Molecular Mechanisms in Activation of Latent HIV-1

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Mechanismen bij activering van latente HIV-1

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To my parents, my husband and my daughter

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Chapter 1

General introduction

Human Immunodeficiency Virus Type 1 (HIV-1)

The human immunodeficiency virus type 1 (HIV-1) was discovered in 1983 (1). HIV-1 primarily infects $CD4^+$ T cells, a subset of the immune system's cells, leading to their depletion and resulting in the progressive decay of the immune system or immune deficiency. According to estimates by WHO and UNAIDS, 35 million people were living with HIV-1 globally at the end of 2013.

During the HIV-1 life cycle, virus attaches to the CD4 receptor and co-receptors (CCR5 or CXCR4) on cell membrane of $CD4^+$ T cells and fuses into the $CD4^+$ T cells (2). The HIV double stranded RNA genome is then reverse transcribed to generate the double stranded DNA provirus, which is the main component of the HIV pre-integration complex (PIC) (3). The PIC enters into the nucleus and integrates to the host genome. The HIV-1 promoter or 5'LTR then directs expression of the integrated provirus using the host transcription machinery and the HIV-1 encoded transactivator protein TAT and transcribes viral mRNA and genomic RNA (4). Viral proteins are then translated from viral mRNA and combined with viral genomic RNA to form new virus particles, which bud from the infected cells and mature (Figure 1). The mature virus can bind and infect other $CD4^+$ cells (2, 3).

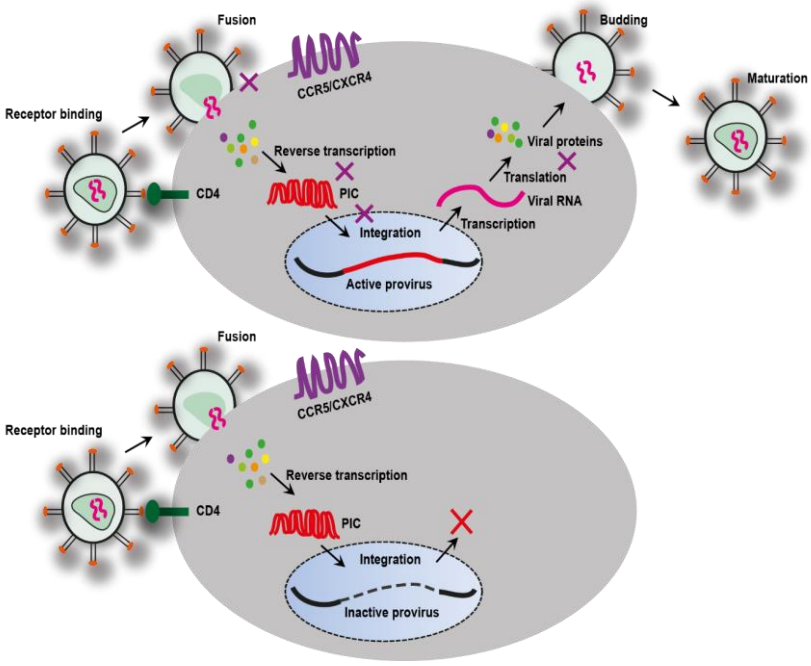

Figure 1. Comparative schematic representation of active and latent HIV-1 replication cycle. In active form, viral DNA integrates into host genome and serves as a transcription template for the synthesis of viral mRNA and genomic RNA. After synthesis of viral proteins, the viral components are assembled together to produce new virions (Top panel). Inactive form of virus integrates into host genome and becomes silenced (Bottom panel).

Combination antiretroviral Therapy (c-ART)

Combination Antiretroviral Therapy (c-ART) stops or interferes with replication of the virus by targeting different stages of the HIV-1's replicative life cycle such as fusion, reverse transcription, integration, proteolysis, or a combination thereof (Figure 1). As a result of successful c-ART, the amount of virus in serum of infected patients is completely suppressed to below detectable levels. This suppression has resulted in an improvement of patient survival (5-7), resulting in HIV-1 infection becoming now managed as a chronic rather than acute disease in countries with access to c-ART. Indeed even in populations with access to c-ART, HIV-1 is not currently curative. Because of this, HIV-1 infected patients must take medication life-long. Lifelong medication causes toxicities and has side effects for the infected patients, including a significantly increased risk of cardiovascular disease (8), atherosclerosis (9), dyslipidemia (10), and insulin resistance (11). Therefore, it is important from a medical, ethical and economic perspective to find a curative therapy for HIV-1. Recently, a significant effort has been put into finding new strategies to eradicate the HIV-1 virus or allow the infected patients to control viral replication in absence of therapy (6, 7).

Post- integration HIV-1 latency

c-ART suppresses viral replication very effectively, such that the virus becomes rapidly undetectable in infected patients by sensitive diagnostic reverse transcription PCR (RT-PCR) tests after a few months of therapy (Figure 2). However, interruption of therapy will result in re-emergence of virus replication and increase in plasma viremia. This re-emergence of virus is due to the presence of a small fraction of HIV-1 infected cells, which are replication competent, but transcriptionally latent (7, 12-16). Latently infected cells persist in the patient despite c-ART treatment. Although transcriptionally silent, this reservoir is fully capable of producing infectious virus, and when therapy is stopped, they can become activated, and support replication of the virus (6). Thus the major barrier towards HIV-1 eradication is the persistence of latent provirus in c-ART treated patients (17, 18).

The common feature of retroviral infection is the integration of viral genomes into the host's genome. Latency is established early during acute infection, likely within days of initial infection. Following infection of the cell, HIV-1 can establish stable latency within resting $CD4^+$ memory T cells, the main reservoir of latent provirus. Although the mechanisms of HIV-1 latency establishment are unclear, one possibility is that HIV-1 infects partially activated $CD4^+$ T cells, which return to a resting memory state (19-22). In this process, as their genome is compacted, the HIV-1 virus, integrated into the genome also becomes silenced. Direct infection of resting memory T cells has also been shown (23-28), although this is thought to be a rare event. The replication-competent latent HIV-1 proviruses that persist in the

genomes of a very small subset of resting memory T cells in infected individuals therefore have a block in transcription (19, 29, 30).

Several different molecular mechanisms have been proposed to be involved in the establishment of HIV-1 post integration latency (31). These include: 1. Transcriptional read-through, where the provirus integrates downstream of an active host gene in the same transcriptional orientation. The host RNA polymerase II displaces essential transcription factors for viral gene expression and may cause read through and splicing out of the virus RNA along with the host gene intronic regions and result in viral silencing (32-35). 2. Another mechanism is transcriptional collision, which occurs when the provirus integrates in the opposite orientation relative to the host gene, leading to collision of the RNA Pol II complexes from the host and viral promoters and early arrest of transcription of both or the weaker promoter (32, 36). 3. Another proposed mechanism for HIV-1 latency when HIV-1 is integrated in opposite orientation of a host promoter is double stranded RNA interference. In this case if both strands of viral DNA are elongated, double-stranded RNA can form, which can cause Dicer-mediated silencing (37-39). 4. Micro RNAs (miRNAs) have been also suggested to be involved in establishing latency via down regulation of HIV-1 transcription or recruiting HDACs on HIV-1 LTR (40, 41). 5. Integration of the virus near silenced or heterochromatic genomic regions is another proposed mechanism for virus silencing that can occur when silent chromatin spreads over onto adjacent genomic regions to cover and transcriptionally silence the virus promoter (41). Indeed, site and orientation of integration have been described to be important for latency establishment (7). HIV-1 latency is the result of multiple molecular mechanisms, which contribute to the transcriptional silencing of the HIV-1 promoter in different integrated genomic locations.

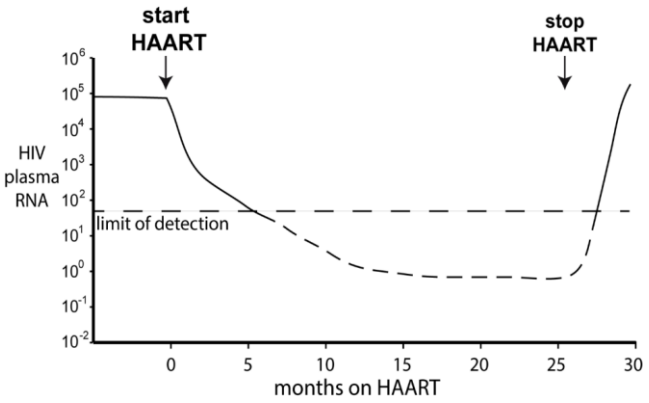

Figure 2. Latent HIV-1 persists in memory CD4$^+$ T cells despite HAART. Upon treatment interruption amount of HIV-1 RNA in plasma of HIV-infected patient suddenly goes up because of activation of the latent reservoir.

HIV-1 transcription

- Basal transcription

The 5'-Long Terminal Repeat (LTR) of HIV-1 functions as the promoter of HIV-1, which contains multiple consensus binding sites for cellular transcription factors and their co-factors, including both activators and repressors (7, 42) (Figure 3). The balance between the binding and activity of these positive and negative transcription factors lead either to the establishment of a productive infection or entry into a repressed latent state (Figure 3).

In the latent provirus, chromatin structure of the 5'-LTR is characterized by the presence of two positioned nucleosomes, Nuc-0 and Nuc-1 and the linker DNA region in between, hyper sensitive site (DHS-1), which is hypersensitive to digestion with nucleases and restriction enzymes (Figure 3) (42-46). The presence of Nuc-0, upstream of the modulatory region, and Nuc-1, immediately downstream of transcription start site, is the hallmark of a repressed 5'-LTR. Nuc-1 is highly repressive to transcription and becomes rapidly and specifically disrupted upon activation (44-46).

Consensus binding sites for many of cellular transcriptional activators, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), Nuclear factor of activated T-cells (NFAT), Lymphoid enhancer-binding factor 1 (LEF1) and specificity protein 1 (SP-1) are scattered on the DHS-1 region of the HIV-1 LTR (7, 45-47). The latent reservoir is thought to be largely formed as a consequence of infection of partially activated $CD4^+$ T cells that return back to a resting state. During this process a number of activators which are essential for HIV-1 expression, such as NF-κB and NFAT transcription factors, are sequestered into the cytoplasm, and unable to activate the HIV-1 transcription in the nucleus, while chromatin condensation further contributes to repression of HIV-1 transcription (48-50). Thus, many activating transcription factors, in resting $CD4^+$ T cells, are recruited to the nucleus from the cytoplasm only after cellular activation (49, 51). On the other hand, several transcriptional repressors including Yin Yang 1 (YY1), Late SV40 factor (LSF), C-REPEAT/DRE BINDING FACTOR 1 (CBF1), and p50 homodimers have been shown to bind to the LTR and suppress HIV-1 transcription (47, 52-55). The balance and local activity of these transcription activators and repressors, and their co-factors including both co-activators and co-repressors, controls expression of the HIV-1 genome.

- TAT-dependent transcription elongation

Following recruitment and binding of transcription factors such as NF-κB in the transcription initiation phase, RNA polymerase II is positioned on the transcription start site (TSS) of the HIV-1 promoter. HIV-1 encodes a strong transactivator protein, TAT. The HIV-1 TAT plays a major role in the elongation phase of transcription. TAT-dependent elongation phase, which promotes much higher levels of HIV-1 transcription (99% of the transcripts are transcribed to their full length) compared to the initiation phase, where high levels of the transcripts

terminate prematurely and are short (7, 18). This premature transcript termination is due to the presence of the negative elongation factors (NELF) and DRB Sensitivity Inducing Factor (DSIF) (56-59). TAT, when expressed, binds to the nascent structured TAR RNA sequence located at the 5' HIV-1 LTR, and causes recruitment of Super Elongation Complex (SEC) to the HIV-1 transcripts (60). The positive transcription elongation complex (P-TEFb) is the key component of SEC and is composed of CyclinT1 and Cyclin-dependent kinase 9 (CDK9) (Luo, 2012). CDK9, a kinase in the P-TEFb complex, phosphorylates NELF and the carboxy-terminal domain of RNA pol II, removes NELF and increases RNA pol II processivity (61, 62). TAT, which is itself subject to extensive post-translational modifications allows the recruitment of additional transcriptional co-activators, such as acetyl transferases and ATP-dependent chromatin-remodeling complexes to activate transcription (60).

P-TEFb Levels in the cells are controlled and promote the switch to productive elongation. When the components of P-TEFb, CyclinT1 and CDK9, interact with 7SK small nuclear ribonucleoprotein complex (7SK snRNP), which contains the inhibitory molecules HEXIM1 or HEXIM2, it is inactive because of inhibition of the kinase activity of CDK9 (63). In actived $CD4^+$ T cells, P-TEFb interacts with the 7SK snRNP complex and TAT activity cause its release (63). In resting $CD4^+$ T cells, the levels of 7SK snRNP are very low, thus the main P-TEFb regulatory mechanism in these cells is likely to be the posttranslational regulation of CyclinT1 levels (64, 65). In these cells, reactivation of HIV-1 happens upon CycT1 accumulation, activation of CDK9, assembly into the 7SK snRNP complex and TAT mediated recruitment to the HIV-1 LTR.

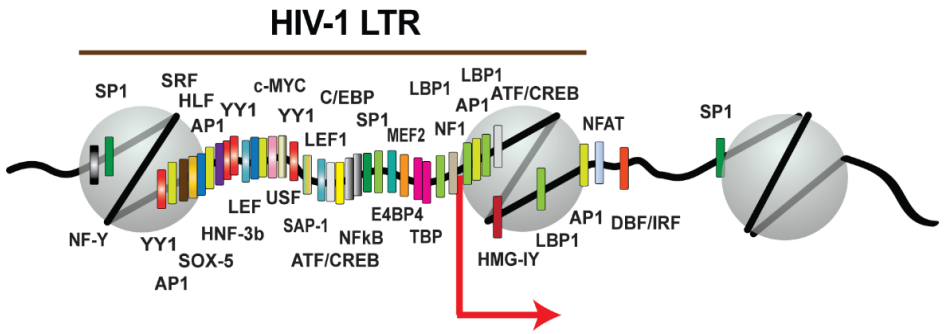

Figure 3. Schematic representation of chromatin structure on the HIV-1 LTR. Chromatin structure of the 5'-LTR is characterized by the presence of two positioned nucleosomes, Nuc-0 and Nuc-1 and the linker DNA region in between, DHS-1. Color bars represent the transcription binding sites on the HIV-1 promoter.

