CD4 silencing, bulk thymocytes were harvested and treated with TAT peptide, TAT-Runx1.d190, or TAT-Rx1d.190K167A for 4 hours. These cells were then harvested, crosslinked, and lysed for chromatin immunoprecipitation (ChIP). CD4 silencer, CD4 enhancer, and CD4 promoter regions were examined for the presence of these specific histone modifications using qPCR. We observed no specific enrichments of activation-associated chromatin marks H3K4me3 and H3K27ac or repression associated marks H3K27me3 and H3K9me3 with Runx1d.190 treatment (Figure 2.15). Our results indicated a slight decrease in activating transcriptional marks H3K4me3 and H3K27ac associated with the CD4 silencer element in the absence of DNA binding. A much more significant decrease was observed in repressive marks H3K27me3 and H3K9me3 associated with the CD4 silencer in the absence of DNA binding. Additionally, H3 incorporation surrounding the CD4 silencer was decreased in the absence of the Runx c-terminus and DNA binding ability (Figure 2.15). This effect may be result of increased transcriptional activity or associated marks which have been observed with these constructs.
Assessing the chromatin marks present at transcriptionally relevant CD4 locus elements such as the CD4 enhancer and promoter indicated no increased chromatin modifications and significant decreases in repressive marks surrounding the promoter element (Figure 2.16). This data is supportive of CD4 transcription in the absence of the Runx c-terminus. Investigation of the histone marks surrounding the enhancer element revealed slight decreases in H3K27me3 as well as H3K4me3 (Figure 2.17). While these marks alone act in opposition, together they can prime a gene for transcriptional activation(167). The absence of these marks or any other substantial epigenetic changes could indicate that the enhancer is not involved in Runx1d.190-mediated CD4 de-repression.

Observation of these treatment-specific global changes encouraged our investigation of specific modifications across all CD4 locus elements. Activating-associated H3K4me3 was significantly enriched in histones surrounding the CD4 promoter and robustly increased in histones associated with the CD4 silencer under all treatment conditions (Figure 2.18). Interestingly, repression-associated H3K9me3 was also significantly enriched in histones surrounding the CD4 promoter and robustly increased in histones associated with the CD4 silencer under most treatment conditions. This specific co-enrichment of activating and repressive histone marks has been reported in other promoter regions as a dynamic epigenetic event(168, 169). It is important to note that H3K27me3 marks were unchanged across CD4 locus elements as this mark has been observed in conjunction with H3K4me3/H3K9me3 coenrichment.
(170). All remaining chromatin marks investigated were unchanged over unique CD4 locus elements.

2.3 Discussion

In this study, we demonstrate that preserving global acetylation of lysines through inhibition of class I HDACs results in CD4 silencing in a dose-dependent manner. This result is confounding as acetylation is commonly associated with transcriptional activation and thus we would expect derepression of CD4. Class I HDAC inhibition was able to overcome the CD4 derepression effect seen with Rx1d.190 truncation as well as silence CD4 in MSCV alone. These results raise the possibility that class I HDAC inhibition is acting on other targets within the lineage commitment network. IL-7 has been demonstrated to upregulate endogenous Runx3 (171) and IL-7 receptor signaling may be bypassed by the addition of TSA (172). Taken together, the CD4 silencing observed in the presence of HDAC inhibition may be resultant of upregulated endogenous Runx3.

Mutation of specific lysine residues within Runx1 demonstrates that Runx must bind to DNA in order to establish CD4 silencing as mutations K83A, K144A, and K167A made within the runt DNA-binding domain resulted in CD4 derepression. Though residues K144, K182, and K188 have been reported to serve as overlapping targets for p300 acetylation of Runx (124), creation of a triple mutant to evaluate this effect on CD4 silencing is not optimal due to the abrogation of DNA-binding seen with the K144A
mutation (155). While residues K24 and K43 have been reported to be acetylated by p300 and their DNA binding and transactivation activity is severely impaired with the disruption of these residues, our findings do not indicate that the modification of these residues is important for CD4 silencing. Mutation of both residues, K43AK24A, does not indicate decreased binding efficiency in the context of CD4 silencing when compared to single mutant K24A which may have very slight derepression of CD4.

Fusion of p300 HAT to either truncated Rx1d.190 or NMTS deletion Rx1d.NMTS did not indicate a role for this enforced association in CD4 silencing as CD4 was derepressed in our BCL-2 transduction system. These fusion proteins demonstrate histone acetylase activity on histone H3, indicating that the HAT domain alone is sufficient to acetylate targets when associated with Runx1. Full length p300 has been reported to have a cooperative effect with pCAF to stimulate rat osteocalcin gene promoter activity, while p300’s intrinsic activity is not required to upregulate this promoter activity (121). Interestingly, binding locations of p300 HAT in CD4^+ T cells correlated with gene expression, Pol II binding, and acetylation levels (173). As the enforced association of p300 HAT with truncated Runx1 constructs resulted in CD4 derepression, p300 HAT activity does not play a role in establishing CD4 silencing. However, full length p300 may still play an architectural role in larger complex for other proteins with intrinsic HAT activity, such as pCAF. This explanation would allow our observations to correlate with data showing increased H3K9 acetylation as well as p300 and Runx1 association with the CD4 silencer in DP cells (118).
The ability of class I HDAC inhibition to silence CD4 indicated that HDAC1 may not have a role in the establishment of CD4 silencing. By enforcing the association of HDAC1 with truncated Rx1d.190 or Rx1d.NMTS we observed the derepression of CD4. HDAC1 is a member of the Sin3A repressor complex though the Sin3A region of association with Runx1 (amino acids 181-210) (52) is not required for CD4 silencing (85). While the HDAC1/Sin3A interaction is indicated in transcriptional repression (52, 174), we do not expect a role for this interaction in the establishment of CD4 silencing. Interestingly, as with p300 HAT, binding locations of HDAC1 in CD4⁺ T cells correlated with gene expression, Pol II binding, and acetylation levels (173). Furthermore, the conditional deletion of HDAC1 in double-negative 3 stage of thymocyte development results in normal thymocyte numbers, indicating no CD4 repression (175). Taken together these findings are supportive of our observation that enforced association of truncated Runx1 constructs with HDAC1 opposes CD4 silencing.

Fusion of SUV39H1 to truncated Rx1d.190 or Rx1d.NMTS did not produce robust levels of protein, though these levels were sufficient to substantially derepress CD4 expression. SUV39H1 is known to bind Runx within the runt domain, resulting in reduced DNA binding capability (139). Our results do not indicate that our SUV39H1 fusion proteins are contacting the Runx portion via the runt domain as we would expect derepressed CD4 expression levels similar to our point mutants in the runt domain. This result is consistent with the hypothesis that Runx binding SUV39H1 outside the runt domain retains DNA-binding capabilities and methyltransferase function (138). While methylation is commonly associated with transcriptional repression; our results do not
indicate that targeting SUV39H1-specific methylation to Runx binding sites, such as the CD4 silencer, is involved in the establishment of CD4 silencing. This result is consistent with reports of H3K9 and H3K27 hypomethylation of the CD4 silencer region in developing thymocyte populations (118). While introduction of a SUV39H1 inhibitor may aid in broadening our understanding of SUV39H1 involvement as this strategy has demonstrated reexpression of silenced genes, these inhibitors are toxic to thymocytes (176).

Regions of the p300 HAT domain have been identified to bind ThPOK and p300-mediated acetylation of specific ThPOK residues has been shown to stabilize the protein (177). Runx also has two consensus binding motifs located in ThPOK and though they are not directly required for ThPOK silencer function, it remains possible for our fusion proteins to bind ThPOK, inducing modifications which lead to upregulated CD4 expression. While these findings support a role for p300 HAT activity in lineage commitment, our data indicate that this role is not through direct association with Runx1 or the modification of specific Runx lysines.

Global decreases of histone marks H3K27ac, H3K27me3, H3K4me3, and H3K9me3 in the presence of Rx1d.190 supports a role for the c-terminus of the Runx protein in establishing a functional epigenetic profile surrounding target genes. The further decreases in histone marks H3K27ac, H3K27me3, H3K4me3, and H3K9me3 in the presence of Rx1d.190K167A indicate that not only the c-terminus of the protein is important for establishing histone profiles, but DNA binding is also critical to this process. In the absence of DNA binding capacity, the overexpressed protein may be
able to interact with and sequester binding partners away from the DNA-binding sites, resulting in decreased global modifications compared to TAT controls. While the ability to introduce full-length Runx proteins with this transduction strategy could answer some questions, attempts to fuse full length Runx1 protein to TAT peptides have been unsuccessful in producing functional Runx1 proteins.