Cellular pathways and targets in HIV-1 latency

Different molecular mechanisms and signaling pathways have been shown to play a role in regulating HIV-1 transcription. The identity of these pathways and the downstream molecule

involved in transcriptionally relaying the signals can provide important insights into mechanisms that can be targeted to induce HIV-1 replication and purge the latent reservoir from infected patients (Figure 4) (18). Below I will discuss the various signaling pathways, and molecular mechanisms that have been implicated in regulation of HIV-1 transcription.

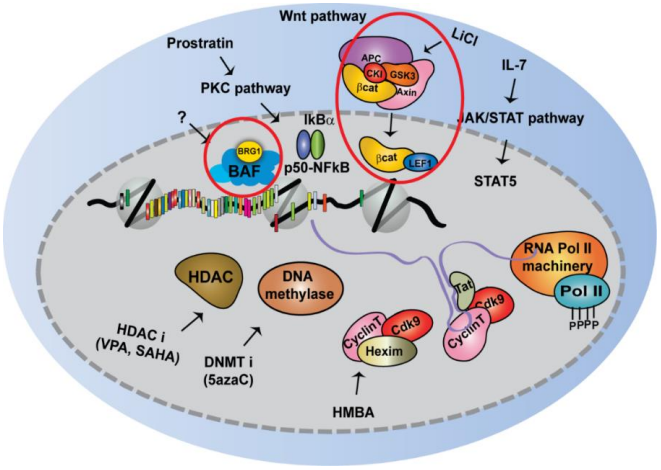

Figure 4. Schematic representation of cellular pathways and targets in HIV-1 latency. Different cellular pathways and targets which are involved in HIV-1 latency are shown. Chapter 2 describes the role of the BAF complex in HIV-1 latency (red circle). Chapter 3 describes the role of the Wnt pathway in activating HIV-1 latency (red circle).

- Targeting the protein kinase C pathway (PKC pathway) for activation activates latent HIV-1

One of the major pathways involved in activation of the HIV-1 promoter is the PKC pathway (66, 67). It has been shown, the transcription factors NF-κB, NFAT and AP-1 are downstream molecular effectors of the PKC pathway and they have several consensus binding sites within the HIV-1 promoter, to which they have been shown to bind (68-70). The inactive form of the NF-κB is the homodimer p50/p50, which binds to the NF-κB binding sites on the HIV-1 promoter in latent and inactive transcriptional states (68). The active form of the transcription factor, the heterodimer p65/p50, p65, is sequestered in the cytoplasm and upon PKC activation, the p65/p50 heterodimer is translocated to the nucleus, where it replaces the inactive dimers (68). Cytoplasmic NFAT, also translocates into the nucleus as a result of calcium/calcineurine induction following PKC activation and binds to its consensus binding sites adjacent to and overlapping the NF-κB sites on the 5'-LTR (69). Both active NF-κB and NFAT recruit histone acetyltransferases, such as p300/CBP, resulting in acetylation of histone tails and opening of the LTR chromatin structure (70, 71). PKC agonists, including phorbol esters (prostratin), diacylglycerol and ingenols (hexanoate), are able to activate latent HIV-1 in latent cell models as well as primary cells from HIV-1 patients (72-74). However, many small

molecule PKC agonists, in addition to activating HIV-1 latency, also result in general T cell activation, and as a result have limited clinical applicability.

- Inhibition of Bromo-domain-containing (BET) factors activates latent HIV-1

In eukaryotic cells, recruitment of P-TEFb to the genes is mediated by BET proteins, in particular the BET factor Bromodomain-containing protein 4 (BRD4) (75, 76). BRD4 acts as a repressor of HIV-1 expression, because it competes with TAT for binding to P-TEFb (77). Therefore, inhibiting BRD4 by small molecules causes increased TAT activity and HIV-1 transcription (78). Recently, TAT-independent activation of latent clones by small molecule BET inhibition was also shown, suggesting other BET proteins may be involved in TAT-independent repression of the HIV-1 LTR (79). Recent studies demonstrated the effectiveness of JQ1 and IBET-151 BET inhibitors in reactivation of latent HIV-1 in cell lines and primary cell models of HIV-1 latency (80-82).

- Inhibition of DNA methyltransferases (DNMTs) activates latent HIV-1

DNA methylation is a mechanism of transcriptional repression initiated by methylation of DNA by DNA methyltransferases at CpG dinucleotides. The methyl-CpG-binding domain protein (MBD2) was identified as a transcriptional repressor which is involved in HIV-1 latency (83). Further investigations determined that MBD2 was associated with the CpG islands and repressed HIV-1 transcription via the recruitment of the Nucleosome Remodeling Deacetylase complex (NURD complex) (83). Inhibition of DNA methylation with the small molecule DNMT inhibitor 5-aza-20deoxycytidine (aza-CdR) compromised repression at the HIV-1 promoter and prevented the recruitment of MBD2 and HDACs and synergized with NF-κB activators to promote a dramatic increase in viral gene expression (83). This CpG methylation pattern was responsible for the maintenance but not establishment of HIV-1 latency (84). These studies has been performed using T cell line model systems for latency. However studies of the effect of DNA methylation in $CD4^+$ T cells obtained from c-ART treated patients indicate that CpG methylation at the HIV-1 LTR is a rare event (84). Therefore the relevance of this modification for HIV -1 latency remains unclear.

- Inhibition of repressive Histone methyltransferases (HMTs) activates latent HIV-1

Histone methylation is another mechanism that has been shown to be involved in transcriptional repression of the HIV-1 LTR. In particular, methylation of Histone 3 (H3) at position lysine 9 and lysine 27 is generally associated with transcriptional repression (85, 86). The G9a and SUV39H1, are two histone lysine methyltransferases (HKMT) that catalyze di-

and tri-methylation of H3K9. The latent HIV-1 LTR is characterized by the presence of these repressive marks, and these two enzymes have been shown to cause repression in latently infected cells from HIV-1 patients (87, 88). H3K27 trimethylation of histones is catalyzed by Histone-lysine N-methyltransferase EZH2, an integral subunit of the Polycomb group repressive complex 2 (PRC2). This enzyme was found to cause transcriptional repression of the HIV-1 promoter (85).

HMT inhibitors have been tested as therapeutic inducers of HIV-1 transcription. In particular, inhibitors targeting the activity of SUV39H1 and G9a, have been tested for their ability to reactivation of latent HIV-1. HMTi's such as Chaetocin, a specific inhibitor of SUV39H1, and BIX-01294 the G9a inhibitor both result in induction of chromatin relaxation and are able to reactive HIV-1 in resting memory $CD4^+$ T cells from HAART-treated patients (89, 90). The main barrier against these compounds is toxicity and their side effects, and further studies are needed to identify additional safer HMTi's for therapeutic use.

- Targeting ATP-dependent chromatin remodelers (BAF) activates latent HIV-1

SWI/SNF is one of four family of mammalian ATP-dependent chromatin remodeling complexes, which contains either Brahma (BRM) or BRG1 as a catalytic subunit (91-96). Different studies have reported that TAT, the potent HIV-1 transactivator, recruits the SWI/SNF chromatin-remodeling complex to the HIV-1 LTR to activate transcription (97-101). It was also shown that SWI/SNF has an additional TAT-independent role in HIV-1 transcription elongation (102). INI-1 (hSNF5 component of SWI/SNF) was identified because of its interaction with the HIV-1 Integrase protein IN (103), and was shown to restrict the early steps of HIV-1 infection (104) and repress basal LTR activity (105). The interaction between the HIV-1 Integrase protein (IN) and SWI/SNF was also shown to be necessary for integration of the HIV-1 PIC into stable nucleosomes (106). Therefore the SWI/SNF chromatin remodeling complex is involved in multiple steps of the HIV-1 life cycle.

Two biochemically distinct SWI/SNF complexes with distinct functions have been described which are called BAF and PBAF. While they share most subunits, the PBAF complex can be distinguished by the exclusive presence of BAF180, BAF200, SAYP, and BRD7 (107-110), and BAF contains the BAF-specific subunit BAF250, but lacks PBAF-specific subunits (111-113). BAF- or PBAF-specific subunits have distinct roles in cell cycle control and mitosis (111, 114), and studies suggest that BAF and PBAF perform distinct functions in transcription regulation (115, 116, 117). Given the role of the SWI/SNF subunit INI-1 in HIV-1 transcriptional repression, in chapter 2 we examined the mechanistic role of SWI/SNF in the establishment and maintenance of HIV-1 latency. We find that depletion of BAF-specific subunits from latent HIV-1 infected cells resulted in re-activation of latent HIV-1. Thus, the biochemically distinct BAF complex is involved in repression of HIV-1 in latent HIV-1 infected $CD4^+$ T cell lines.

Interestingly, comparison of the predicted nucleosome affinity of the HIV-1 LTR sequence to the known *in vivo* latent nucleosome structure indicated that the LTR nucleosomes are not present *in vivo* in thermodynamically optimal positions. We find that BAF actively moves a preferred DHS-1 nucleosome over to less favorable sequences over the Nuc-1 region in order to position Nuc-1 and repress HIV-1 transcription, leading to the establishment and maintenance of HIV-1 latency (97). Upon HIV-1 activation, BAF is displaced from the latent HIV-1 LTR, and the biochemically distinct PBAF complex is recruited by TAT to the HIV-1 LTR to activate transcription. Given its function in Nuc-1 positioning and transcription repression at the HIV-1 LTR, the ATP-dependent chromatin remodeling complex BAF is a promising candidate for therapeutic inhibition in HIV-1 re-activation approaches.

Recently, small molecule inhibitors of the BAF complex have been identified in a screen for activation of Bmi1, a BAF-repressed gene in ES cells (118). Future studies are needed to investigate whether these molecules are capable of re-activating latent HIV-1.

- Targeting the Wnt pathway for activation activates latent HIV-1

The Wnt pathway is a highly conserved signaling pathway controlling a variety of biological processes, including normal T cell development (119). In the absence of Wnt ligands, β-catenin, the key mediator of Wnt signaling, is subsequently phosphorylated, ubiquitinated, and then proteosomally degraded by the cytoplasmic β-catenin destruction complex, and TCF/LEF factors bind to and repress Wnt target genes in the absence of β-cateninin the nucleus (120). Upon binding of Wnt ligands to the receptors on cell surface, ubiquitination of phosphorylated β-catenin is blocked, newly synthesized β-catenin accumulates and is transported to the nucleus where it complexes with LEF/TCF transcription factors to activate Wnt target genes (119, 120).

Lymphoid enhancing factor 1 (LEF1), one of the downstream molecular effectors of Wnt pathway, has several consensus binding sites on the HIV-1 LTR. LEF1 was identified after purification from Jurkat T cells by binding to its sites on the HIV-1 LTR (121, 122). It has been shown that LEF1 bound to the HIV-1 LTR, and countered nucleosome repression in in vitro reconstituted HIV-1 chromatin (122). Implicating Wnt signaling in HIV-1 transcription activation, it was shown that siRNA depletion of AXIN1, one of the components of the β-catenin destruction complex, resulted in HIV-1 transcription activation (123).

On the other hand, several studies have also shown that in astrocytes, β-catenin/Wnt may play a repressive role in HIV-1 transcription and replication (124-127). The primary cell targets for productive HIV-1 replication in the brain are monocyte/macrophages and microglia. Astrocytes constitute 40 to 70% of brain cells and perform critical functions in central nervous system. Recent data suggest that astrocytes are an important target for HIV-1 latency and under certain conditions support productive HIV-1 replication. Suppression of HIV-1

replication by β-catenin/Wnt was mediated by the activity of TCF4 (128), suggested to overcome the LEF-driven activation during HIV-1 infection in astrocytes, where it could represent a latency driving mechanism (126, 127). It was shown that treatment with Lithium, the GSK3β inhibitor, inhibit HIV-1 replication in peripheral blood mononuclear cells (129).

In chapter 3, we examined the effect of induction of the Wnt pathway on transcription of latent HIV-1 infected $CD4^+$ T cells. We found that activation of the Wnt pathway by natural ligands (Wnt3A-Rspondin), chemical inhibitors (Lithium) or exogenous expression of a constitutively active β-catenin activates latent HIV-1. Wnt activation of latent HIV-1 resulted in recruitment of the molecular effectors β-catenin and LEF1 to the HIV-1 LTR. High resolution MNase nucleosomal mapping assays showed the drastic nucleosome remodeling of the latent HIV-1 LTR in response to Wnt stimulation. Synergistic activation of latent HIV-1 LTR was achieved by combining Wnt pathway activation with HDAC inhibitors, a class of drugs currently under clinical investigation for activation of latent HIV-1. Finally, therapeutically relevant doses of the Wnt agonist Lithium activated latent HIV-1 from *ex-vivo* infected primary $CD4^+$ T cells. Our data suggest that activation of Wnt pathway may be an attractive strategy in a combinatorial therapy for activation of latent HIV-1 infected cells followed by their elimination in the presence of c-ART.

T-cell factor 1 (TCF1) and lymphoid enhancing factor 1 (LEF1) in CD4+ T cells leukemia

The molecular effectors of the Wnt pathway are TCF/LEF factors (130-134), of which there are four members in vertebrates, LEF1, TCF1, TCF3 and TCF4 (133, 135-137). TCF/LEF factors, because they share high homology in their highly conserved HMG box DNA binding domains are thought to be largely functionally redundant and tissue specifically expressed (138). Interestingly, in T cells, the two TCF/LEF members, TCF1 and LEF1 are co-expressed, and genetic studies in mice showed that they play both redundant and opposing roles in T cell development (132, 139-141); TCF1 functions as a tumor suppressor gene, while in the absence of TCF1, LEF1 functions as a proto-oncogene.