Further studies will be needed to elucidate specific modifications present on histones surrounding the CD4 silencer in the presence of full length Runx and how this correlates with Runx-mediated transactivation and repression. This and future studies continue to broaden our understanding of thymocyte lineage commitment, the vast array of molecules involved, and the critical roles they play in this process.
Figure 2.1. Inhibition of HDAC increases the kinetics of Runx3-mediated CD4 silencing in positively signaled thymocytes in a dose-dependent manner.

Simulating positively selecting signals through the TCR in thymocytes with phorbol myristyl acetate (PMA) and ionomycin (IM) leads to the upregulation of Runx3 expression and binding to the CD4 silencer. Thymocytes were positively signaled by treatment with 0.2ng/mL PMA and 0.2ng/mL IM for 18 hours before treatment with a class I HDAC inhibitor, trichostatin A (TSA), at nanomolar concentrations as indicated (1-10ng/mL) or DMSO control (0ng/mL).  

A. Immunofluorescent staining of TSA treated thymocytes expressing high levels of CD4.  

B. Overlaid histograms of thymocyte populations immunofluorescently stained for CD4 after treatment with TSA at concentrations of 3ng/mL (black line), 6ng/mL (grey line), or DMSO control (grey fill).
**Figure 2.2.** Runx1 lysine mutant proteins are expressed robustly and at similar levels.

HEK 293T cells were transiently transfected with the indicated Runx lysine point mutants and cultured for 36 hours before analysis of Runx expression. **A.** Cells were fixed and permeabilized before staining with anti-HA Tag antibody and an APC-conjugated goat anti-rabbit secondary antibody. Cells were first gated for viability, followed by GFP expression, and HA Tag expression. Contour plots are depicted with the indicated axis. The black box indicates the efficiency of transfection, representing the percentage of cells expressing GFP. **B.** Graphs indicate mean fluorescent intensity of HA Tag expression.
Figure 2.3. Experimental outline of CD4-CD8- (DN) thymocyte isolation and retroviral infection strategy

A retroviral transduction strategy is used to determine the effects of enforcing the expression of various Runx1 constructs during thymocyte development. Runx1 cDNAs are cloned into the MSCV-IRES-GFP retroviral vector, upstream of the internal ribosome entry site (IRES). This allows the expression of Runx1 constructs to be monitored by the level of green fluorescent protein (GFP). Retroviral supernatants to be used for infection are generated in 293T cells using FuGENE HD. Thymocytes from BCL-2+ transgenic mice are used for this assay because the presence of the BCL-2 anti-apoptotic factor allows the cells to remain viable during purification and in vitro culture compared to wildtype thymocytes. Thymocytes are purified using Miltenyi MACS negative selection with anti-CD4 and anti-CD8 magnetic beads, resulting in thymocytes that are CD4-CD8- (DN). These DN cells are not only synchronized for culture, but are easily infected as retroviruses may only stably infect proliferating cells.
Figure 2.4. Isolation of double positive thymocytes which have silenced CD4 expression upon Runx1 infection.

CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with murine Runx1 constructs or MSCV empty vector control, both expressing a GFP marker. **A.** Cells were first gated for viability. This population was then gated for cells expressing high levels of GFP corresponding with Runx1 protein expression. This GFP⁺ population was then gated on cells with hi levels of CD8 expression. This gating scheme results in cells which are CD4⁺CD8⁺ (DP) and cells that have downregulated CD4 expression to varying extents. Percentages indicate the percent of gated cells that express low levels of CD4 when gated as indicated by the solid bar. **B.** Once infected, there is a delay in expression of the Runx proteins, allowing CD4 to be expressed during earlier time points such as 16hrs, and silenced as Runx protein expression increases at 36 and 60hr timepoints.
Figure 2.5. Runx1 Lysine modifications associated with enhanced DNA binding do not contribute to CD4 silencing.
Figure 2.5. Runx1 Lysine modifications associated with enhanced DNA binding do not contribute to CD4 silencing.

CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with retroviral constructs encoding full-length Runx1; Runx1 lysine point mutants K24A, K43A, K43AK24A, and empty vector control (MSCV). Schematics of the constructs indicate the runt DNA-binding domain (grey box) and locations of the lysine point mutations (red X). The MSCV (grey fill) and Runx1 constructs (red line) are shown as overlaid histograms with CD4 expression on the x-axis at 16, 36, and 60 hours post-infection. Percentages indicate the percent of gated cells that express low levels of CD4 when gated as indicated by the solid bar. Graphs show the percent change CD4 expression at 16 hours (white fill), 36 hours (red fill), and 60 hours (grey fill) post-infection as compared to MSCV control CD4 expression levels, as set to axis, 0% change. Data is representative of multiple experiments (n=4, except in K43AK24A where n=2). CD4 expression levels with MSCV infection are set to axis, 0% change. Error is represented as standard error of the mean between experiments.
Figure 2.6. Runx DNA binding is required to establish CD4 silencing.
Figure 2.6. Runx DNA binding is required to establish CD4 silencing.

CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with retroviral constructs encoding full-length Runx1; Runx1 lysine point mutants K83A, K144A, K167A, and empty vector control (MSCV). Schematics of the constructs indicate the runt DNA-binding domain (grey box) and locations of the lysine point mutations (red X). The MSCV (grey fill) and Runx1 constructs (red line) are shown as overlaid histograms with CD4 expression on the x-axis at 16, 36, and 60 hours post-infection. Percentages indicate the percent of gated cells that express low levels of CD4 when gated as indicated by the solid bar. Graphs show the percent change CD4 expression at 16 hours (white fill), 36 hours (red fill), and 60 hours (grey fill) post-infection as compared to MSCV control CD4 expression levels, as set to axis, 0% change. Data is representative of multiple experiments (n=4). CD4 expression levels with MSCV infection are set to axis, 0% change. Error is represented as standard error of the mean between experiments. Significant differences between full length Runx1 and indicated mutants, as determined by t-test (one-tailed), are marked by asterisks (* = p < 0.05, ** = p < 0.005, *** = p < 0.0005).
Figure 2.7. CD4 silencing is unaltered by protecting Runx from ubiquitin-mediated degradation.
Figure 2.7. CD4 silencing is unaltered by protecting Runx from ubiquitin-mediated degradation.

CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with retroviral constructs encoding full-length Runx1; Runx1 lysine point mutants K182A, K188A, K144A, and empty vector control (MSCV). Schematics of the constructs indicate the runt DNA-binding domain (grey box) and locations of the lysine point mutations (red X). The MSCV (grey fill) and Runx1 constructs (red line) are shown as overlaid histograms with CD4 expression on the x-axis at 16, 36, and 60 hours post-infection. Percentages indicate the percent of gated cells that express low levels of CD4 when gated as indicated by the solid bar. Graphs show the percent change CD4 expression at 16 hours (white fill), 36 hours (red fill), and 60 hours (grey fill) post-infection as compared to MSCV control CD4 expression levels, as set to axis, 0% change. Data is representative of multiple experiments (n=4). CD4 expression levels with MSCV infection are set to axis, 0% change. Error is represented as standard error of the mean between experiments. Significant differences between full length Runx1 and indicated mutants, as determined by t-test (one-tailed), are marked by asterisks (* = p < 0.05, ** = p < 0.005, *** = p < 0.0005).
Figure 2.8. Inhibition of HDAC works cooperatively with the enforced expression of Runx1 constructs to establish CD4+CD8- (DN) BCL-2+ transgenic thymocytes were infected with murine Runx1 constructs, Runx1 (full length), Runx1 truncated at amino acid 190, Runx1 lysine point mutants K24A, K43A, and K43AK24A, as well as empty vector (MSCV) at a nanomolar concentration (3ng/mL) or DMSO control (0ng/mL) was added to the culture media beginning at 20 hours to allow ample time for the proliferating thymocytes to become infected. Cells were first gated for viability, followed by expression, and CD8 expression. Thymocytes were transduced with indicated Runx1 constructs, treated with 3ng/mL TSA, and cultured up to 60 hours post-infection. Schematics of the constructs indicate the runt DNA-binding domain (black of the lysine point mutations ( grey X). MSCV empty vector ( grey fill), 0ng/mL TST (black line), and TSA-treated, 3ng/mL shown as overlaid histograms with CD4 expression on the x-axis. Graph indicates the geometric mean fluorescent intensity (MFI) of CD4 (0ng/mL TSA = black text, 3ng/mL TSA = red text). Data is representative of multiple experiments (n...
Figure 2.9. Construction and expression of Runx1 fusion partners p300 HAT domain, HDAC1, and HMT SUV39H1.

Intracellular staining of 293T cells transfected with Runx1 full length, Rx1d.NMTS, Rx1d.190, and Runx1 fusion Rx1d.NMTS-p300 HAT, Rx1d.190-p300 HAT, Rx1d.NMTS-HDAC1, Rx1d.190-HDAC1, Rx1d.NMTS-SUV39H1, Rx1d.190-SUV39H1 analyzed for protein expression at 36 hours. Fusion protein schematics illustrate the runt DNA binding domain with intracellular staining using a HA tag antibody. Cells were gated for GFP and HA tag positivity. Histograms with MSCV (grey fill) are overlaid with histograms of cells transfected with the indicated Runx1 constructs (black fill).
Figure 2.10. Enzymatic activity of Runx1 fusion partners p300 HAT domain, HDAC1, and HMT SUV39H1.

A. Histones were acid extracted at 36 hours from 293T cells transfected with the indicated constructs and immuno-precipitated with anti-acetylated lysine, loading a cellular equivalence of 2.5 x 10^5 cells/lane. Histone H3 acetylated lysine shown. Immunoblots were stripped and re-probed for total histone H3 as a loading control. B. Histones were acid extracted at 36 hours from 293T cells transfected with the indicated constructs and immunoblotted for pan-methyl H3K9 at a cellular equivalence of 2.5 x 10^5 cells/lane. Immunoblots were stripped and re-probed for total histone H3 as a loading control. C,D. Relative intensities of bands were determined and modification intensities per histone H3 calculated (acetylated lysine band intensity/total normalized protein value) and results depicted with bar graphs. Error is represented as standard error of the mean. 1:10 independent experiments (n=3). Significant differences between indicated constructs were determined by t-test and are marked by asterisks (* = p < 0.05, ** = p < 0.005, *** = p < 0.0005).
Figure 2.11. p300 HAT association with truncated Runx1 proteins promotes CD4 transactivation.

CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with murine Runx1 constructs fused to the p300 histone acetyltransferase domain. The MSCV (grey fill) and indicated Runx1 constructs fused to chromatin modifying enzymes (red) are shown as overlaid histograms at 60 hours post-infection with the geometric MFI of CD4 expression noted. Dashed lines denote Runx1 truncated constructs Rx1d.NMTS or Rx1d.190 as indicated. Graphs show the percent change CD4 expression at 16 hours (no fill), 36 hours (red fill), and 60 hours (grey fill) post-infection as compared to MSCV control CD4 expression levels, as set to axis, 0% change. Data is representative of multiple experiments (n=3, except (A) where n=4). Error is represented as standard error of the mean between experiments. Significant differences between indicated constructs were determined by t-test (one-tailed) and are marked by asterisks (*** = p < 0.0005).
Figure 2.12. HDAC1 association with truncated Runx1 proteins is not sufficient for CD4 silencing.

CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with murine Runx1 constructs fused to histone deacetylase 1. The MSCV (grey fill) and indicated Runx1 constructs fused to chromatin modifying enzymes (red) are shown as overlaid histograms at 60 hours post-infection with the geometric MFI of CD4 expression noted. Dashed lines denote Runx1 truncated constructs Rx1d.NMTS or Rx1d.190 as indicated. Graphs show the percent change CD4 expression at 16 hours (no fill), 36 hours (red fill), and 60 hours (grey fill) post-infection as compared to MSCV control CD4 expression levels, as set to axis, 0% change. Data is representative of multiple experiments (n=3, except (A) where n=4). Error is represented as standard error of the mean between experiments. Significant differences between indicated constructs were determined by t-test (one-tailed) and are marked by asterisks (* = p < 0.05, ** = p < 0.005).
Figure 2.13. HMT SUV39H1 association with truncated Runx1 proteins is not sufficient for CD4 silencing

CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with murine Runx1 constructs fused to histone methyltransferase SUV39H1. The MSCV (grey fill) and indicated Runx1 constructs fused to chromatin modifying enzymes (red) are shown as overlaid histograms at 60 hours post-infection with the geometric MFI of CD4 expression noted. Dashed lines denote Runx1 truncated constructs Rx1d.NMTS or Rx1d.190 as indicated. Graphs show the percent change CD4 expression at 16 hours (no fill), 36 hours (red fill), and 60 hours (grey fill) post-infection as compared to MSCV control CD4 expression levels, as set to axis, 0% change. Data is representative of multiple experiments (n=3, except (A) where n=4). Error is represented as standard error of the mean between experiments. Significant differences between indicated constructs were determined by t-test (one-tailed) and are marked by asterisks (** = p < 0.005, *** = p < 0.0005).
Figure 2.14 CD4 mRNA is derepressed upon treatment of thymocytes with TAT-Rx1d.190 protein.

Bulk thymocytes were treated with Runx1d.190-TAT (lt. gray), Runx1d.190K167A-TAT (dk. gray), or TAT peptide control (black) for 4 hours. qPCR was performed using primers specific for CD4. Data is represented relative to GAPDH in triplicate (n=3). The mean of each data set is indicated by the bar and error is represented as standard error of the mean. Significant differences between indicated constructs were determined by student's t-test and are marked by asterisks (\(** = p < 0.005\)).
Figure 2.15 Runx1d.190 does not influence epigenetic marks incorporated into histones surrounding the CD4 silencer.

Bulk thymocytes were treated with Runx1d.190-TAT (squares), Runx1d.190K167A-TAT (triangles), or TAT peptide control (circles) for 4 hours before harvesting for chromatin immunoprecipitation (ChIP) assay. Chromatin was precipitated using anti-IgG, anti-H3, anti-H3K27ac, anti-H3K27me3, anti-H3K4me3, or anti-H3K9me3 antibodies. qPCR was performed using primers specific for the CD4 silencer element. Data is represented relative to the input of independent ChIP experiments (n=3) and triplicate qPCR experiments (n=3). The mean of each data set is indicated by the bar and error is represented as standard error of the mean. Significant differences between indicated constructs were determined by one way ANOVA and are marked by asterisks (* = p < 0.05, ** = p < 0.005, *** = p < 0.0005, **** = p < 0.00005).
Figure 2.16 Runx1d.190 does not influence epigenetic marks incorporated into histones surrounding the CD4 promoter.

Bulk thymocytes were treated with Runx1d.190-TAT (squares), Runx1d.190K167A-TAT (triangles), or TAT peptide control (circles) for 4 hours before harvesting for chromatin immunoprecipitation (ChIP) assay. Chromatin was precipitated using anti-IgG, anti-H3, anti-H3K27ac, anti-H3K27me3, anti-H3K4me3, or anti-H3K9me3 antibodies. qPCR was performed using primers specific for the CD4 promoter element. Data is represented relative to the input of independent ChIP experiments (n=3) and triplicate qPCR experiments (n=3). The mean of each data set is indicated by the bar and error is represented as standard error of the mean. Significant differences between indicated constructs were determined by 2 way ANOVA and are marked by asterisks (* = p < 0.05, ** = p < 0.005, *** = p < 0.0005, **** = p < 0.00005).
Figure 2.17 Runx1d.190 does not influence epigenetic marks incorporated into histones surrounding the CD4 enhancer.

Bulk thymocytes were treated with Runx1d.190-TAT (squares), Runx1d.190K167A-TAT (triangles), or TAT peptide control (circles) for 4 hours before harvesting for chromatin immunoprecipitation (ChIP) assay. Chromatin was precipitated using anti-IgG, anti-H3, anti-H3K27ac, anti-H3K27me3, anti-H3K4me3, or anti-H3K9me3 antibodies. qPCR was performed using primers specific for the CD4 enhancer element. Data is represented relative to the input of independent ChIP experiments (n=3) and triplicate qPCR experiments (n=3). The mean of each data set is indicated by the bar and error is represented as standard error of the mean. Significant differences between indicated constructs were determined by 2 way ANOVA and are marked by asterisks (* = p < 0.05, ** = p < 0.005, *** = p < 0.0005, **** = p < 0.00005).
Figure 2.18. Chromatin marks H3K4me3 and H3K9me3 are enriched surrounding the CD4 promoter and CD4 silencer.