Studies on T cell development have described that, deregulations of Wnt signaling is involved in formation of different leukemias including acute lymphoblastic leukemia (ALL) (142, 143)). In T- ALL cell lines, epigenetic silencing (such as methylation) of Wnt inhibitors, result in aberrant nuclear localization of β-catenin and activation of Wnt target genes (144). In T-ALL $CD4^+$ T cell lines, Jurkat and SupT1 illegitimate partial activation of the Wnt pathway has been reported (144-146). However, in these systems, Wnt pathway components are intact and can be induced by Wnt stimulation (146, 147).

In chapter 4, we explored the molecular mechanisms behind the functional specialization observed in vivo between TCF1 and LEF1 in leukemia. We found that, TCF1 and LEF1 have distinct isoform expression profiles in T-ALL leukemic cells and $CD4^+$ primary T cells; while TCF1 is expressed mainly in its dominant negative form, LEF1 is predominantly expressed in its Wnt-responsive form. One mechanism by which TCF1 and LEF1 can exert their distinct functions is if they bind to DNA and regulate transcription in the context of different protein complexes, using distinct co-factors. We performed immunoprecipitation coupled with mass spectrometry analysis of LEF1 and TCF1 in the presence or absence of Wnt signaling to identify the Wnt- responsive and Wnt-dependent TCF1 and LEF1 complexes. As expected we found shared but also unique cofactors for TCF1 and LEF1 in the presence or absence of Wnt signaling in SupT1 cells. We next determined the TCF1-specific and LEF1-specific regulated genes in the presence or absence of Wnt stimulation using siRNA depletion of each factor. Indeed, TCF1 and LEF1 display distinct target gene profiles. Focusing on two of the novel Wnt target genes identified by RNA sequencing we performed chromatin immunoprecipitation analysis, and showed that LEF1 and TCF1 are differentially recruited to the regulatory regions of these targets in the presence or absence of Wnt. Our data provides mechanistic evidence for the functionally distinct roles played by TCF1 and LEF1 in T cell leukemia.

- Inhibition of Histone deacetylases (HDACs) activates latent HIV

Histone deacetylases (HDACs) are the main players among the cellular factors that maintain HIV-1 in a transcriptionally silent state. Histone acetyl transferases (HATs) add acetyl group to the histone tails resulting in more open chromatin structure, and in this way facilitate transcription. HDACs remove the acetyl groups from the histone tails, resulting in the generation of more closed and rigid chromatin, causing transcriptional repression. Several HDACs including HDAC1, HDAC2, HDAC3, and HDAC4 have been shown to be recruited to the HIV-1 promoter via interaction with different transcription factors. This recruitment results in repression of HIV-1 transcription (7, 18, 148-151). HDAC1 is recruited to the Nuc-1 region of the HIV-1 LTR via cooperative binding of the transcription factors Late SV40 factor (LSF) and Yin Yang 1 (YY1). The repressive p50/p50 homodimers bound to the NF-κB sequences to recruit HDAC1 to the HIV-1 LTR (151). C promoter binding factor 1 (CBF-1) the downstream molecular effector of the Notch pathway, also has been shown to recruit HDACs to the latent HIV-1 LTR (55).

Therefore, using HDAC inhibitors to block histone deacetylase activity, would create a less-repressive chromatin state and would facilitate HIV-1 transcription. Indeed small molecule inhibition of HDACs is currently the most successful avenue in approaches in therapeutics for activation of latent HIV-1. HDAC inhibition was shown to disrupt Nuc-1 and increase HIV-1 transcription (152-154). Valproic acid (VPA), an HDACi used in epilepsy and bipolar disorders,

was the first compound tested in the latency reactivation in HIV-1 infected suppressed patients. Very early studies of this compound, showed a significant decrease in the size of reservoirs after VPA treatment coupled to HAART intensification (155), however further studies failed to replicate this finding (156, 157). Recent studies in this field showed stronger effects with the HDAC inhibitors Vorinostat, Givinostat, Droxinostat, Panobinostat, Romidepsin and Entinostat both *in vitro* and *ex vivo* (158, 159). Importantly, vorinostat (suberoylanilide hydroxamic acid/SAHA) was shown to induce HIV-1 transcription from latently infected cells obtained from HAART treated patients (160). A clinical study in which HIV-1 patients were treated with vorinostat showed that vorinostat treatment induced increase in HIV-1 cellular associated RNA only after the first exposure (160).

Therefore, HDAC inhibition is a promising avenue in HIV-1 therapy, due to activation of different HIV-1 subtypes without inducing massive T cell activation.

Role of HDAC7 in B lymphocyte differentiation

Class II HDACs, which include HDACs 4, 5, 7 and 9, unlike class I HDACs (described above in repression of HIV-1) are expressed in a tissue specific manner (161-166) and have been shown to play an important role in processes of development and differentiation (167-169). A distinguishing feature of Class II HDACs that sets them apart from Class I HDACs is that in addition to their catalytic C-terminus, they possess an N-terminal domain, which contains highly conserved Serine residues. When unphosphorylated at these serine residues, Class II HDACs are localized in the nucleus, where they interact with sequence specific transcription factors to repress the expression of their target genes. Upon signal-dependent phosphorylation, Class II HDACs are shuttled to the cytoplasm resulting in de-repression of their target genes in the nucleus.

In the immune system, the Class II HDAC, HDAC7 is highly expressed in thymocytes (170). HDAC7 was found to be involved in the regulation of expression of a number of genes involved in both positive and negative selection in the developing thymocytes, including *Nur77* (170, 171). In T cells HDAC7 has also been shown to be regulated in a signal-dependent manner (172). Phosphorylation of HDAC7 leads to its nuclear export, and to the derepression of its gene targets in T cells (170, 172, 173). Thus HDAC7, regulated by nucleo-cytoplasmic shuttling plays a critical role in T cell development by repressing its downstream target genes.

In chapter 5, we found that HDAC7 is also highly expressed in B lymphocytes and plays an important role in repressing the expression of lineage inappropriate target genes. While highly expressed in pre-B cells, HDAC7 expression was dramatically down-regulated during differentiation into the macrophage lineage. Re-expression of HDAC7 interfered with the gene transcriptional program of macrophages during cell differentiation. HDAC7 caused a block in induction of key genes for macrophage function, such as immune, inflammatory, and

defense response, cellular response to infections, positive regulation of cytokines production, and phagocytosis. Chromatin immunoprecipitation experiments indicated that HDAC7 was recruited to the regulatory regions of MEF2 target genes by specific interaction with the transcription factor MEF2C in pre-B cells. Therefore, HDAC7 repressed expression of MEF2 target genes critical for macrophage function. Thus, HDAC7 is essential in transcriptional repression of undesirable genes in B cells.

HIV-1 subtypes and diversity

HIV-1 displays extreme genetic diversity, generated by two viral mechanisms. One mechanism is mutations in viral genome during replication, and second mechanism is mutations that occur because of recombination between viral genome. There are four groups of HIV-1: group M (Main or Major) (174, 175), group O (Outlier) (176, 177), group N (Non-M and Non-O) (178, 179), and finally group P (180, 181). HIV-1 group M can be subdivided into nine subtypes, A, B, C, D, F, G, H, J, and K, based on their phylogenetic relatedness (182). Several studies on proviral transcriptional latency properties of different HIV-1 subtypes have shown that their LTRs have unique assemblies of transcription factor binding sites. Using constructed isogenic recombinant viral genomes with the subtype-specific promoters inserted in the common backbone of the subtype B provided an assay to investigate HIV-1 proviral latency in T cell lines (183). Although, the potential contribution of distinct subtypes to latency establishment remains incompletely understood, one study showed that there are no gross differences among the subtypes in the initial latency levels or activation response to tumor necrosis factor α (TNFα). The exception was subtype AE that exhibited an increased level of basal transcription with a reduced TNFα response, because of the presence of a GA-binding protein (GABP) instead of NF-κB binding site in the LTR. Further studies are necessary to investigate the potential for latency establishment and activation to different LRAs.

Model systems for the study of HIV-1 latency

Both *in vivo* and *in vitro* models have been used to study the mechanisms of HIV-1 latency. *In vivo* models developed in non-human primates have provided valuable information. Although, these SIV Macaque models could play an important role in characterization of latent reservoirs and eradication of HIV-1, there are several limitations to the use of these models. First, for ethical reasons it is difficult to perform experiments of primates. Second, SIV, the main virus used in studies involving nonhuman primates, is different from HIV-1 in its promoter activity, propensity for latency establishment, profile of transcription factor binding

site, and re-activation. Therefore the relevance of this model system to HIV-1 latency in humans is limited.

Another model system for the study of HIV-1 latency is the humanized mouse model, which contain an immune system made from hematopoietic stem cells (HSCs) obtained from human fetal liver (184). HIV-1 infection in humanized mice differs from HIV-1 infection in humans in a number of important respects including a lower viral load, and a near absence of antibody-mediated immune responses, and therefore there is no pre-existing selective pressure on the envelope. Importantly, although the immune system is humanized, the liver, which metabolized putative latency reversal drugs or c-ART, and other organs such as brain, which constitutes a main reservoir in humans, is not human. Therefore, this model too has limitations in the study of HIV-1 reservoirs and latency.

A useful *in vitro* cell line model system that reflects the state of HIV-1 latency was established by Verdin and colleauges. They generated a number of latently infected Jurkat cell line-derived clones carrying single infection cycle latent HIV-1 in single integration sites, and *GFP* or *luciferase* reporter in place of *Nef* as a measure of viral transcriptional activity (J-Lat) (185, 186). J-Lat cells have been used in numerous molecular studies in the area of viral latency. In contrast to the other cell models, where have mutations in the HIV-1 *TAT* gene or the TAR element, the J-Lat cell model contains wild-type *TAT.* A major advantage of these latent HIV-1 model system is that it yield itself to large scale experiments required to probe the molecular determinants of HIV-1 latency such as chromatin immunoprecipitation, high resolution MNase mapping, immunoprecipitation-mass spectrometry, etc. (187). While these transformed cell lines are useful in the molecular characterization of HIV-1 latency, an important limitation of the model is that, in patients latent HIV-1 is primarily found in central memory and transitional memory $CD4^+$ T cells (188).

Several primary $CD4^+$ T cell model systems have also been established to study HIV-1 latency (189). These are generated either from direct spin infection of resting memory $CD4^+$ T cells (28, 190), or by infection of naïve $CD4^+$T cells (191, 192) that have been activated but are in the process of entering a memory state due to removal of the T cell activating signal. This model generates latent infections in cells, which are thought to resemble $CD4^+$ central memory T cells. While these primary models of latency maybe more relevant to the latent HIV-1 in patients, the system is very inefficient in that a small percentage of the cells are infected with virus. In addition, primary T cell cultures are limited in that they can be maintained for only a few weeks. Therefore the primary model systems of latency do not yield themselves to molecular analysis that requires a lot of cells and long-term analysis (189). They are mainly used to confirm mechanisms identified using the cell line models described above.

Recently, to evaluate the effect of anti-latency reagents on latent infection *in vivo*, material from HIV-1 infected patients receiving stable c-ART were used in different studies (14, 19, 193-195). Purified populations of resting $CD4^+$ T cells can be obtained from patients on suppressive therapy by continuous-flow leukopheresis followed by purification of $CD4^+$ T cells. Cells can then be cultured and treated with various candidate molecules, and expression of cell associated HIV-1 RNA can be detected by sensitive qRT-PCR assays.

Combinatorial approaches to activate latent HIV-1

In order to eradicate the HIV-1 virus from infected patients, it is important to unravel the complex molecular nature of HIV-1 latency and identify the key molecular mechanisms responsible for establishment and maintenance of HIV-1 latency. Identifying the responsible mechanisms, the signaling pathways and the molecules involved in silencing the HIV-1 virus will be necessary to devise strategies to reverse HIV-1 latency and purge replication competent virus from the latent reservoir. This so called “shock and kill” approach for purging the latent HIV-1 infected reservoir consists of two parts. The goal is that the “shock” or activation of latent HIV-1 would cause sufficient expression of viral RNA and viral proteins to make the re-activated cell recognizable to the host immune system, and to allow for the “kill”, or elimination of the re-activated cell as a result of viral cytopathic effects and host immune responses (196-199). The continuation of c-ART treatment during latency reversal or activation of latent HIV-1 would ensure the prevention of new rounds of HIV-1 infection and replication.

Better understanding of the diverse molecular mechanisms involved in HIV-1 latency is aimed at enhancing strategies to accomplish robust re-activation of latenly infected cells. Many factors can influence the effectiveness of individual anti-latency reagents in activating latent HIV-1. These include the site of virus integration (200), differences in promoter activity among different HIV-1 subtypes (183, 201, 202), and the specific biological features of each cellular reservoir (189). To this end, and because many mechanisms are involved in HIV-1 latency, a combinations of anti-latency reagents targeting different pathways could more efficiently induce the expression of replication-competent proviral genomes within biologically diverse cellular reservoirs *in vivo.* Recent studies have shown that, the combination of HIV-1 transcription activators and inhibition of HIV-1 repressors at the same time may be the most effective way in HIV-1 reactivation (203-206). These studies indicate that the use of multiple latency reversing compounds can result in additive or synergistic activation of HIV-1 transcription (74, 207-209). Under conditions where individual compounds synergistically work together, lower concentrations of each compound would be necessary to induce HIV-1 activation. This is an important therapeutic consideration because the toxic side effects and pleiotropic consequence of each molecule on gene expression would in this way be diminished. In addition, combinatorial treatment can provide a level of specificity for

activation of the HIV-1 LTR, again limiting the pleiotropic effects on gene expression. Therefore, to achieve optimal latency reversal it is critical to identify and target the functionally important factors involved in latency and to identify compounds, which specifically interfere with their function in order to activate HIV-1.