A. CD4 locus elements and their locations relative to each other and within CD4. B. Bulk thymocytes were treated with Runx1d.190-TAT, Runx1d.190K167A-TAT, or TAT peptide (control) for 4 hours before harvesting for ChIP assay. Chromatin was precipitated using anti-IgG, anti-H3K4me3, or anti-H3K9me3 antibodies. qPCR was performed using primer sets specific to the CD4 enhancer (gray circle), CD4 Promoter (black square), or the CD4 silencer (red triangle). Data is represented relative to input of independent ChIP experiments (n=3) and triplicate qPCR experiments (n=3). Significant differences between indicated constructs were determined by 2 way ANOVA and are marked by asterisks (* = p < 0.05, ** = p < 0.005, *** = p < 0.0005, **** = p < 0.00005)
CHAPTER 3

FUTURE DIRECTIONS

3.1 cKrox

cKrox has been reported to have an antagonistic effect on Runx-mediated CD4 silencing (178). In order to ascertain whether this effect could be observed and investigated in our retroviral culture system, cKrox was generously provided by R. Bosselut (BC046533) and cloned into an MSCV-DsRed retroviral vector. This construct was co-transduced into Bcl-2+ transgenic DN thymocytes along with Rx1d.190 and cultured for 36 hours. FACS analysis of CD4 indicates that Rx1d.190 does affect CD4 expression levels when cotransduced with cKrox (Figure 3.1). This result supports previous reports where cKrox and Rx1d.190 have been shown to derepress CD4. In addition to derepression of CD4, instances of cKrox downregulating CD8 expression have also been reported (178). These effects appear to be active in our system as CD8 expression is downregulated and is not rescued by Rx1d.190 (Figure 3.1). Decreased levels of CD4 mean fluorescence intensity observed are most likely due to poor infection efficiency of MSCV-DsRed retroviral supernatants.

While this data wasn’t expanded during the tenure of this project, preparation of retroviral supernatants with improved efficiency as well as supernatants of Runx1 full length constructs similar to those used in previous cKrox reports may expand our understanding of cKrox antagonism of Runx proteins and how this pertains to CD4
silencing in our Bcl-2+ transgenic thymocyte culture system. Given cKrox's proposed interaction with p300 proteins, coinfection with a Rx1d.190-p300 HAT may yield results broadening our understanding of the elaborate interplay between cKrox, p300, and Runx proteins.

3.2 Lentiviral Infection

Our primary system of delivering the full length Runx protein into thymocytes has been retroviral transduction. While this system is adept at introducing Runx into dividing cells at adequate amounts for analysis by flow cytometry, infection efficiency can be a limitation. In order to enhance our current infection efficiency, a lentiviral strategy has been proposed. This lentiviral strategy is anticipated to enhance the current retroviral system as lentivirus may integrate into the genome of cells which are not undergoing cell division (179, 180), β-selection in the case of thymocytes. This is due to a lentivirus's ability to pass through the nuclear membrane pores whereas retrovirus can only infect dividing cells as it gets taken up into the nucleus of dividing cells upon nuclear membrane breakdown and reformation.

A lentiviral vector (pLEIGW)(181) has been prepared to receive chromatin modifying enzymes fused to truncated Runx proteins through replacement of the multi-cloning site (MCS), adding appropriate restriction enzyme sites. These fusion proteins may be packaged in HEK 293T cells and used to infect bulk thymocyte populations,
assessing CD4 silencing by flow cytometry as with the retroviral strategy. We have successfully

It is anticipated that this strategy will increase our infection efficiency; allowing chromatin immunoprecipitation (ChIP) to be preformed targeting specific epigenetic modifications in the CD4 silencer, promoter, and enhancer regions under the influence of larger proteins such as full length Runx. These experiments would provide further support for the functionality of our fusion proteins, as well as expanding our data set concerning a possible mechanism of CD4 derepression observed upon overexpression of full length Runx and our Runx fusion proteins in thymocytes.

Possible pitfalls for this strategy include a difficult cloning strategy as restriction sites are limited in the retroviral vector. Additionally, pLEIGW is quite large (10.5kb) and the inserts range in size from 1.8kb to 3.5kb. These mechanics could present challenges for efficient cloning and for production of adequate viral titers. As Runx expression through lentiviral infection has not been used to silence CD4, it is unknown if DP cells will be susceptible to CD4 silencing by Runx overexpression. In the event that DP cells are not susceptible to CD4 silencing by Runx overexpression, DN cells can be enriched for and infected as done in the retroviral strategy.

3.3 Runx-independent CD4 silencing

CD4 silencing observed in the presence of HDAC inhibition may be resultant of upregulated endogenous Runx3 as class I HDAC inhibition could be acting on additional targets within the lineage commitment network. IL-7 has been demonstrated to
upregulate endogenous Runx3 (171) and IL-7 receptor signaling may be bypassed by the addition of TSA (172). To explore this possibility, bulk thymocytes can be treated with TSA and mRNA may be examined for changes in Runx transcripts using primers designed to the Runt domain (84). This strategy is expected to detect any changes in endogenous expression of the Runx family members as a result of TSA treatment. Observation of increased Runx mRNA would indicate that increased amounts of endogenous Runx play a role in TSA-induced CD4 silencing. Alternatively, if no change in Runx expression is observed with TSA treatment, CD4 silencing induced by class I HDAC inhibition is not due to increased endogenous Runx expression. This result would suggest the involvement of an entirely different pathway by which TSA treatment is able to overcome Rx1d.190’s CD4 derepression. Additionally, these observations would indicate that IL-7 receptor signaling bypassed by TSA treatment does not play a role in CD4 silencing.
Figure 3.1. Rx1d.190 cotransduction with cKrox does not affect CD4 silencing.

CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with MSCV-GFP (green), MSCV-Ds.Red (red), Rx1d.190-MSCV-GFP (blue), cKrox-MSCV-Ds.Red (purple), or co-transduced with Rx1.190-MSCV-GFP & cKrox-MSCV-Ds.Red (orange). Thymocytes were cultured for 36 hours and analyzed for CD4 and CD8 expression via FACS.
Figure 3.2. Lentiviral Infection of BCL2+ bulk thymocytes

Bulk BCL2+ thymocytes were infected with full length murine Runx1 (Runx1), Runx1 c-terminally truncated (Rx1d.190), and Runx1d.190 fused to histone deacetylase 1 (Rx1d.190-HDAC1) constructs or empty lentiviral vector control (pLEIGW). Lentiviral infection was performed by spin infection using 48hr and 72hr lentiviral supernatants with lipofectamine. GFP expression was analyzed with FACS at 48 hour and 72 hour timepoints. Graphs indicate the percentage of infected culture cells expressing GFP at 48 hour (gray fill) and 72 hour (red fill) timepoints.
CHAPTER 4
MATERIALS & METHODS

4.1 Materials

4.1.1 Animals

BCL-2+ and C57Bl/6 mice were obtained for use from The Jackson Laboratory in Bar Harbor, Maine. Breeding pairs were established at the University of Massachusetts, Amherst and the progeny were genotyped using an ear punch tissue sample. Primers designed to amplify the bcl-2 gene were used for PCR of genomic DNA. Animals were housed in an animal care facility at the University of Massachusetts, Amherst in accordance with federal guidelines. Animal use was approved by IACUC.

IRC mice were generously provided by Dr. Kim Tremblay, University of Massachusetts, Amherst, MA.

4.1.2 Antibodies

Specific antibodies for FACS analysis were purchased from the manufacturers indicated in Table. HA Tag antibody used for intracellular staining was purchased from Cell Signaling Technology. Secondary antibodies used for intracellular staining were purchased from the companies indicated in Table.

Anti-Acetylated-Lysine and Anti-Histone H3 antibodies used for immunoblotting were purchased from Cell Signaling Technology, while Anti-Pan-methyl H3K9 antibody was purchased from Active Motif. Goat Anti-Rabbit Ig-HRP secondary antibody was purchased from Southern Biotechnology Associates.
Antibodies used for chromatin immunoprecipitation were purchased from the companies listed in Table.

4.1.3 Cells

HEK-293T cells were purchased from ATCC. Thymocytes were harvested from mice promptly after CO₂-induced euthanasia. The thymus was dissociated to a single cell suspension using a curved scissor to first cut the organ into small pieces and following; the corrugated end of a syringe plunger was ground against a sterile culture plate to disrupt the cell matrices. Cells were then sterile filtered to remove undissociated cells and aggregates.

4.1.4 Competent Cells

Stbl2 competent cells were purchased from Invitrogen. DH10 competent cells were prepared from a 5ml LB inoculation grown up overnight. The following morning the culture was diluted 1/100 with fresh LB and grown to an OD₆₀₀ of 0.2 - 0.5. The culture was split into two tubes and incubated on ice for 10 minutes before centrifuging the cells at 3000rpm and 4°C for 10 min. The pellets were resuspended in a 10% culture volume of chilled TSS buffer (LB with 10%w/v PEG, 5%v/v DMSO, 20mM MgCl₂, pH6.5). Cells were aliquoted, flash frozen, and stored at 80°C.

4.1.5 Plasmids

pBluescript II KS was provided by Dr. Dominique Alfandari. Retroviral vector MSCV including the HA Tag was provided by Dr. Janice C. Telfer. Lentiviral vector
pLEIGW was obtained from Dr. Christoph Schaniel, Princeton University, Princeton, NJ.

MSCV.DS.Red was provided by Dr. Barbara Osborne.

4.1.6 Primers

Primers used for mutagenesis and sequencing were synthesized and HPLC purified by Integrated DNA Technologies (Coralville, IA). Primers used for PCR or qPCR amplification were synthesized with standard desalting by Integrated DNA Technologies. Primers were maintained in 10mM Tris pH 8.0 at a 20uM concentration. Primer sets used are listed in Table 1-Table 3, and Table 5.

4.1.7 Rx1d.190 Biotin-labeled Probe

Rx1d.190 Biotin-labeled Probe for use in plate lifts was synthesized using the North2South Biotin Random Prime Labeling Kit (Pierce, Rockford, IL). Rx1d.190 template was prepared through restriction enzyme digest from Rx1d.190-MSCV using BglII and Xhol. Restriction digests were run on a 1% agarose gel stained with Ethidium Bromide and specific products were extracted with the Gel Extraction Kit (QIagen) according to manufacturer’s instructions. Template was used to generate a Biotin labeled probe according to manufacturer’s instructions. Probe yield was quantitated measuring absorbance at 260nm with a spectrophotometer.
4.2 Methods

4.2.1 Site-directed Mutagenesis

Point mutations of nine individual and two combined lysine residues to alanines were made in full length Runx1 (pBluescriptSK vector) using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used for mutagenesis are as indicated (Table 1). Two lysine point mutations were made in K43AK24A by using the mutated K43A as a template for the mutation of lysine residue K24 to alanine. Mutagenesis was carried out according to manufacturer's instructions.

4.2.2 Retroviral Constructs

4.2.2.1 Point Mutations in Runx1

Runx1 point mutant inserts were generated with PCR using an editing DNA polymerase, Pfx (Invitrogen Life Technologies, Carlsbad, CA) to amplify template DNA from a pBluescriptSK vector. BgIII restriction sites were generated in all inserts with a 5’ primer (GAAGATCTCCGCCATGCCGTATCCCCGGTAGATGCGAGCAGCGCCG)(Table 2), also including a Kozak sequence upstream of the initiation methionine. XhoI restriction sites were added to the inserts with a 3’ primer (CGGCTCGAGGTAGGCCGACGCGGCTCTCTCC). Runx1 deletion constructs are as described (85). These mutants were inserted into the murine stem cell virus retroviral vector (MSCV) at BgIII and XhoI sites through ligation (Takara Bio Inc., Otsu, Shiga, Japan) at a 10:1 insert:vector molar ratio at 16°C, overnight. Ligation reactions were used to transform Stbl2 competent cells (Invitrogen) according to manufacturer's
instructions. Clones were grown up at 30°C and DNA was prepared from the bacteria for screening of the presence of appropriate insert using a Qiagen Mini-prep kit (Qiagen). Restriction digests with BglII and Xhol were used to screen for inserts. Point mutations were commercially sequenced (Genewiz, South Plainfield, NJ) to confirm that they were in frame.

Retrovirus was packaged in 293T cells by transient co-transfection with pECL-ECO using FuGENE-HD (Roche, Basel, Switzerland) with 2µg DNA, as directed by manufacturer. Retroviral supernatants were harvested and sterile filtered at 48 and 72 hours before storage at -80°C.

4.2.2.2 HDAC1 & SUV39H1 fused to truncated Runx1

HDAC1 (NM_004964.2; OriGene, Rockville, MD) or HMT SUV39H1 (NM_003173.1; OriGene) were obtained as cDNA clones commercially and used as template to amplify specific products using PCR primers (Table 2) incorporating EcoRI restriction sites. HDAC1 and HMT SUV39H1 products were fused to Rx1.190 through the EcoRI restriction sites and HMT SUV39H1 was fused to Rx1d.NMTS. These PCR products were ligated (Takara Bio Inc.) into MSCV at a 3:3:1 insert:insert:vector molar ratio at 16°C, overnight. Ligation reactions were used to transform Stbl2 competent cells (Invitrogen) according to manufacturer’s instructions. Clones were grown up on LB plates at 30°C and screened for the presence of appropriate insert using a plate lifts and biotin-labeled probe designed to Rx1d.190.
Plate lifts were carried out by pre-cooling the plates at 4°C for 30min followed by gentle placement of the Hybond-N+ (Amersham Biosciences, Piscataway, NJ) membrane over the DNA for 30-60 seconds. The plates were then returned to 30°C for 4hours. The membranes were processed by placement over blotting paper saturated with 10% SDS for initial lysis. The membranes were then processed with Denaturation Buffer(1.5M NaCl, 500mM NaOH) for 2-5 minutes followed by Neutralization Buffer(1.5M NaCl, 500mM Tris, pH 7.5) for 3min. The neutralization step was repeated once and the membranes were vigorously washed twice in saline-sodium citrate (SSC)(20X: 3M NaCl, 300mM Tri-sodium citrate, pH 7.0) to remove proteinous debris. Membranes were air dried and the DNA was fixed using an optimized UV crosslinker. Rxd.190 biotin-labeled probe was hybridized to the membrane using the North2South Chemiluminescent Hybridization and Detection kit (Pierce) according to manufacturer’s instructions.

Colonies which were positive for Rxd.190 were selected for and grown up in LB at 30°C shaking at 250rpm. DNA was prepared from the bacteria for screening of the presence of appropriate insert using a Qiagen Mini-prep kit (Qiagen). Restriction digests with BglII and Xhol were used to screen for inserts. Fusion proteins were commercially sequenced (Genewiz) to confirm that they were in frame.

Rxd.NMTS-HDAC1 was obtained commercially (GenScript, Piscataway, NJ). Due to the similar size of vector and insert, the PUC57 cloning vector was digested with KpnI and HindIII to shorten the vector. This product was gel purified with a Gel Extraction Kit (Qiagen) according to manufacturer’s instructions. The product was then digested with BglII and Xhol to extract the Rxd.NMTS-HDAC1 fusion. This product was gel purified
and inserted into the MSCV vector at BgIII and XhoI sites through ligation (Takara Bio Inc.) at a 10:1 insert:vector molar ratio at 16°C, overnight. Ligation reactions were used to transform Stbl2 competent cells (Invitrogen) according to manufacturer’s instructions. Clones were grown up at 30°C and DNA was prepared from the bacteria for screening of the presence of appropriate insert using a Qiagen Mini-prep kit (Qiagen). Restriction digests with BgIII and XhoI were used to screen for inserts. Fusion proteins were commercially sequenced (Genewiz) to confirm that they were in frame.

Retrovirus was packaged in 293T cells by transient co-transfection with pECL-ECO using FuGENE-HD (Roche) with 2µg DNA, as directed by manufacturer. Retroviral supernatants were harvested and sterile filtered at 48 and 72 hours before storage at -80°C.

4.2.2.3 p300 HAT fused to truncated Runx1

p300 HAT Domain (aa. 934-1652, (162)) was constructed from PCR-generated fragments of two separate clones (BU188211/BC053889, CV570591; Open Biosystems, Huntsville, AL) with splice overlap extension PCR (SOE-PCR)(182-184). Primers used to amplify the inserts from the clones, introducing 5’ HindIII and 3’ XhoI sites, as well as for the SOE-PCR reaction are as listed (Table 3).

The p300 HAT SOE-PCR product was cloned into pBluescriptSK at HindIII and XhoI sites. p300 HAT was amplified from the pBluescript template with PCR and fused to Rx1d.190 or Rx1d.NMTS through a HindIII site introduced with the 5’ and 3’ PCR primers (Table 3). These mutants were inserted into MSCV at BgIII and XhoI sites.
through ligation (Takara) at a 3:3:1 insert:insert:vector molar ratio for 4°C, overnight. Ligation reactions were used to transform Stbl2 competent cells (Invitrogen) according to manufacturer’s instructions. Clones were grown up on LB plates at 30°C and screened for the presence of appropriate insert using a plate lifts and Rx1d.190 biotin-labeled probe.