References

1. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220:868-871.
2. Cann AJ, Karn J. 1989. Molecular biology of HIV: new insights into the virus life-cycle. Aids 3 Suppl 1:S19-34.
3. Buchbinder A. 1990. Virology of the human immunodeficiency virus type 1. Ear, nose, & throat journal 69:376, 379-384.
4. McCutchan JA. 1990. Virology, immunology, and clinical course of HIV infection. Journal of consulting and clinical psychology 58:5-12.
5. Pau AK, George JM. 2014. Antiretroviral therapy: current drugs. Infectious disease clinics of North America 28:371-402.
6. Margolis DM. 2014. How Might We Cure HIV? Current infectious disease reports 16:392.
7. Ruelas DS, Greene WC. 2013. An integrated overview of HIV-1 latency. Cell 155:519-529.
8. Mendes L, Silva D, Miranda C, Sa J, Duque L, Duarte N, Brito P, Bernardino L, Pocas J. 2014. Impact of HIV infection on cardiac deformation. Revista portuguesa de cardiologia : orgao oficial da Sociedade Portuguesa de Cardiologia = Portuguese journal of cardiology : an official journal of the Portuguese Society of Cardiology 33:501-509.
9. Pirs M, Erzen B, Sabovic M, Karner P, Vidmar L, Poljak M, Jug B, Mikac M, Tomazic J. 2014. Early atherosclerosis in HIV-infected patients below the age of 55 years: Slovenian national study. Wiener klinische Wochenschrift 126:263-269.
10. Leyes P, Martinez E, Larrousse M, Cofan M, Trabal J, Perez-Heras AM, Forga MT, Ros E. 2014. Effects of ezetimibe on cholesterol metabolism in HIV-infected patients with protease inhibitor-associated dyslipidemia: a single-arm intervention trial. BMC infectious diseases 14:497.
11. Araujo S, Banon S, Machuca I, Moreno A, Perez-Elias MJ, Casado JL. 2014. Prevalence of insulin resistance and risk of diabetes mellitus in HIV-infected patients receiving current antiretroviral drugs. European journal of endocrinology / European Federation of Endocrine Societies 171:545-554.
12. Chun TW, Finzi D, Margolick J, Chadwick K, Schwartz D, Siliciano RF. 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nature medicine 1:1284-1290.
13. Wong JK, Hezareh M, Gunthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 278:1291-1295.
14. Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, Quinn TC, Chadwick K, Margolick J, Brookmeyer R, Gallant J, Markowitz M, Ho DD, Richman DD, Siliciano RF. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science 278:1295-1300.
15. Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, Baseler M, Lloyd AL, Nowak MA, Fauci AS. 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proceedings of the National Academy of Sciences of the United States of America 94:13193-13197.
16. Lori F, Jessen H, Lieberman J, Finzi D, Rosenberg E, Tinelli C, Walker B, Siliciano RF, Lisziewicz J. 1999. Treatment of human immunodeficiency virus infection with hydroxyurea, didanosine, and a protease inhibitor before seroconversion is associated with normalized immune parameters and limited viral reservoir. The Journal of infectious diseases 180:1827-1832.
17. Mahmoudi T. 2012. The BAF complex and HIV latency. Transcription 3:171-176.
18. Mbonye U, Karn J. 2014. Transcriptional control of HIV latency: cellular signaling pathways, epigenetics, happenstance and the hope for a cure. Virology 454-455:328-339.
19. Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, Hermankova M, Chadwick K, Margolick J, Quinn TC, Kuo YH, Brookmeyer R, Zeiger MA, Barditch-Crovo P, Siliciano RF. 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature 387:183-188.
20. Lassen KG, Bailey JR, Siliciano RF. 2004. Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting CD4+ T cells in vivo. Journal of virology 78:9105-9114.
21. Zhou Y, Zhang H, Siliciano JD, Siliciano RF. 2005. Kinetics of human immunodeficiency virus type 1 decay following entry into resting CD4+ T cells. Journal of virology 79:2199-2210.
22. Pierson TC, Zhou Y, Kieffer TL, Ruff CT, Buck C, Siliciano RF. 2002. Molecular characterization of preintegration latency in human immunodeficiency virus type 1 infection. Journal of virology 76:8518-8531.
23. Eckstein DA, Penn ML, Korin YD, Scripture-Adams DD, Zack JA, Kreisberg JF, Roederer M, Sherman MP, Chin PS, Goldsmith MA. 2001. HIV-1 actively replicates in naive CD4(+) T cells residing within human lymphoid tissues. Immunity 15:671-682.

24. Wightman F, Solomon A, Khoury G, Green JA, Gray L, Gorry PR, Ho YS, Saksena NK, Hoy J, Crowe SM, Cameron PU, Lewin SR. 2010. Both CD31(+) and CD31(-) naive CD4(+) T cells are persistent HIV type 1-infected reservoirs in individuals receiving antiretroviral therapy. The Journal of infectious diseases 202:1738-1748.
25. Li Q, Lifson JD, Duan L, Schacker TW, Reilly C, Carlis J, Estes JD, Haase AT. 2005. Potential roles of follicular dendritic cell-associated osteopontin in lymphoid follicle pathology and repair and in B cell regulation in HIV-1 and SIV infection. The Journal of infectious diseases 192:1269-1276.
26. Nishimura Y, Brown CR, Mattapallil JJ, Igarashi T, Buckler-White A, Lafont BA, Hirsch VM, Roederer M, Martin MA. 2005. Resting naive CD4+ T cells are massively infected and eliminated by X4-tropic simian-human immunodeficiency viruses in macaques. Proceedings of the National Academy of Sciences of the United States of America 102:8000-8005.
27. Ostrowski MA, Chun TW, Justement SJ, Motola I, Spinelli MA, Adelsberger J, Ehler LA, Mizell SB, Hallahan CW, Fauci AS. 1999. Both memory and CD45RA+/CD62L+ naive CD4(+) T cells are infected in human immunodeficiency virus type 1-infected individuals. Journal of virology 73:6430-6435.
28. Swiggard WJ, Baytop C, Yu JJ, Dai J, Li C, Schretzenmair R, Theodosopoulos T, O'Doherty U. 2005. Human immunodeficiency virus type 1 can establish latent infection in resting CD4+ T cells in the absence of activating stimuli. Journal of virology 79:14179-14188.
29. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373:123-126.
30. Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH, et al. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373:117-122.
31. Colin L, Van Lint C. 2009. Molecular control of HIV-1 postintegration latency: implications for the development of new therapeutic strategies. Retrovirology 6:111.
32. Crampton N, Bonass WA, Kirkham J, Rivetti C, Thomson NH. 2006. Collision events between RNA polymerases in convergent transcription studied by atomic force microscopy. Nucleic acids research 34:5416-5425.
33. Greger IH, Demarchi F, Giacca M, Proudfoot NJ. 1998. Transcriptional interference perturbs the binding of Sp1 to the HIV-1 promoter. Nucleic acids research 26:1294-1301.
34. Lenasi T, Contreras X, Peterlin BM. 2008. Transcriptional interference antagonizes proviral gene expression to promote HIV latency. Cell host & microbe 4:123-133.
35. Han Y, Lin YB, An W, Xu J, Yang HC, O'Connell K, Dordai D, Boeke JD, Siliciano JD, Siliciano RF. 2008. Orientation-dependent regulation of integrated HIV-1 expression by host gene transcriptional readthrough. Cell host & microbe 4:134-146.
36. Lewinski MK, Bisgrove D, Shinn P, Chen H, Hoffmann C, Hannenhalli S, Verdin E, Berry CC, Ecker JR, Bushman FD. 2005. Genome-wide analysis of chromosomal features repressing human immunodeficiency virus transcription. Journal of virology 79:6610-6619.
37. Hu WY, Bushman FD, Siva AC. 2004. RNA interference against retroviruses. Virus research 102:59-64.
38. Voinnet O. 2005. Induction and suppression of RNA silencing: insights from viral infections. Nature reviews. Genetics 6:206-220.
39. Obbard DJ, Finnegan DJ. 2008. RNA interference: endogenous siRNAs derived from transposable elements. Current biology : CB 18:R561-563.
40. Bartel DP. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281-297.
41. Morris JPt, McManus MT. 2005. Slowing down the Ras lane: miRNAs as tumor suppressors? Science's STKE : signal transduction knowledge environment 2005:pe41.
42. Pereira LA, Bentley K, Peeters A, Churchill MJ, Deacon NJ. 2000. A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. Nucleic acids research 28:663-668.
43. Verdin E. 1991. DNase I-hypersensitive sites are associated with both long terminal repeats and with the intragenic enhancer of integrated human immunodeficiency virus type 1. Journal of virology 65:6790-6799.
44. Van Lint C, Emiliani S, Ott M, Verdin E. 1996. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. The EMBO journal 15:1112-1120.
45. Verdin E, Paras P, Jr., Van Lint C. 1993. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. The EMBO journal 12:3249-3259.
46. Verdin E, Van Lint C. 1995. Internal transcriptional regulatory elements in HIV-1 and other retroviruses. Cellular and molecular biology 41:365-369.
47. Bentley KS, Kirkland D, Murphy M, Marshall R. 2000. Evaluation of thresholds for benomyl- and carbendazim-induced aneuploidy in cultured human lymphocytes using fluorescence in situ hybridization. Mutation research 464:41-51.
48. Donahue DA, Wainberg MA. 2013. Cellular and molecular mechanisms involved in the establishment of HIV-1 latency. Retrovirology 10:11.

49. Pan C, Mezei M, Mujtaba S, Muller M, Zeng L, Li J, Wang Z, Zhou MM. 2007. Structure-guided optimization of small molecules inhibiting human immunodeficiency virus 1 Tat association with the human coactivator p300/CREB binding protein-associated factor. Journal of medicinal chemistry 50:2285-2288.
50. Pan X, Baldauf HM, Keppler OT, Fackler OT. 2013. Restrictions to HIV-1 replication in resting CD4+ T lymphocytes. Cell research 23:876-885.
51. Kinoshita S, Chen BK, Kaneshima H, Nolan GP. 1998. Host control of HIV-1 parasitism in T cells by the nuclear factor of activated T cells. Cell 95:595-604.
52. Ylisastigui L, Kaur R, Johnson H, Volker J, He G, Hansen U, Margolis D. 2005. Mitogen-activated protein kinases regulate LSF occupancy at the human immunodeficiency virus type 1 promoter. Journal of virology 79:5952-5962.
53. Coull JJ, Romerio F, Sun JM, Volker JL, Galvin KM, Davie JR, Shi Y, Hansen U, Margolis DM. 2000. The human factors YY1 and LSF repress the human immunodeficiency virus type 1 long terminal repeat via recruitment of histone deacetylase 1. Journal of virology 74:6790-6799.
54. Bernhard W, Barreto K, Raithatha S, Sadowski I. 2013. An upstream YY1 binding site on the HIV-1 LTR contributes to latent infection. PloS one 8:e77052.
55. Tyagi M, Karn J. 2007. CBF-1 promotes transcriptional silencing during the establishment of HIV-1 latency. The EMBO journal 26:4985-4995.
56. Yamaguchi Y, Shibata H, Handa H. 2013. Transcription elongation factors DSIF and NELF: promoter-proximal pausing and beyond. Biochimica et biophysica acta 1829:98-104.
57. Jadlowsky JK, Wong JY, Graham AC, Dobrowolski C, Devor RL, Adams MD, Fujinaga K, Karn J. 2014. Negative elongation factor is required for the maintenance of proviral latency but does not induce promoter-proximal pausing of RNA polymerase II on the HIV long terminal repeat. Molecular and cellular biology 34:1911-1928.
58. Ping YH, Rana TM. 2001. DSIF and NELF interact with RNA polymerase II elongation complex and HIV-1 Tat stimulates P-TEFb-mediated phosphorylation of RNA polymerase II and DSIF during transcription elongation. The Journal of biological chemistry 276:12951-12958.
59. Natarajan M, Schiralli Lester GM, Lee C, Missra A, Wasserman GA, Steffen M, Gilmour DS, Henderson AJ. 2013. Negative elongation factor (NELF) coordinates RNA polymerase II pausing, premature termination, and chromatin remodeling to regulate HIV transcription. The Journal of biological chemistry 288:25995-26003.
60. Sobhian B, Laguette N, Yatim A, Nakamura M, Levy Y, Kiernan R, Benkirane M. 2010. HIV-1 Tat assembles a multifunctional transcription elongation complex and stably associates with the 7SK snRNP. Molecular cell 38:439-451.
61. Isel C, Westhof E, Massire C, Le Grice SF, Ehresmann B, Ehresmann C, Marquet R. 1999. Structural basis for the specificity of the initiation of HIV-1 reverse transcription. The EMBO journal 18:1038-1048.
62. Kim YK, Bourgeois CF, Isel C, Churcher MJ, Karn J. 2002. Phosphorylation of the RNA polymerase II carboxyl-terminal domain by CDK9 is directly responsible for human immunodeficiency virus type 1 Tat-activated transcriptional elongation. Molecular and cellular biology 22:4622-4637.
63. Yik JH, Chen R, Nishimura R, Jennings JL, Link AJ, Zhou Q. 2003. Inhibition of P-TEFb (CDK9/Cyclin T) kinase and RNA polymerase II transcription by the coordinated actions of HEXIM1 and 7SK snRNA. Molecular cell 12:971-982.
64. Tyagi M, Pearson RJ, Karn J. 2010. Establishment of HIV latency in primary CD4+ cells is due to epigenetic transcriptional silencing and P-TEFb restriction. Journal of virology 84:6425-6437.
65. Budhiraja S, Famiglietti M, Bosque A, Planelles V, Rice AP. 2013. Cyclin T1 and CDK9 T-loop phosphorylation are downregulated during establishment of HIV-1 latency in primary resting memory CD4+ T cells. Journal of virology 87:1211-1220.
66. Brooks DG, Hamer DH, Arlen PA, Gao L, Bristol G, Kitchen CM, Berger EA, Zack JA. 2003. Molecular characterization, reactivation, and depletion of latent HIV. Immunity 19:413-423.
67. Tong-Starkesen SE, Luciw PA, Peterlin BM. 1989. Signaling through T lymphocyte surface proteins, TCR/CD3 and CD28, activates the HIV-1 long terminal repeat. Journal of immunology 142:702-707.
68. Coiras M, Lopez-Huertas MR, Rullas J, Mittelbrunn M, Alcami J. 2007. Basal shuttle of NF-kappaB/I kappaB alpha in resting T lymphocytes regulates HIV-1 LTR dependent expression. Retrovirology 4:56.
69. Cron RQ, Bartz SR, Clausell A, Bort SJ, Klebanoff SJ, Lewis DB. 2000. NFAT1 enhances HIV-1 gene expression in primary human CD4 T cells. Clinical immunology 94:179-191.
70. Garcia-Rodriguez C, Rao A. 1998. Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). The Journal of experimental medicine 187:2031-2036.
71. Zhong H, May MJ, Jimi E, Ghosh S. 2002. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Molecular cell 9:625-636.
72. Korin YD, Brooks DG, Brown S, Korotzer A, Zack JA. 2002. Effects of prostratin on T-cell activation and human immunodeficiency virus latency. Journal of virology 76:8118-8123.
73. Williams SA, Chen LF, Kwon H, Fenard D, Bisgrove D, Verdin E, Greene WC. 2004. Prostratin antagonizes HIV latency by activating NF-kappaB. The Journal of biological chemistry 279:42008-42017.