Plate lifts were carried out by pre-cooling the plates at 4°C for 30min followed by gentle placement of the Hybond-N+ (Amersham Biosciences) membrane over the DNA for 30-60 seconds. The plates were then returned to 30°C for 4hours. The membranes were processed by placement over blotting paper saturated with 10% SDS for initial lysis. The membranes were then processed with Denaturation Buffer (1.5M NaCl, 500mM NaOH) for 2-5 minutes followed by Neutralization Buffer (1.5M NaCl, 500mM Tris, pH 7.5) for 3min. The neutralization step was repeated once and the membranes were vigorously washed twice in saline-sodium citrate (SSC) (20X; 3M NaCl, 300mM Trisodium citrate, pH 7.0) to remove proteinous debris. Membranes were air dried and the DNA was fixed using an optimized UV crosslinker. Rxd.190 biotin-labeled probe was hybridized to the membrane using the North2South Chemiluminescent Hybridization and Detection kit (Pierce) according to manufacturer’s instructions.

Colonies which were positive for Rx1d.190 were selected for and grown up in LB at 30°C shaking at 250rpm. DNA was prepared from the bacteria for screening of the presence of appropriate insert using a Qiagen Mini-prep kit (Qiagen). Restriction digests with BgIII and XhoI were used to screen for inserts. Fusion proteins were commercially sequenced (Genewiz) to confirm that they were in frame.
Retrovirus was packaged in 293T cells by transient co-transfection with pECL-ECO using FuGENE-HD (Roche) with 2µg DNA, as directed by manufacturer. Retroviral supernatants were harvested and sterile filtered at 48 and 72 hours before storage at -80°C.

4.2.2.4 cKrox

cKrox in pcDNA3 vector was obtained from R. Bosselut (BC046533). cKrox was amplified with PCR using Pfx editing polymerase (Invitrogen) and primers adding a '5 EcoRI site (EcoRI.cKrox.fwd 5' : CGCGAATTCATGGGAGCCCG) and a 3' BamHI site (BamHI.cKrox.rev 5' : CGCGGATCCTAAGAGGACTCCATG). The PCR products were run on a 1% agarose gel stained with Ethidium bromide and gel purified using a gel extraction kit (Qiagen) according to manufacturer’s instructions. cKrox was then inserted into the MSCVDS.Red retroviral vector at EcoRI and BamHI sites through ligation (Takara Bio Inc.) at a 10:1 insert:vector molar ratio for 16°C, overnight. Colonies were picked and grown up in LB at 30°C shaking at 250rpm. DNA was prepared from the bacteria for screening of the presence of appropriate insert using a Qiagen Mini-prep kit (Qiagen). Restriction digests with EcoRI and HindIII were used to screen for inserts. Fusion proteins were commercially sequenced (Genewiz) to confirm that they were in frame.

Retrovirus was packaged in 293T cells by transient co-transfection with pECL-ECO using FuGENE-HD (Roche) with 2µg DNA in 6mm cell culture plates, as directed by
manufacturer. Retroviral supernatants were harvested and sterile filtered at 48 and 72 hours before storage at -80°C.

4.2.3 Lentiviral constructs

The MCS of pLEIGW (courtesy of Dr. Christoph Shaniel, Princton University)(18) was removed at EcoRI and BamHI sites and replaced with oligos designed to reposition four remaining restriction sites in reverse order (pLEIGW.1) (pLEIGW.MCS.5’: AATTGAAGGATCCAAATCGATAAAAAGCTAGCAAGAATTCAA, pLEIGW.MCS.3’: TTCCTAGGTTCAGCTATTTCGATCCTTTAAAGTTTCTAG).

Runx1 full length, Rx1d.NMTS, Rx1d.190, and fusion proteins Rx1d.NMTS-p300HAT, Rx1d.190-p300HAT, Rx1d.NMTS-HDAC1, Rx1d.190-HDAC1, Rx1d.NMTS-SUV39H1, Rx1d.190-SUV39H1 were amplified from MSCV including the HA tag using an editing DNA polymerase, Pfx (Invitrogen Life Technologies). Clal restriction sites were generated in all inserts with a 5’ primer (GAATCGATCCGCCATGCATCTCCCCGTAGATGCCAGCACGAGCCG), also including a Kozak sequence upstream of the initiation methionine. Nhel restriction sites were added to the inserts with a 3’ primer (CGCGTAGCTCAAGCGTAATCTGGGACGCTCGATG). PCR products were gel purified from a 1% agarose gel using a Gel Extraction Kit (Qiagen). These mutants were then inserted into the pLEIGW.1 lentiviral vector at Clal and Nhel sites through ligation (Takara Bio Inc.) at a 10:1 insert:vector molar ratio for 16°C, overnight.
Colonies were picked and grown up in LB at 30°C shaking at 250rpm. DNA was prepared from the bacteria for screening of the presence of appropriate insert using a Qiagen Mini-prep kit (Qiagen). Restriction digests with EcoRI were used to screen for inserts. Fusion proteins were commercially sequenced (Genewiz) to confirm that they were in frame.

4.2.4 Transfection of mammalian cells

Retrovirus was packaged using HEK-293T cells by transient co-transfection with pECL-ECO using FuGENE-HD (Roche), as directed by manufacturer. 24 hours before transfection, HEK-293T cells were seeded at a density of 1 x 10^6 cells per plate in 6mm cell culture plate. Cells were grown to ~70-80% confluency in IMDM (1X Hepes, 1X MEM-Non-essential amino acids, 1X Sodium Pyruvate (Invitrogen Life Technologies)) at 37°C, 7% CO₂ prior to transfection. The ratio of DNA to FuGENE-HD (Roche) was maintained at 2:7 (µg:µl). 2µg DNA (1µg pECL-ECO and 1µg each MSCV construct) was dissolved in 100µl serum-free media with 7µl FuGENE-HD (Roche) and incubated at room temperature for 20 minutes. This complex was diluted with 900µl antibiotic-free medium and added to the HEK-293T cultures drip wise. Medium was replenished at 24 hours and GFP expression was observed qualitatively. Retroviral supernatants were harvested and sterile filtered at 48 and 72 hours post-transfection and stored at -80°C.

Lentivirus was packaged in 293T cells by transient co-transfection with packaging plasmids pCMV-VSV-G and pCMVΔ8.9. 1µg of each pLEIGW construct was mixed with 0.25µg pCMV-VSV-G and 0.75µg pCMVΔ8.9 using FuGENE-HD (Roche) in 6mm cell
culture plates, as directed by manufacturer. 24 hours before transfection, HEK-293T cells were seeded at a density of $1 \times 10^6$ cells per plate in 6mm cell culture plate. Cells were grown to ~70-80% confluency in IMDM at 37°C, 7% CO2 prior to transfection. The ratio of DNA to FuGENE-HD (Roche) was maintained at 2:7 (ug:ul). 2ug DNA (1ug pECL-ECO and 1ug each MSCV construct) was dissolved in 100ul serum-free media with 7ul FuGENE-HD (Roche) and incubated at room temperature for 20 minutes. This complex was diluted with 900ul antibiotic-free medium and added to the HEK-293T cultures drip wise. Medium was replenished at 24 hours and GFP expression was observed qualitatively. Lentiviral supernatants were harvested and sterile filtered at 48 and 72 hours before short-term storage at 4°C. Lentiviral supernatant was stored long-term at -80°C.

HEK-293T cells transfected with Runx lysine point mutants or Runx fusion proteins using FuGENE-HD (Roche) as directed by manufacturer. 24 hours before transfection, HEK-293T cells were seeded at a density of $1 \times 10^6$ cells per well in 6-well cell culture plates. Cells were grown to ~70-80% confluency in IMDM at 37°C, 7% CO2 prior to transfection. The ratio of DNA to FuGENE-HD (Roche) was maintained at 1:3.5 (ug:ul). 1ug DNA (MSCV constructs) was dissolved in 50ul serum-free media with 3.5ul FuGENE-HD (Roche) and incubated at room temperature for 20 minutes. This complex was diluted with 150ul antibiotic-free medium and added to the HEK-293T cultures drip wise. Fresh antibiotic-free media was supplied at 24 hours post-transfection and cells were harvested for analysis at 48 hours post-transfection.
4.2.5 Bcl-2 transgenic thymocyte culture

Thymocytes were harvested from bcl-2+ transgenic mice (The Jackson Laboratory, Bar Harbor, ME). They were then enriched for immature thymocytes by negative selection using anti-CD4 and anti-CD8 antibodies directly conjugated to microbeads and passed over a MACS column (Miltenyi Biotech, Bergisch Gladbach, Germany) as directed. The immature thymocytes (DN, CD4-CD8-) were then plated in a twenty-four-well cell culture plates in DMEM-FTOC media (10% charcoal-stripped FCS, 1X nonessential amino acids (Invitrogen Life Technologies), 1X Hepes, 50 μm 2-ME, 100μg/μl penicillin-streptomycin (Invitrogen Life Technologies)).

Cells were infected by centrifuging with 1.2mL retroviral supernatants plus 5μg/mL lipofectamine (Invitrogen Life Technologies) at 2300rpm for 1 hr at 30°C in a Thermo IEC Centra-CL3R. Lentiviral infection was performed by centrifuging with 2mL lentiviral supernatants plus 5μg/mL lipofectamine (Invitrogen Life Technologies) at 2300rpm for 1 hr at 30°C in a Thermo IEC Centra-CL3R.

Depleted media was aspirated and fresh media was added to the cells post-infection. Trichostatin A (TSA) (Millipore, Billerica, MA), where indicated, was added via media replacement 24 hours post-infection. The thymocytes were cultured in DMEM-FTOC at 37°C, 7%CO₂ for up to 60 hours post-transduction.

4.2.6 FACS analysis

Cells were harvested at indicated time points and washed with 5.5 mM KCl, 0.4 mM KH2PO4, 140 mM NaCl, 5.6 mM glucose, 0.3 mM Na2HPO4, 3.9 mM NaHCO3, 0.2%
BSA, 1 mM EDTA. Non-antigen-specific binding was blocked with anti-mouse CD16/32 (FC Block) (eBioscience, San Diego, CA). Cells were stained with PE conjugated anti-mouse CD4 (L3T4) (eBioscience), PerCP-Cy5.5 conjugated anti-mouse CD8α (Ly-2) (eBioscience), and APC conjugated anti-mouse TCRβ chain (BD Pharmingen). Cells were run on a LSR II (BD Biosciences, Franklin Lakes, NJ) and FACS data were analyzed with FlowJo (Tree Star, Ashland, OR) software.

4.2.7 Intracellular Staining

293T cells were transiently transfected with 2μg of DNA using FuGENE-HD (Roche) as directed for 6-well plates. Fresh antibiotic-free media was supplied at 24 hours post-transfection and cells were harvested at 48 hours post-transfection. Cells were counted and washed 2 times with Phosphate buffered saline + 100ng/ml TSA and protease inhibitors (1μg/ml Aprotinin, 1μg/ml Pepstatin A, 1mM PMSF).

Cells were fixed in 2% methanol-free formaldehyde and incubated at 37°C for 10 minutes, followed by 1 minute on ice. Cells were centrifuged and resuspended in 90% methanol and incubated on ice for 30 minutes. Cells were washed 2 times and blocked for 10 minutes in Incubation Buffer (1X Phosphate buffered saline & 0.05%W/V Bovine serum albumin (BSA)). Cells were incubated with HA Tag antibody (Cell Signaling Technology) at a 1:50 dilution for 30min with rotation. Cells were washed in incubation buffer and incubated with goat anti-mouse APC (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:50 dilution for 30min with rotation. Cells were analyzed on a LSR II (BD Biosciences) and FACS data were analyzed with FlowJo (Tree Star) software.
4.2.8 Histone Extraction

293T cells were transiently transfected with 2μg of fusion protein DNA using FuGENE-HD (Roche) as directed for 6-well plates. Fresh antibiotic-free media was supplied at 24 hours post-transfection and cells were harvested at 48 hours post-transfection. Cells were counted and washed 2 times with Phosphate buffered saline + 100ng/ml TSA and protease inhibitors (1μg/ml Aprotinin, 1μg/ml Pepstatin A, 1mM PMSF). Cells were divided for further intracellular staining or histone extraction.

Histone extraction was performed by lysis of the cells with Triton Extraction Buffer (Phosphate buffered saline, 100ng/ml TSA, 0.5% Triton-X-100, 2mM PMSF, 0.02% NaN3) and centrifugation for 10min at 2000 rpm (165, 166). Pellet was resuspended in 0.2N HCl and acid extraction was carried out overnight at 4°C and supernatant was saved. Sample buffer was added 2:1 to the histones and 5 x 10⁵ cell equivalence was loaded per well on a 15% SDS-PAGE gel.

4.2.9 Western Blotting

Samples were transferred to PVDF membranes (Millipore) and blocked in Tris buffered saline 0.5% Tween-20 (TBS-T) with 5% nonfat milk overnight at 4°C. Membranes were incubated with appropriate antibodies (Anti-Acetylated-Lysine (Cell Signaling Technology) or Anti-Pan-methyl H3K9 (Active Motif, Carlsbad, CA)) in TBS-T with 5% BSA overnight at 4°C with rotation. The membranes were then washed 9 times for 5 minutes in TBS-T before incubation with Goat Anti-Rabbit Ig-HRP (Southern Biotechnology Associates, Birmingham, AL) in TBS-T with 1% nonfat milk for 1 hour.
Following nine washes for 5 minutes each in TBS-T the membranes were developed with ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). Photoshop (Adobe, San Jose, CA) was used to quantitate band signals.

Membranes were stripped in Stripping Buffer (100mM 2-Mercaptoethanol, 2% w/v SDS, 62.5mM Tris-HCL pH 6.7) for 30 min at 70°C and blocked in 5% nonfat milk overnight at 4°C. Membranes were incubated with Anti-Histone H3 (Cell Signaling Technology) in TBS-T with 5% nonfat milk overnight at 4°C with rotation. The membranes were then washed nine times for 5 minutes in TBS-T before incubation with Goat Anti-Rabbit Ig-HRP (Southern Biotechnology Associates, Birmingham, AL) in TBS-T with 1% nonfat milk for 1 hour. Following nine washes for 5 minutes in TBS-T the membranes were developed with ECL Western Blotting Detection Reagents (GE Healthcare). Photoshop (Adobe) was used to quantitate band signals.

4.2.10 Purification of TAT Proteins

A single colony of BL21 competent cells transformed with Runx1d.190-TAT or Runx1d.190K167A-TAT was inoculated into modified terrific broth (with 8mL/L glycerol) with 50ng/mL kanamycin. The cultures were grown up 16 hours at 37°C with shaking at 250rpm before 1:10 dilution into 1L terrific broth. 500uM IPTG was added to the diluted cultures before continuing incubation and shaking for 5 hours. The cultures were centrifuged at 5,000rpm for 5 minutes at 4°C and washed 2X with PBS pH 7.4. The cells were resuspended in 10mLs of Buffer Z(8M urea, 100mM NaCl, 20mM HEPES) pH8.0 before sonication on ice three times at 30% power for 15 second pulses using a
Sonicator Ultrasonic Processor XL (Qsonica, Newtown, CT). The lysates were centrifuged at 13,200rpm for 10 minutes at 4°C and supernatant was removed and adjusted to contain 20mM imidazole. Lysates were added to 5mL of 50% slurry Nickel Sepharose 6 Fast Flow beads (GE Healthcare) and incubated overnight at 4°C with rotation. The beads were washed three times with 5 volumes of Buffer Z with 20mM imidazole for 15 minutes at 4°C with rotation. Protein was eluted in 1 volume Buffer Z with 200mM for 4 hours at 4°C with rotation. A second elution in 1 volume Buffer Z with 500mM imidazole was performed overnight at 4°C with rotation.

The protein elutions were diluted with 1 volume of 20mM HEPES and concentrated using Amicon Ultra-10K MWCO (Millipore) according to manufacturer’s instructions. The high salt/imidazole buffer was exchanged for PBS pH7.4 with 10% glycerol using PD-10 (GE Healthcare) desalting columns according to manufacturer’s instructions. LPS was removed from proteins through incubation with 30µl polymyxin B- Agarose bead suspension (Sigma) per mL of protein at 4°C for 5 minutes with rotation. Purified proteins were centrifuged at 13,200rpm for 10 minutes and sterile filtered at 0.2 microns before flash freezing and storage at -80°C.