74. Abreu CM, Price SL, Shirk EN, Cunha RD, Pianowski LF, Clements JE, Tanuri A, Gama L. 2014. Dual role of novel ingenol derivatives from Euphorbia tirucalli in HIV replication: inhibition of de novo infection and activation of viral LTR. PloS one 9:e97257.
75. Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K, Zhou Q. 2005. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Molecular cell 19:535-545.
76. Wu SY, Chiang CM. 2007. The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. The Journal of biological chemistry 282:13141-13145.
77. Bisgrove DA, Mahmoudi T, Henklein P, Verdin E. 2007. Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription. Proceedings of the National Academy of Sciences of the United States of America 104:13690-13695.
78. Zhu J, Gaiha GD, John SP, Pertel T, Chin CR, Gao G, Qu H, Walker BD, Elledge SJ, Brass AL. 2012. Reactivation of latent HIV-1 by inhibition of BRD4. Cell reports 2:807-816.
79. Boehm D, Calvanese V, Dar RD, Xing S, Schroeder S, Martins L, Aull K, Li PC, Planelles V, Bradner JE, Zhou MM, Siliciano RF, Weinberger L, Verdin E, Ott M. 2013. BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism. Cell cycle 12:452-462.
80. Banerjee C, Archin N, Michaels D, Belkina AC, Denis GV, Bradner J, Sebastiani P, Margolis DM, Montano M. 2012. BET bromodomain inhibition as a novel strategy for reactivation of HIV-1. Journal of leukocyte biology 92:1147-1154.
81. Bartholomeeusen K, Xiang Y, Fujinaga K, Peterlin BM. 2012. Bromodomain and extra-terminal (BET) bromodomain inhibition activate transcription via transient release of positive transcription elongation factor b (P-TEFb) from 7SK small nuclear ribonucleoprotein. The Journal of biological chemistry 287:36609-36616.
82. Li Z, Guo J, Wu Y, Zhou Q. 2013. The BET bromodomain inhibitor JQ1 activates HIV latency through antagonizing Brd4 inhibition of Tat-transactivation. Nucleic acids research 41:277-287.
83. Kauder SE, Bosque A, Lindqvist A, Planelles V, Verdin E. 2009. Epigenetic regulation of HIV-1 latency by cytosine methylation. PLoS pathogens 5:e1000495.
84. Blazkova J, Trejbalova K, Gondois-Rey F, Halfon P, Philibert P, Guiguen A, Verdin E, Olive D, Van Lint C, Hejnar J, Hirsch I. 2009. CpG methylation controls reactivation of HIV from latency. PLoS pathogens 5:e1000554.
85. Friedman J, Cho WK, Chu CK, Keedy KS, Archin NM, Margolis DM, Karn J. 2011. Epigenetic silencing of HIV-1 by the histone H3 lysine 27 methyltransferase enhancer of Zeste 2. Journal of virology 85:9078-9089.
86. Imai K, Togami H, Okamoto T. 2010. Involvement of histone H3 lysine 9 (H3K9) methyltransferase G9a in the maintenance of HIV-1 latency and its reactivation by BIX01294. The Journal of biological chemistry 285:16538-16545.
87. du Chene I, Basyuk E, Lin YL, Triboulet R, Knezevich A, Chable-Bessia C, Mettling C, Baillat V, Reynes J, Corbeau P, Bertrand E, Marcello A, Emiliani S, Kiernan R, Benkirane M. 2007. Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. The EMBO journal 26:424-435.
88. Marban C, Suzanne S, Dequiedt F, de Walque S, Redel L, Van Lint C, Aunis D, Rohr O. 2007. Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. The EMBO journal 26:412-423.
89. Bernhard W, Barreto K, Saunders A, Dahabieh MS, Johnson P, Sadowski I. 2011. The Suv39H1 methyltransferase inhibitor chaetocin causes induction of integrated HIV-1 without producing a T cell response. FEBS letters 585:3549-3554.
90. Bouchat S, Gatot JS, Kabeya K, Cardona C, Colin L, Herbein G, De Wit S, Clumeck N, Lambotte O, Rouzioux C, Rohr O, Van Lint C. 2012. Histone methyltransferase inhibitors induce HIV-1 recovery in resting CD4(+) T cells from HIV-1-infected HAART-treated patients. Aids 26:1473-1482.
91. Katsani KR, Mahmoudi T, Verrijzer CP. 2003. Selective gene regulation by SWI/SNF-related chromatin remodeling factors. Current topics in microbiology and immunology 274:113-141.
92. de la Serna IL, Ohkawa Y, Higashi C, Dutta C, Osias J, Kommajosyula N, Tachibana T, Imbalzano AN. 2006. The microphthalmia-associated transcription factor requires SWI/SNF enzymes to activate melanocyte-specific genes. The Journal of biological chemistry 281:20233-20241.
93. Hargreaves DC, Crabtree GR. 2011. ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell research 21:396-420.
94. Klochendler-Yeivin A, Muchardt C, Yaniv M. 2002. SWI/SNF chromatin remodeling and cancer. Current opinion in genetics & development 12:73-79.
95. Ho L, Crabtree GR. 2010. Chromatin remodelling during development. Nature 463:474-484.
96. Wang W, Xue Y, Zhou S, Kuo A, Cairns BR, Crabtree GR. 1996. Diversity and specialization of mammalian SWI/SNF complexes. Genes & development 10:2117-2130.
97. Rafati H, Parra M, Hakre S, Moshkin Y, Verdin E, Mahmoudi T. 2011. Repressive LTR nucleosome positioning by the BAF complex is required for HIV latency. PLoS biology 9:e1001206.

98. Mahmoudi T, Parra M, Vries RG, Kauder SE, Verrijzer CP, Ott M, Verdin E. 2006. The SWI/SNF chromatin-remodeling complex is a cofactor for Tat transactivation of the HIV promoter. The Journal of biological chemistry 281:19960-19968.
99. Agbottah E, Deng L, Dannenberg LO, Pumfery A, Kashanchi F. 2006. Effect of SWI/SNF chromatin remodeling complex on HIV-1 Tat activated transcription. Retrovirology 3:48.
100. Ariumi Y, Serhan F, Turelli P, Telenti A, Trono D. 2006. The integrase interactor 1 (INI1) proteins facilitate Tat-mediated human immunodeficiency virus type 1 transcription. Retrovirology 3:47.
101. Treand C, du Chene I, Bres V, Kiernan R, Benarous R, Benkirane M, Emiliani S. 2006. Requirement for SWI/SNF chromatin-remodeling complex in Tat-mediated activation of the HIV-1 promoter. The EMBO journal 25:1690-1699.
102. Mizutani T, Ishizaka A, Tomizawa M, Okazaki T, Yamamichi N, Kawana-Tachikawa A, Iwamoto A, Iba H. 2009. Loss of the Brm-type SWI/SNF chromatin remodeling complex is a strong barrier to the Tat-independent transcriptional elongation of human immunodeficiency virus type 1 transcripts. Journal of virology 83:11569-11580.
103. Kalpana GV, Marmon S, Wang W, Crabtree GR, Goff SP. 1994. Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. Science 266:2002-2006.
104. Maroun M, Delelis O, Coadou G, Bader T, Segeral E, Mbemba G, Petit C, Sonigo P, Rain JC, Mouscadet JF, Benarous R, Emiliani S. 2006. Inhibition of early steps of HIV-1 replication by SNF5/Ini1. The Journal of biological chemistry 281:22736-22743.
105. Boese A, Sommer P, Holzer D, Maier R, Nehrbass U. 2009. Integrase interactor 1 (Ini1/hSNF5) is a repressor of basal human immunodeficiency virus type 1 promoter activity. The Journal of general virology 90:2503-2512.
106. Lesbats P, Botbol Y, Chevereau G, Vaillant C, Calmels C, Arneodo A, Andreola ML, Lavigne M, Parissi V. 2011. Functional coupling between HIV-1 integrase and the SWI/SNF chromatin remodeling complex for efficient in vitro integration into stable nucleosomes. PLoS pathogens 7:e1001280.
107. Yan Z, Cui K, Murray DM, Ling C, Xue Y, Gerstein A, Parsons R, Zhao K, Wang W. 2005. PBAF chromatin-remodeling complex requires a novel specificity subunit, BAF200, to regulate expression of selective interferon-responsive genes. Genes & development 19:1662-1667.
108. Xue Y, Canman JC, Lee CS, Nie Z, Yang D, Moreno GT, Young MK, Salmon ED, Wang W. 2000. The human SWI/SNF-B chromatin-remodeling complex is related to yeast rsc and localizes at kinetochores of mitotic chromosomes. Proceedings of the National Academy of Sciences of the United States of America 97:13015-13020.
109. Lemon B, Inouye C, King DS, Tjian R. 2001. Selectivity of chromatin-remodelling cofactors for ligand-activated transcription. Nature 414:924-928.
110. Chalkley GE, Moshkin YM, Langenberg K, Bezstarosti K, Blastyak A, Gyurkovics H, Demmers JA, Verrijzer CP. 2008. The transcriptional coactivator SAYP is a trithorax group signature subunit of the PBAP chromatin remodeling complex. Molecular and cellular biology 28:2920-2929.
111. Nagl NG, Jr., Wang X, Patsialou A, Van Scoy M, Moran E. 2007. Distinct mammalian SWI/SNF chromatin remodeling complexes with opposing roles in cell-cycle control. The EMBO journal 26:752-763.
112. Kaeser MD, Aslanian A, Dong MQ, Yates JR, 3rd, Emerson BM. 2008. BRD7, a novel PBAF-specific SWI/SNF subunit, is required for target gene activation and repression in embryonic stem cells. The Journal of biological chemistry 283:32254-32263.
113. Nie Z, Xue Y, Yang D, Zhou S, Deroo BJ, Archer TK, Wang W. 2000. A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. Molecular and cellular biology 20:8879-8888.
114. Moshkin YM, Mohrmann L, van Ijcken WF, Verrijzer CP. 2007. Functional differentiation of SWI/SNF remodelers in transcription and cell cycle control. Molecular and cellular biology 27:651-661.
115. Hsiao PW, Fryer CJ, Trotter KW, Wang W, Archer TK. 2003. BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatin-remodeling complex for transactivation. Molecular and cellular biology 23:6210-6220.
116. Mohrmann L, Verrijzer CP. 2005. Composition and functional specificity of SWI2/SNF2 class chromatin remodeling complexes. Biochimica et biophysica acta 1681:59-73.
117. Inoue H, Furukawa T, Giannakopoulos S, Zhou S, King DS, Tanese N. 2002. Largest subunits of the human SWI/SNF chromatin-remodeling complex promote transcriptional activation by steroid hormone receptors. The Journal of biological chemistry 277:41674-41685.
118. Dykhuizen EC, Carmody LC, Tolliday N, Crabtree GR, Palmer MA. 2012. Screening for inhibitors of an essential chromatin remodeler in mouse embryonic stem cells by monitoring transcriptional regulation. Journal of biomolecular screening 17:1221-1230.
119. Clevers H, Nusse R. 2012. Wnt/beta-catenin signaling and disease. Cell 149:1192-1205.
120. Li VS, Ng SS, Boersema PJ, Low TY, Karthaus WR, Gerlach JP, Mohammed S, Heck AJ, Maurice MM, Mahmoudi T, Clevers H. 2012. Wnt signaling through inhibition of beta-catenin degradation in an intact Axin1 complex. Cell 149:1245-1256.