4.2.11 Quantitation of mRNA transcripts

Thymocytes were harvested from IRC transgenic mice, counted to obtain 1x10^6 cells per sample, resuspended in DMEM-FTCO media (w/o phenol red) with TAT proteins Rx1d190-TAT, Rx1d190K167A-TAT, or TAT peptide, and plated in twelve-well cell culture plates. Cells were incubated for 4 hours at 37°C. Cells were harvested and mRNA was
extracted using Qiazol (Qiagen) reagent followed by the RNeasy kit according to manufacturer's instructions. RNA was quantitated in DEPC water using 260nm absorbance. RNA samples were treated with DNAse (New England Biolabs) according to manufacturer's instructions. cDNA synthesis was carried out using an iScript kit (Biorad) according to manufacturer's instructions using the following thermocycler protocol: 25°C for 5min, 42°C for 30min, 85°C for 5min. Quantitative Real Time PCR of samples was performed in triplicate using specific primers (Table).

4.2.12 Chromatin Immunoprecipitation (ChIP) - Agarose Beads

Thymocytes were harvested from bcl-2 or IRC transgenic mice, counted to obtain 1x10^6 cells per sample, resuspended in DMEM-FOC media (w/o phenol red) with TAT proteins Rx1d190-TAT, Rx1d190K167A-TAT, or TAT peptide, and plated in twelve-well cell culture plates. Cells were incubated for 30 minutes at 4°C followed by 3.5 hours at 37°C. Cells were harvested and DNA was crosslinked to histones by addition of formaldehyde to a final concentration of 0.37% for 10 minutes at 37°C. Cells were washed in PBS pH 7.4 and Protease Inhibitor Cocktail (1mM PMSF, 1μg/ml aprotinin, & 1μg/ml pepstatin A). Samples were resuspended in SDS Lysis Buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0) and sonicated at 30% maximum power for 10 seconds a total of four times using a Sonicator Ultrasonic Processor XL (Qsonica). Samples were centrifuged for 10 minutes at 13,000 rpm at 4°C and supernatants were diluted with ChIP Dilution Buffer (0.01% SDS, 1.1% Triton-X-100, 1.2mM EDTA, 16.7mM Tris pH 8.0, 167mM NaCl) with Protease Inhibitor Cocktail. Samples were pre-cleared with 80μl
Salmon Sperm DNA/Protein G Agarose-50% Slurry (Millipore) for 30 minutes at 4°C and centrifuged. The sample supernatants were incubated with manufacturer's recommended dilutions of precipitating antibodies (Table 4) or control IgG1 antibody (Southern Biotech) at 4°C overnight with rotation. 60μl of Salmon Sperm DNA/Protein G Agarose-50% Slurry was added to collect the antibody/histone complex for one hour at 4°C. The protein G agarose/antibody/histone complex was washed for five minutes each wash in Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20mM Tris pH 8.0, 150mM NaCl), Medium Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20mM Tris pH 8.0, 300mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl). Histone complex was eluded from the antibody with Elution Buffer (1% SDS, 0.1M NaHCO3) twice and the histone-DNA crosslinks were reversed with 5M NaCl addition and incubation at 65°C for 4 hours. DNA was recovered with the Qiagen II Gel Extraction Kit (Qiagen, Valencia, CA) as directed.

PCR products were amplified using GoTaq Hotstart Polymerase(Promega), 1μl of template DNA, and primers amplifying specific DNA regions (Table ). PCR reactions were run on a 2% agarose gel stained with Ethidium Bromide and results visualized with GBox Image Station. Photoshop (Adobe) was used to quantitate band signals.

4.2.13 Chromatin Immunoprecipitation (ChIP) - Magnetic Beads

Thymocytes were harvested from IRC transgenic mice, counted to obtain 1x10^6 cells per sample, resuspended in DMEM-FTOC media (w/o phenol red) with TAT proteins
Rx1d190-TAT, Rx1d190K167A-TAT, or TAT peptide, and plated in twelve-well cell culture plates. Cells were incubated for 30 minutes at 4°C followed by 3.5 hours at 37°C. Cells were harvested and DNA was crosslinked to histones by addition of formaldehyde to a final concentration of 0.37% for 10 minutes at 37°C. Steps and buffers from this point were part of a commercially obtained Magna-ChIP kit (Millipore). Cells were washed in PBS pH 7.4 and Protease Inhibitor Cocktail. Samples were resuspended in Lysis Buffer and sonicated at 30% maximum power for 10 seconds a total of four times using a Sonicator Ultrasonic Processor XL (Qsonica). Samples were centrifuged for 10 minutes at 13,000 rpm at 4°C and supernatants were diluted with ChIP Dilution Buffer with Protease Inhibitor Cocktail. The sample supernatants were incubated with manufacturer's recommended dilutions of precipitating antibodies (Table 4) or control IgG1 antibody (Southern Biotech) as well as 20ul Magna-ChIP beads at 4°C overnight with rotation. The magnetic bead/antibody/histone complex was washed for five minutes each wash in Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Wash Buffer, and TE Buffer. The histone complex was decrosslinked and eluded from the antibody with Elution Buffer incubation for 2hrs at 62°C with rotation. DNA was recovered using DNA binding spin columns included in the Magna ChIP kit as directed.

4.2.14 Quantitative Real Time PCR (qPCR)

Quantitative Real Time PCR was performed on chromatin immunoprecipitation products when subtle differences could not be resolved using gel electrophoresis.
methods. DNA was amplified in triplicate using SYBR® Advantage® qPCR Premix (Clontech, Mountain View, CA). Specific primers designed to CD4 Silencer, Promoter, or Enhancer regions were used (Table). Data was analyzed using MxPro software.
### Table 2 Primers for Site-directed Mutagenesis

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*Amino acid specificity is based on distal Runx1 sequence.*
Table 3 Primers for construction of HDAC1 & SUV39H1 enzyme fusion proteins

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*Italics indicate restriction enzyme location.
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<td>CD4 Promoter</td>
<td>Forward</td>
<td>GACTCCTGAGGCTGGCTTA</td>
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<tr>
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<td>Reverse</td>
<td>GAGTCTTGCTTGCCTTCACAG</td>
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<tr>
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<td>GGGTGAGGATGGTGTC</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GGGCTGGGTCAAAAGGCTT</td>
</tr>
<tr>
<td>Specificity</td>
<td>Dilution/Amount used</td>
<td>Manufacturer</td>
</tr>
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<td>IgG1</td>
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<td>1:200</td>
<td>Cell signaling Technology (Danvers MA)</td>
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<td>SUMO-1</td>
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<td>Cell Signaling Technology</td>
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<tr>
<td>Anti-Histone H3</td>
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<td>Anti-Histone H3 (acetyl K27)</td>
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<td>Anti-Histone H3 (tri-methyl K4)</td>
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<tr>
<td>Specificity*</td>
<td>Primer Orientation</td>
<td>Sequence (5’-3’)</td>
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<td>CD4 Silencer</td>
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<td>GAGGGACCACAAAGGATGTTAT</td>
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<td>Reverse</td>
<td>TCTACGTTGCCCAGGAAATGCAAG</td>
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* Specificities are likely misrepresented or incompletely transcribed.
**Appendix 1. Evaluation of magnetic bead sorting efficiency.**

Bulk thymocytes are harvested from BCL2+ transgenic animals and purified using Miltenyi MACS negative selection. Incubation of the thymocytes with anti-CD4 and anti-CD8 magnetic beads before passage over a magnetic column allows for the retention of CD4+ and CD8+ populations: CD4SP, CD8SP, & DP cells. This strategy results in thymocytes which are CD4−CD8− (DN). FACS staining with anti-CD4 (y-axis) and anti-CD8 (x-axis) confirms the presence of CD4+ and CD8+ cells in the retained fraction (black) while the flow through fraction (red) is devoid of these cells. Lack of substantial CD4 staining in the retained fraction is likely due to the affinity of the magnetic beads for CD4, blocking antibody binding sites.
APPENDIX 2

EVALUATION OF CD4 AND CD8 EXPRESSION IN GFP+ AND GFP- POPULATIONS OF MSCV EMPTY VECTOR INFECTED THYMOCYTE CULTURE

Appendix 2. Evaluation of CD4 and CD8 expression in GFP+ and GFP- populations of MSCV empty vector infected thymocyte culture.

CD4-CD8-(DN) BCL-2+ transgenic thymocytes were infected with MSCV empty vector control, expressing a GFP marker. Cells were first gated for viability. This population was then gated for cells expressing high levels of GFP corresponding with protein expression or the absence of GFP expression, indicating the uninfected cells. FACS staining with anti-CD4 (y-axis) and anti-CD8 (x-axis) confirms the presence of similar amounts of CD4+ and CD8+ cells between the two populations. This data indicates that our retroviral infection strategy has no effect on CD4 or CD8 expression.
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