121. Waterman ML, Jones KA. 1990. Purification of TCF-1 alpha, a T-cell-specific transcription factor that activates the T-cell receptor C alpha gene enhancer in a context-dependent manner. New Biol 2:621-636.
122. Sheridan PL, Sheline CT, Cannon K, Voz ML, Pazin MJ, Kadonaga JT, Jones KA. 1995. Activation of the HIV-1 enhancer by the LEF-1 HMG protein on nucleosome-assembled DNA in vitro. Genes & development 9:2090-2104.
123. Kameoka M, Kameoka Y, Utachee P, Kurosu T, Sawanpanyalert P, Ikuta K, Auwanit W. 2009. Short communication: RNA interference directed against Axin1 upregulates human immunodeficiency virus type 1 gene expression by activating the Wnt signaling pathway in HeLa-derived J111 cells. AIDS research and human retroviruses 25:1005-1011.
124. Henderson LJ, Sharma A, Monaco MC, Major EO, Al-Harthi L. 2012. Human immunodeficiency virus type 1 (HIV-1) transactivator of transcription through its intact core and cysteine-rich domains inhibits Wnt/beta-catenin signaling in astrocytes: relevance to HIV neuropathogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:16306-16313.
125. Narasipura SD, Henderson LJ, Fu SW, Chen L, Kashanchi F, Al-Harthi L. 2012. Role of beta-catenin and TCF/LEF family members in transcriptional activity of HIV in astrocytes. Journal of virology 86:1911-1921.
126. Wortman B, Darbinian N, Sawaya BE, Khalili K, Amini S. 2002. Evidence for regulation of long terminal repeat transcription by Wnt transcription factor TCF-4 in human astrocytic cells. Journal of virology 76:11159-11165.
127. Carroll-Anzinger D, Kumar A, Adarichev V, Kashanchi F, Al-Harthi L. 2007. Human immunodeficiency virus-restricted replication in astrocytes and the ability of gamma interferon to modulate this restriction are regulated by a downstream effector of the Wnt signaling pathway. Journal of virology 81:5864-5871.
128. Henderson LJ, Narasipura SD, Adarichev V, Kashanchi F, Al-Harthi L. 2012. Identification of novel T cell factor 4 (TCF-4) binding sites on the HIV long terminal repeat which associate with TCF-4, beta-catenin, and SMAR1 to repress HIV transcription. Journal of virology 86:9495-9503.
129. Kumar A, Zloza A, Moon RT, Watts J, Tenorio AR, Al-Harthi L. 2008. Active beta-catenin signaling is an inhibitory pathway for human immunodeficiency virus replication in peripheral blood mononuclear cells. Journal of virology 82:2813-2820.
130. Bienz M. 2005. beta-Catenin: a pivot between cell adhesion and Wnt signalling. Current biology : CB 15:R64-67.
131. Cadigan KM, Waterman ML. 2012. TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harbor perspectives in biology 4.
132. Tiemessen MM, Baert MR, Schonewille T, Brugman MH, Famili F, Salvatori DC, Meijerink JP, Ozbek U, Clevers H, van Dongen JJ, Staal FJ. 2012. The nuclear effector of Wnt-signaling, Tcf1, functions as a T-cell-specific tumor suppressor for development of lymphomas. PLoS biology 10:e1001430.
133. Steinke FC, Xue HH. 2014. From inception to output, Tcf1 and Lef1 safeguard development of T cells and innate immune cells. Immunologic research 59:45-55.
134. Albano F, Zagaria A, Anelli L, Coccaro N, Impera L, Minervini CF, Minervini A, Rossi AR, Tota G, Casieri P, Specchia G. 2013. Gene expression profiling of chronic myeloid leukemia with variant t(9;22) reveals a different signature from cases with classic translocation. Molecular cancer 12:36.
135. Ma J, Wang R, Fang X, Sun Z. 2012. beta-catenin/TCF-1 pathway in T cell development and differentiation. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology 7:750-762.
136. Xue HH, Zhao DM. 2012. Regulation of mature T cell responses by the Wnt signaling pathway. Annals of the New York Academy of Sciences 1247:16-33.
137. Kuhnl A, Gokbuget N, Kaiser M, Schlee C, Stroux A, Burmeister T, Mochmann LH, Hoelzer D, Hofmann WK, Thiel E, Baldus CD. 2011. Overexpression of LEF1 predicts unfavorable outcome in adult patients with B-precursor acute lymphoblastic leukemia. Blood 118:6362-6367.
138. Mao CD, Byers SW. 2011. Cell-context dependent TCF/LEF expression and function: alternative tales of repression, de-repression and activation potentials. Critical reviews in eukaryotic gene expression 21:207-236.
139. Staal FJ, Luis TC, Tiemessen MM. 2008. WNT signalling in the immune system: WNT is spreading its wings. Nature reviews. Immunology 8:581-593.
140. Roozen PP, Brugman MH, Staal FJ. 2012. Differential requirements for Wnt and Notch signaling in hematopoietic versus thymic niches. Annals of the New York Academy of Sciences 1266:78-93.
141. Bigas A, Guiu J, Gama-Norton L. 2013. Notch and Wnt signaling in the emergence of hematopoietic stem cells. Blood cells, molecules & diseases 51:264-270.
142. Saha A, Dandekar S, Milla S, Roman E, Bhatla T. 2014. Bilateral parotid gland enlargement and palpable nephromegaly in infant acute lymphoblastic leukemia: case report and review of the literature. Journal of pediatric hematology/oncology 36:246-248.
143. Dandekar S, Romanos-Sirakis E, Pais F, Bhatla T, Jones C, Bourgeois W, Hunger SP, Raetz EA, Hermiston ML, Dasgupta R, Morrison DJ, Carroll WL. 2014. Wnt inhibition leads to improved chemosensitivity in paediatric acute lymphoblastic leukaemia. British journal of haematology 167:87-99.

144. Roman-Gomez J, Cordeu L, Agirre X, Jimenez-Velasco A, San Jose-Eneriz E, Garate L, Calasanz MJ, Heiniger A, Torres A, Prosper F. 2007. Epigenetic regulation of Wnt-signaling pathway in acute lymphoblastic leukemia. Blood 109:3462-3469.
145. Derksen PW, Tjin E, Meijer HP, Klok MD, MacGillavry HD, van Oers MH, Lokhorst HM, Bloem AC, Clevers H, Nusse R, van der Neut R, Spaargaren M, Pals ST. 2004. Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. Proceedings of the National Academy of Sciences of the United States of America 101:6122-6127.
146. Groen RW, Oud ME, Schilder-Tol EJ, Overdijk MB, ten Berge D, Nusse R, Spaargaren M, Pals ST. 2008. Illegitimate WNT pathway activation by beta-catenin mutation or autocrine stimulation in T-cell malignancies. Cancer research 68:6969-6977.
147. Schmitt AM, Shi J, Wolf AM, Lu CC, King LA, Zou Y. 2006. Wnt-Ryk signalling mediates medial-lateral retinotectal topographic mapping. Nature 439:31-37.
148. Palmisano I, Della Chiara G, Schiaffino MV, Poli G. 2012. Passport control for foreign integrated DNAs: An unexpected checkpoint by class II HDAC4 revealed by amino acid starvation. Mobile genetic elements 2:233-238.
149. Keedy KS, Archin NM, Gates AT, Espeseth A, Hazuda DJ, Margolis DM. 2009. A limited group of class I histone deacetylases acts to repress human immunodeficiency virus type 1 expression. Journal of virology 83:4749-4756.
150. Margolis DM. 2011. Histone deacetylase inhibitors and HIV latency. Current opinion in HIV and AIDS 6:25-29.
151. Williams SA, Chen LF, Kwon H, Ruiz-Jarabo CM, Verdin E, Greene WC. 2006. NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. The EMBO journal 25:139-149.
152. Edelstein LC, Micheva-Viteva S, Phelan BD, Dougherty JP. 2009. Short communication: activation of latent HIV type 1 gene expression by suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor approved for use to treat cutaneous T cell lymphoma. AIDS research and human retroviruses 25:883-887.
153. Shirakawa K, Chavez L, Hakre S, Calvanese V, Verdin E. 2013. Reactivation of latent HIV by histone deacetylase inhibitors. Trends in microbiology 21:277-285.
154. Sheridan PL, Mayall TP, Verdin E, Jones KA. 1997. Histone acetyltransferases regulate HIV-1 enhancer activity in vitro. Genes & development 11:3327-3340.
155. Lehrman G, Hogue IB, Palmer S, Jennings C, Spina CA, Wiegand A, Landay AL, Coombs RW, Richman DD, Mellors JW, Coffin JM, Bosch RJ, Margolis DM. 2005. Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. Lancet 366:549-555.
156. Sagot-Lerolle N, Lamine A, Chaix ML, Boufassa F, Aboulker JP, Costagliola D, Goujard C, Pallier C, Delfraissy JF, Lambotte O, study AE. 2008. Prolonged valproic acid treatment does not reduce the size of latent HIV reservoir. Aids 22:1125-1129.
157. Steel A, Clark S, Teo I, Shaunak S, Nelson M, Gazzard B, Kelleher P. 2006. No change to HIV-1 latency with valproate therapy. Aids 20:1681-1682.
158. Rasmussen TA, Schmeltz Sogaard O, Brinkmann C, Wightman F, Lewin SR, Melchjorsen J, Dinarello C, Ostergaard L, Tolstrup M. 2013. Comparison of HDAC inhibitors in clinical development: effect on HIV production in latently infected cells and T-cell activation. Human vaccines & immunotherapeutics 9:993-1001.
159. Wei DG, Chiang V, Fyne E, Balakrishnan M, Barnes T, Graupe M, Hesselgesser J, Irrinki A, Murry JP, Stepan G, Stray KM, Tsai A, Yu H, Spindler J, Kearney M, Spina CA, McMahon D, Lalezari J, Sloan D, Mellors J, Geleziunas R, Cihlar T. 2014. Histone deacetylase inhibitor romidepsin induces HIV expression in CD4 T cells from patients on suppressive antiretroviral therapy at concentrations achieved by clinical dosing. PLoS pathogens 10:e1004071.
160. Archin NM, Bateson R, Tripathy MK, Crooks AM, Yang KH, Dahl NP, Kearney MF, Anderson EM, Coffin JM, Strain MC, Richman DD, Robertson KR, Kashuba AD, Bosch RJ, Hazuda DJ, Kuruc JD, Eron JJ, Margolis DM. 2014. HIV-1 expression within resting CD4+ T cells after multiple doses of vorinostat. The Journal of infectious diseases 210:728-735.
161. Parra M. 2014. Class IIa HDACs: New insights into their functions in physiology and pathology. The FEBS journal.
162. Yang XJ, Gregoire S. 2005. Class II histone deacetylases: from sequence to function, regulation, and clinical implication. Molecular and cellular biology 25:2873-2884.
163. Verdin E, Dequiedt F, Kasler HG. 2003. Class II histone deacetylases: versatile regulators. Trends in genetics : TIG 19:286-293.
164. McKinsey TA, Zhang CL, Olson EN. 2001. Control of muscle development by dueling HATs and HDACs. Current opinion in genetics & development 11:497-504.
165. Grozinger CM, Hassig CA, Schreiber SL. 1999. Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proceedings of the National Academy of Sciences of the United States of America 96:4868-4873.
166. Fischle W, Emiliani S, Hendzel MJ, Nagase T, Nomura N, Voelter W, Verdin E. 1999. A new family of human histone deacetylases related to Saccharomyces cerevisiae HDA1p. The Journal of biological chemistry 274:11713-11720.
167. Barneda-Zahonero B, Roman-Gonzalez L, Collazo O, Rafati H, Islam AB, Bussmann LH, di Tullio A, De Andres L, Graf T, Lopez-Bigas N, Mahmoudi T, Parra M. 2013. HDAC7 is a repressor of myeloid genes whose downregulation is required for transdifferentiation of pre-B cells into macrophages. PLoS genetics 9:e1003503.

168. Martin M, Kettmann R, Dequiedt F. 2009. Class IIa histone deacetylases: conducting development and differentiation. The International journal of developmental biology 53:291-301.
169. Parra M, Verdin E. 2010. Regulatory signal transduction pathways for class IIa histone deacetylases. Current opinion in pharmacology 10:454-460.
170. Dequiedt F, Kasler H, Fischle W, Kiermer V, Weinstein M, Herndier BG, Verdin E. 2003. HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. Immunity 18:687-698.
171. Kasler HG, Verdin E. 2007. Histone deacetylase 7 functions as a key regulator of genes involved in both positive and negative selection of thymocytes. Molecular and cellular biology 27:5184-5200.
172. Parra M, Kasler H, McKinsey TA, Olson EN, Verdin E. 2005. Protein kinase D1 phosphorylates HDAC7 and induces its nuclear export after T-cell receptor activation. The Journal of biological chemistry 280:13762-13770.
173. Kao HY, Verdel A, Tsai CC, Simon C, Juguilon H, Khochbin S. 2001. Mechanism for nucleocytoplasmic shuttling of histone deacetylase 7. The Journal of biological chemistry 276:47496-47507.
174. Hemelaar J, Gouws E, Ghys PD, Osmanov S, Isolation W-UNfH, Characterisation. 2011. Global trends in molecular epidemiology of HIV-1 during 2000-2007. Aids 25:679-689.
175. Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK, Gao F, Hahn BH, Kalish ML, Kuiken C, Learn GH, Leitner T, McCutchan F, Osmanov S, Peeters M, Pieniazek D, Salminen M, Sharp PM, Wolinsky S, Korber B. 2000. HIV-1 nomenclature proposal. Science 288:55-56.
176. Yamaguchi J, Bodelle P, Vallari AS, Coffey R, McArthur CP, Schochetman G, Devare SG, Brennan CA. 2004. HIV infections in northwestern Cameroon: identification of HIV type 1 group O and dual HIV type 1 group M and group O infections. AIDS research and human retroviruses 20:944-957.
177. Mauclere P, Loussert-Ajaka I, Damond F, Fagot P, Souquieres S, Monny Lobe M, Mbopi Keou FX, Barre-Sinoussi F, Saragosti S, Brun-Vezinet F, Simon F. 1997. Serological and virological characterization of HIV-1 group O infection in Cameroon. Aids 11:445-453.
178. Roques P, Robertson DL, Souquiere S, Apetrei C, Nerrienet E, Barre-Sinoussi F, Muller-Trutwin M, Simon F. 2004. Phylogenetic characteristics of three new HIV-1 N strains and implications for the origin of group N. Aids 18:1371-1381.
179. Bodelle P, Vallari A, Coffey R, McArthur CP, Beyeme M, Devare SG, Schochetman G, Brennan CA. 2004. Identification and genomic sequence of an HIV type 1 group N isolate from Cameroon. AIDS research and human retroviruses 20:902-908.
180. Plantier JC, Leoz M, Dickerson JE, De Oliveira F, Cordonnier F, Lemee V, Damond F, Robertson DL, Simon F. 2009. A new human immunodeficiency virus derived from gorillas. Nature medicine 15:871-872.
181. Vallari A, Holzmayer V, Harris B, Yamaguchi J, Ngansop C, Makamche F, Mbanya D, Kaptue L, Ndembi N, Gurtler L, Devare S, Brennan CA. 2011. Confirmation of putative HIV-1 group P in Cameroon. Journal of virology 85:1403-1407.
182. Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N, Hakim JG, Kumwenda J, Grinsztejn B, Pilotto JH, Godbole SV, Mehendale S, Chariyalertsak S, Santos BR, Mayer KH, Hoffman IF, Eshleman SH, Piwowar-Manning E, Wang L, Makhema J, Mills LA, de Bruyn G, Sanne I, Eron J, Gallant J, Havlir D, Swindells S, Ribaudo H, Elharrar V, Burns D, Taha TE, Nielsen-Saines K, Celentano D, Essex M, Fleming TR, Team HS. 2011. Prevention of HIV-1 infection with early antiretroviral therapy. The New England journal of medicine 365:493-505.
183. van der Sluis RM, Pollakis G, van Gerven ML, Berkhout B, Jeeninga RE. 2011. Latency profiles of full length HIV-1 molecular clone variants with a subtype specific promoter. Retrovirology 8:73.
184. Klein F, Halper-Stromberg A, Horwitz JA, Gruell H, Scheid JF, Bournazos S, Mouquet H, Spatz LA, Diskin R, Abadir A, Zang T, Dorner M, Billerbeck E, Labitt RN, Gaebler C, Marcovecchio PM, Incesu RB, Eisenreich TR, Bieniasz PD, Seaman MS, Bjorkman PJ, Ravetch JV, Ploss A, Nussenzweig MC. 2012. HIV therapy by a combination of broadly neutralizing antibodies in humanized mice. Nature 492:118-122.
185. Jordan A, Bisgrove D, Verdin E. 2003. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. The EMBO journal 22:1868-1877.
186. Jordan A, Defechereux P, Verdin E. 2001. The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. The EMBO journal 20:1726-1738.
187. Bonczkowski P, De Spiegelaere W, Bosque A, White CH, Van Nuffel A, Malatinkova E, Kiselinova M, Trypsteen W, Witkowski W, Vermeire J, Verhasselt B, Martins L, Woelk CH, Planelles V, Vandekerckhove L. 2014. Replication competent virus as an important source of bias in HIV latency models utilizing single round viral constructs. Retrovirology 11:70.
188. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, Boucher G, Boulassel MR, Ghattas G, Brenchley JM, Schacker TW, Hill BJ, Douek DC, Routy JP, Haddad EK, Sekaly RP. 2009. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nature medicine 15:893-900.
189. Spina CA, Anderson J, Archin NM, Bosque A, Chan J, Famiglietti M, Greene WC, Kashuba A, Lewin SR, Margolis DM, Mau M, Ruelas D, Saleh S, Shirakawa K, Siliciano RF, Singhania A, Soto PC, Terry VH, Verdin E, Woelk C, Wooden S,

Xing S, Planelles V. 2013. An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4+ T cells from aviremic patients. PLoS pathogens 9:e1003834.
190. Lassen KG, Hebbeler AM, Bhattacharyya D, Lobritz MA, Greene WC. 2012. A flexible model of HIV-1 latency permitting evaluation of many primary CD4 T-cell reservoirs. PloS one 7:e30176.
191. Bosque A, Planelles V. 2011. Studies of HIV-1 latency in an ex vivo model that uses primary central memory T cells. Methods 53:54-61.
192. Bosque A, Planelles V. 2009. Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. Blood 113:58-65.
193. Archin NM, Eron JJ, Palmer S, Hartmann-Duff A, Martinson JA, Wiegand A, Bandarenko N, Schmitz JL, Bosch RJ, Landay AL, Coffin JM, Margolis DM. 2008. Valproic acid without intensified antiviral therapy has limited impact on persistent HIV infection of resting CD4+ T cells. Aids 22:1131-1135.
194. Archin NM, Liberty AL, Kashuba AD, Choudhary SK, Kuruc JD, Crooks AM, Parker DC, Anderson EM, Kearney MF, Strain MC, Richman DD, Hudgens MG, Bosch RJ, Coffin JM, Eron JJ, Hazuda DJ, Margolis DM. 2012. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. Nature 487:482-485.
195. Blankson JN, Finzi D, Pierson TC, Sabundayo BP, Chadwick K, Margolick JB, Quinn TC, Siliciano RF. 2000. Biphasic decay of latently infected CD4+ T cells in acute human immunodeficiency virus type 1 infection. The Journal of infectious diseases 182:1636-1642.
196. Deere JD, Schinazi RF, North TW. 2011. Simian immunodeficiency virus macaque models of HIV latency. Current opinion in HIV and AIDS 6:57-61.
197. Shan L, Deng K, Shroff NS, Durand CM, Rabi SA, Yang HC, Zhang H, Margolick JB, Blankson JN, Siliciano RF. 2012. Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. Immunity 36:491-501.
198. Siliciano JD, Siliciano RF. 2014. Recent developments in the search for a cure for HIV-1 infection: targeting the latent reservoir for HIV-1. The Journal of allergy and clinical immunology 134:12-19.
199. Siliciano JD, Siliciano RF. 2013. HIV-1 eradication strategies: design and assessment. Current opinion in HIV and AIDS 8:318-325.
200. Sherrill-Mix S, Lewinski MK, Famiglietti M, Bosque A, Malani N, Ocwieja KE, Berry CC, Looney D, Shan L, Agosto LM, Pace MJ, Siliciano RF, O'Doherty U, Guatelli J, Planelles V, Bushman FD. 2013. HIV latency and integration site placement in five cell-based models. Retrovirology 10:90.
201. Burnett JC, Lim KI, Calafi A, Rossi JJ, Schaffer DV, Arkin AP. 2010. Combinatorial latency reactivation for HIV-1 subtypes and variants. Journal of virology 84:5958-5974.
202. Duverger A, Wolschendorf F, Zhang M, Wagner F, Hatcher B, Jones J, Cron RQ, van der Sluis RM, Jeeninga RE, Berkhout B, Kutsch O. 2013. An AP-1 binding site in the enhancer/core element of the HIV-1 promoter controls the ability of HIV-1 to establish latent infection. Journal of virology 87:2264-2277.
203. Reuse S, Calao M, Kabeya K, Guiguen A, Gatot JS, Quivy V, Vanhulle C, Lamine A, Vaira D, Demonte D, Martinelli V, Veithen E, Cherrier T, Avettand V, Poutrel S, Piette J, de Launoit Y, Moutschen M, Burny A, Rouzioux C, De Wit S, Herbein G, Rohr O, Collette Y, Lambotte O, Clumeck N, Van Lint C. 2009. Synergistic activation of HIV-1 expression by deacetylase inhibitors and prostratin: implications for treatment of latent infection. PloS one 4:e6093.
204. Perez M, de Vinuesa AG, Sanchez-Duffhues G, Marquez N, Bellido ML, Munoz-Fernandez MA, Moreno S, Castor TP, Calzado MA, Munoz E. 2010. Bryostatin-1 synergizes with histone deacetylase inhibitors to reactivate HIV-1 from latency. Current HIV research 8:418-429.
205. Archin NM, Keedy KS, Espeseth A, Dang H, Hazuda DJ, Margolis DM. 2009. Expression of latent human immunodeficiency type 1 is induced by novel and selective histone deacetylase inhibitors. Aids 23:1799-1806.
206. Archin NM, Espeseth A, Parker D, Cheema M, Hazuda D, Margolis DM. 2009. Expression of latent HIV induced by the potent HDAC inhibitor suberoylanilide hydroxamic acid. AIDS research and human retroviruses 25:207-212.
207. Ying H, Zhang Y, Zhou X, Qu X, Wang P, Liu S, Lu D, Zhu H. 2012. Selective histonedeacetylase inhibitor M344 intervenes in HIV-1 latency through increasing histone acetylation and activation of NF-kappaB. PloS one 7:e48832.
208. Micheva-Viteva S, Kobayashi Y, Edelstein LC, Pacchia AL, Lee HL, Graci JD, Breslin J, Phelan BD, Miller LK, Colacino JM, Gu Z, Ron Y, Peltz SW, Dougherty JP. 2011. High-throughput screening uncovers a compound that activates latent HIV-1 and acts cooperatively with a histone deacetylase (HDAC) inhibitor. The Journal of biological chemistry 286:21083-21091.
209. Pandelo Jose D, Bartholomeeusen K, da Cunha RD, Abreu CM, Glinski J, da Costa TB, Bacchi Rabay AF, Pianowski Filho LF, Dudycz LW, Ranga U, Peterlin BM, Pianowski LF, Tanuri A, Aguiar RS. 2014. Reactivation of latent HIV-1 by new semi-synthetic ingenol esters. Virology 462-463:328-339.

Chapter 2

Repressive LTR Nucleosome Positioning by the BAF Complex Is Required for HIV Latency

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Abstract

Persistence of a reservoir of latently infected memory T cells provides a barrier to HIV eradication in treated patients. Several reports have implicated the involvement of SWI/SNF chromatin remodeling complexes in restricting early steps in HIV infection, in coupling the processes of integration and remodeling, and in promoter/LTR transcription activation and repression. However, the mechanism behind the seemingly contradictory involvement of SWI/SNF in the HIV life cycle remains unclear. Here we addressed the role of SWI/SNF in regulation of the latent HIV LTR before and after transcriptional activation. We determined the predicted nucleosome affinity of the LTR sequence and found a striking reverse correlation when compared to the strictly positioned in vivo LTR nucleosomal structure; sequences encompassing the DNase hypersensitive regions displayed the highest nucleosome affinity, while the strictly positioned nucleosomes displayed lower affinity for nucleosome formation. To examine the mechanism behind this reverse correlation, we used a combinatorial approach to determine DNA accessibility, histone occupancy, and the unique recruitment and requirement of BAF and PBAF, two functionally distinct subclasses of SWI/SNF at the LTR of HIV-infected cells before and after activation. We find that establishment and maintenance of HIV latency requires BAF, which removes a preferred nucleosome from DHS1 to position the repressive nucleosome-1 over energetically sub-optimal sequences. Depletion of BAF resulted in de-repression of HIV latency concomitant with a dramatic alteration in the LTR nucleosome profile as determined by high resolution MNase nucleosomal mapping. Upon activation, BAF was lost from the HIV promoter, while PBAF was selectively recruited by acetylated Tat to facilitate LTR transcription. Thus BAF and PBAF, recruited during different stages of the HIV life cycle, display opposing function on the HIV promoter. Our data point to the ATP-dependent BRG1 component of BAF as a putative therapeutic target to deplete the latent reservoir in patients.

Introduction

After host cell infection and entry into the nucleus, the Human immunodeficiency virus (HIV-1) DNA integrates into the host genome as a chromatin template. Through unclear mechanisms, a very small percentage of infected T cells become latent. Despite the successes of modern Highly Active Anti-Retroviral Therapy (HAART) in suppressing viral replication, the presence of latently infected resting memory CD4+T cells provides the main impediment to curing HIV [1–3]. Infected patients must receive continuous HAART, as treatment interruption results in rapid rebound of viremia [4]. Latent HIV-1 infected resting memory CD4+T cells harbor replication competent virus, which is blocked at the level of transcription.

Transcription of the HIV-1 virus is driven by the LTR and is restricted in vivo. Regardless of the position of virus integration in the host genome, within the 59LTR, the nucleosomes are strictly deposited at specific positions [5–7]. Chromatin organization of the HIV-1 provirus characterized by nuclease digestion of intact nuclei of infected cells under basal conditions demonstrates the presence of at least three precisely positioned nucleosomes, nuc-0, nuc-1, and nuc-2 and their intervening nucleosome-free regions [5,6]. In particular, nuc-1, the nucleosome positioned immediately downstream of the transcription start site, is repressive to transcription and is surrounded by two large domains of nucleosome-free DNA. Following activation, nuc-1 becomes rapidly and specifically disrupted [5,8].

To overcome nucleosome mediated repression, the cell uses at least two mechanisms to increase the accessibility of DNA sequences embedded within nucleosomes. The first is through the action of enzymatic complexes which covalently modify histones. Histone modifying complexes are thought to regulate transcription at the HIV LTR. For example, HDAC1 is recruited to and represses transcription at the LTR [9–11]. Following activation, histone acetylation surrounding nuc-1 has been demonstrated to increase significantly, concomitant with removal of HDAC [7,10,12,13]. Many histone-modifying enzymes have been shown to be recruited to the LTR by the HIV transactivator Tat and/or by host cell transcription factors, whose consensus binding sites are present on the LTR. Tat itself is subject to distinct modifications by various factors (including p300/CBP, PCAF, hGCN5, SIRT1, PRMT5, SETDB1, SETDB2, SET7/9 KMT7) [14,15], a mechanism to modulate its interaction with the many cofactors Tat recruits to the LTR.

The second mechanism for altering DNA accessibility within repressive nucleosomes is via enzymatic complexes, which use energy from ATP hydrolysis to alter the structure of chromatin [16,17]. One family of remodeling complexes, SWI/SNF, contains either Brahma (BRM) or the closely related BRG1 as its catalytic subunit and shares most common subunits [16–22]. At least two biochemically distinct SWI/SNF complexes with different functions have been described and are called BAF and PBAF. The PBAF complex contains either BRG1 or BRM together with the PBAF-specific subunits BAF180, BAF200, SAYP, and Brd7, but lacks BAF250

[23–27]. The BAF complex contains either BRG1 or BRM together with the BAF-specific subunit BAF250, but lacks PBAF-specific subunits (Figure 1A) [28,29]. The presence of distinct isoforms of subunits such as BAF60 also increases the number of possible complexes [16,17]. BAF- or PBAF-specific subunits have been implicated in transcriptional activation by selective nuclear hormone receptors [24,28,30,31], in distinct roles in cell cycle control and mitosis [29,32], and the expression of interferon-responsive genes [26]. OSA, the Drosophila BAFspecific subunit, is required for repression of Wingless target genes [33]. BAF and PBAF subunit-specific polytene staining of Drosophila salivary glands indicates that the complexes are recruited to distinct targets [34]. These and other studies suggest that BAF and PBAF complexes perform distinct functions in transcription regulation.

In the immediate-early phase of HIV infection, cellular transcription factors activate transcription from the viral promoter in the 5'-LTR, leading to accumulation of viral Tat protein, a potent transactivator. Tat binds TAR, an RNA stem-loop in the nascent viral RNA, and recruits a positive transcription elongation factor complex (pTEFb) containing CDK9 and cyclinT1. This recruitment leads to the phosphorylation of the carboxyl-terminal domain of RNA PolII and increased transcriptional elongation. In turn, more efficient transcription of the HIV genome, including Tat, generates a Tat-dependent positive feedback loop [15].

Tat also leads to the remodeling of nuc-1 [5,35], the nucleosome positioned immediately downstream of the transcription start site. We and others have reported that Tat recruits the SWI/SNF chromatin-remodeling complex to the HIV LTR to activate transcription [36–39]. SWI/SNF was also shown to promote HIV transcription elongation via a Tat-independent mechanism [40]. INI-1 (hSNF5), a core subunit to all SWI/SNF complexes, was first identified because of its interaction with HIV IN [41]. In addition to its direct involvement in Tat-mediated LTR activation [38,39], INI-1 was shown to restrict early steps of HIV infection [42] and to repress basal LTR activity [43]. Recently, the interaction between the HIV IN and SWI/SNF was suggested to functionally couple the processes of integration and remodeling necessary for integration into stable nucleosomes [44]. However, despite these studies on regulation of the HIV life cycle by SWI/SNF, the mechanism behind the seemingly contradictory involvement of SWI/SNF in regulating various stages of the HIV life cycle (i.e. integration, transcription activation, as well as repression) is not understood. Here we examined the mechanistic role played by SWI/SNF in the establishment and maintenance of HIV latency and its re-activation.

Results

The SWI/SNF and MI2 Family of ATP-Dependent Chromatin Remodeling Enzymes Are Involved in LTR Repression

We and other laboratories previously reported the requirement of SWI/SNF chromatin remodeling complexes on activation of HIV-1 LTR [36–40]. To investigate further the role of ATPdependent remodelers on LTR regulation, we examined the effect of cellular ATP depletion by sodium azide (NaN3) on LTR activity and chromatin remodeling. We treated a Jurkat cell line containing an integrated LTR-GFP virus (Figure S1B and S1C) [45] as well as J-Lat A2 [46], containing an integrated latent LTRTat-IRES-GFP virus (Figure S1D and S1E) with increasing concentrations of NaN_3. Surprisingly, in the absence of Tat expression, addition of NaN_3 was associated with derepression of basal HIV promoter activity (Figure S1C and S1E). To determine whether this derepression was associated with chromatin remodeling of the HIV promoter, we used a restriction enzyme accessibility assay coupled to indirect end-labeling, as described [5]. In this assay, nuc-1 remodeling leads to increased accessibility of the restriction enzyme AflII to its recognition site, generating a novel restriction fragment (Figure S1A). Nuc-1 remodeling was assayed in untreated cells, in cells treated with PMA as a positive control, and in cells treated with NaN_3 (Figure S1B and S1D). We observed nuc-1 remodeling in response to NaN_3 at the same concentrations that induced HIV promoter derepression (Figure S1C and S1E). Thus, with the caveat that toxicity resulting from cellular ATP depletion by NaN3 may be accompanied by nonspecific effects, these observations suggested that ATP dependent chromatin remodeling activity is required to suppress basal promoter activity.

To determine which family of ATP-dependent chromatin remodeling enzymes may be involved in LTR repression, we used siRNAs to deplete the expression of the catalytic subunit of each class of mammalian remodelers. There are four major families of remodelers, each named after their central ATPase: SWI/SNF (BRG1 or BRM), ISWI, CHD/MI2, and INO80 (Figure 1A) [16,17]. Specific siRNAs directed against each catalytic subunit were transfected via nucleofection into J-Lat A2 cells (Figure 1B) leading to the efficient depletion of each factor as shown by Western blot analysis for CHD3, ISWI, BRG1, and BRM and by RT-PCR for INO80 (Figure 1C). Depletion of CHD3 resulted in derepression of latent HIV LTR activity as measured by an increase in GFP expression (Figure 1B). In support of this observation, the CHD3 containing NuRD complex as well as the methyl CpG binding protein MBD2, which is another component of the NuRD complex, were shown to be involved in LTR repression [47,48]. The related CHD1 protein was also shown to repress the HIV LTR [49]. siRNA nucleofection had no non-specific effect on LTR-driven GFP expression. While depletion of INO80, ISWI, or BRM had no affect on LTR activity, we found robust de-repression of latent LTR upon BRG1 depletion.

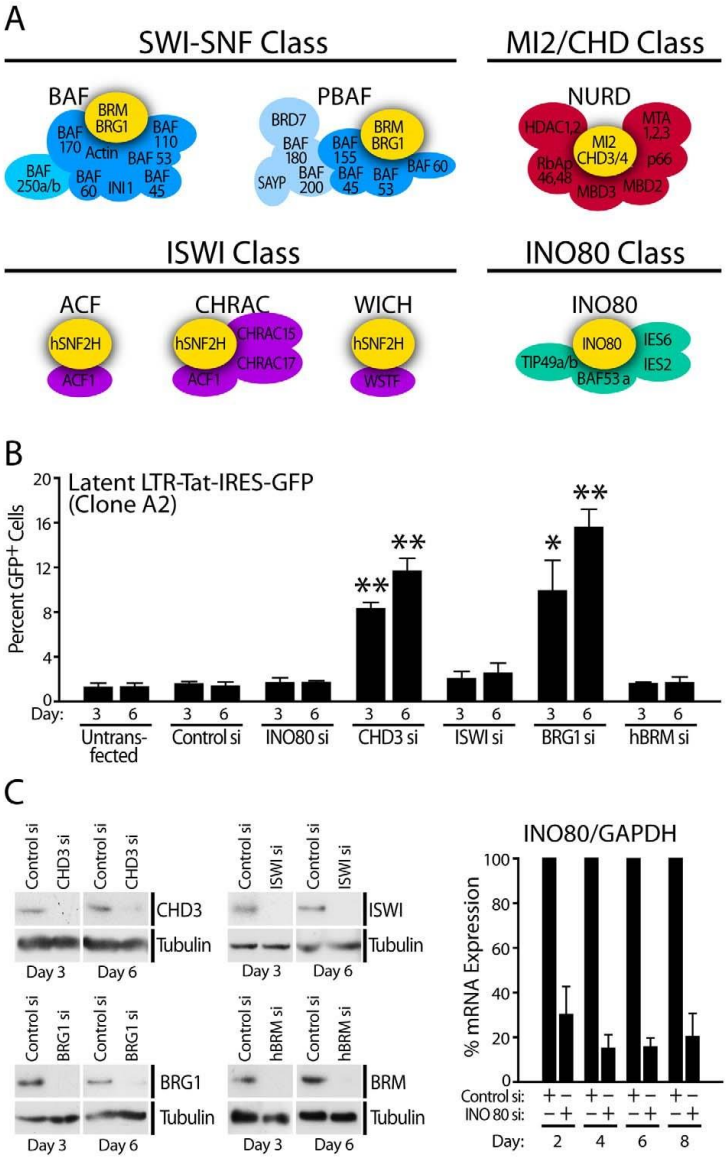

Figure 1. SWI/SNF and MI2 family of ATP-dependent chromatin remodeling enzymes are involved in LTR repression. (A) Four distinct classes of mammalian ATP-dependent chromatin remodeling complexes distinguished by their catalytic subunits, BRG-1/BRM (SWI/SNF), ISWI, INO80, or CHD3/4 (MI2). (B) RNAi depletion of ATPase subunit of each class of ATP-dependent chromatin remodeling complexes as indicated in J-Lat A2 cells containing an integrated latent LTR-Tat-IRES-GFP virus. Cells were nucleofected with control siRNA or siRNAs targeting INO80, CHD3, ISWI, BRG1, and BRM as indicated. GFP expression was monitored by FACS at indicated times post-transfection and is presented as % GFP-positive cells. (C) Western blot analysis to demonstrate depletion of individual ATPase subunits

as indicated 3 and 6 d after transfection of siRNAs. RT-PCR analysis indicated stable depletion of INO80 mRNA up to 8 d after siRNA transfection. Error bars represent the SEM of three independent experiments. * p,0.05, ** p,0.01.

This suggested that a BRG1 containing SWI/SNF complex represses basal HIV promoter activity and may be required for maintaining latency.

Repression of Basal HIV Promoter Activity by the BAF Complex

The HIV transactivator Tat recruits the SWI/SNF ATPdependent chromatin-remodeling complex to the LTR to activate transcription [36–39]. The observation that the HIV preintegration complex interacts with the core SWI/SNF subunit INI-1/ hSNF5 in the cytoplasm before integration [41,44,50] suggested that the SWI/SNF complex might also regulate the activity of the HIV LTR immediately after integration, before Tat accumulation. In support of this notion, INI-1 has been implicated in both LTR activation and repression [38,39,43].

To examine this possibility, and determine which of the compositionally distinct SWI/SNF complexes, BAF or PBAF (Figure 2A), may be responsible for repressing HIV LTR, we used siRNAs to deplete the expression of selected subunits, in two clonal Jurkat cell lines (clones D and E) that contain single integrations of the minimal HIV genome, LTR-GFP virus lacking Tat, and expressing low levels of GFP (Figure 2B and 2C). To dissect the requirements of distinct BAF/PBAF complexes in basal LTRdriven, Tat-independent transcription, siRNAs directed against the core subunits BRG1, BRM, INI-1, the PBAF-specific subunit BAF180, and the BAF-specific subunit BAF250a were transfected via nucleofection leading to the efficient depletion of each factor as shown for Jurkat clone D by Western blot analysis (Figure 2D). We used BAF180 depletion to examine the effect of PBAF on LTRdriven transcription as depletion of the PBAF-specific BAF200 was previously reported to result in disruption of the complex [26]. To ensure that the stability of the complex is not compromised when one component of the complex is depleted by siRNA, we examined the protein levels of the other SWI/SNF subunit by Western blot analysis after depletion of each subunit. SWI/SNF subunit protein levels were unaffected by siRNA depletion of specific subunits (Figure S2). HIV promoter activity was monitored using flow cytometry by measuring changes in Mean Fluorescent Intensity (MFI) of the low basal GFP fluorescence in Jurkat clones D and E for 2 wk after siRNA depletion of SWI/SNF subunits (Figure 2B and 2C). Depletion of BRG1, INI-1, and the BAF-specific BAF250a resulted in derepression of HIV promoter activity as measured by an increase in GFP expression monitored over 14 d. In contrast, depletion of BRM or the PBAFspecific BAF180 did not affect LTR activity (Figures 2B, 2C, and 1B). Derepression of HIV LTR activity peaked at approximately 8 d followed by de novo repression. LTR derepression was tightly correlated with depletion of SWI/SNF subunits as shown by Western blot analysis (Figure 2D). Derepression of basal HIV promoter activity in response to BAF250a depletion suggested that the BAF complex, which is defined by the unique BAF250 subunit, is required for repression of

basal LTR activity. In agreement with this model, depletion of BAF180, unique to PBAF, had no effect on basal repressed HIV promoter activity. Identical results observed with both Jurkat clones containing integrated HIV LTR-GFP demonstrated that this effect occurs independent of the two distinct integration sites.

Latent HIV Is Derepressed Upon BAF Depletion

Suppression by the BAF complex was also observed during a latent HIV infection. SWI/SNF subunits were depleted as described above by siRNA in two Jurkat clonal cell lines, J-Lat A2 and J-Lat 11.1, which harbor latent HIV [46]. J-Lat A2 contains an integrated latent LTR-Tat-IRES-GFP virus (Figure 3A), and J-Lat 11.1 contains a full-length HIV-1 genome expressing GFP in place of Nef (Figure 3B). J-Lat cells are not transcribing, and are therefore GFP negative in the latent state. Because of the absence of Tat expression, this system is ideal to examine the role of chromatin modulators in derepression of latent LTR, which leads to an increase in the percentage of LTR-driven GFP expressing cells. We depleted core and BAF/PBAF-specific SWI/SNF subunits from J-Lat cells by siRNA transfection. To ensure that the BRG-1 complex remains intact following depletion of BAF- or PBAF-specific subunits, we immunoprecipitated BRG1 from J-Lat A2 cell lysates, which were either undepleted or depleted of BAF250 or BAF180 and probed for the presence of BAF/PBAF subunits by Western blot analysis (Figure 3C). Depletion of BAF180 or BAF250 did not affect the binding of the other core or specific SWI/SNF subunits to the BRG-1 immunoprecipitated complexes, supporting the notion that there is no cross-talk between these distinct SWI/SNF complexes. In both J-Lat cell lines, depletion of BRG1, INI-1, or the BAF-specific subunit BAF250a resulted in derepression of HIV expression, as demonstrated by an increase in percent GFP-positive cells. GFP expression peaked 6–10 d after siRNA nucleofection (Figure 3A and 3B) and inversely correlated with expression of BRG1, INI-1, and BAF250a (unpublished data). To address the perceived lag between factor depletion and GFP detection by FACS analysis, which peaked at 6–10 d following siRNA transfection, we performed RT-qPCR time-course analysis of GFP mRNA expression in J-Lat A2 cells after siRNA depletion of BAF250 and BRG-1 (Figure S3A). GFP mRNA was significantly induced as early as 2 d post-siRNA transfection, at the same time point in which significant protein depletion is achieved, and peaked at 4– 5 d following siRNA transfection. Thus the delay in GFP detection by FACS appears to be a matter of accumulation of GFP protein over background levels. We also examined the effect of depletion of BAF250b, a BAF-specific complex component also expressed in Jurkat cells, which is functionally and biochemically distinct from the BAF250a complex [51], on LTR regulation in J-Lat A2 cells (Figure S3B). While depletion of BAF250a and BRG-1 caused robust expression of GFP mRNA, depletion of BAF250b had no effect on LTR transcription. These results demonstrated that the BAF250a-containing BAF complex is specifically required for maintenance of repression of latent HIV.

K50,51 Acetylated Tat Coimmunoprecipitates with and Requires PBAF for Transactivation of HIV Promoter

We and others reported that a SWI/SNF complex associates with Tat to activate the HIV promoter [36–39]. A recent study found that depletion of neither the PBAF-specific BAF180 nor BAF250a had any effect on Tat-mediated LTR activation, while another PBAF-specific component BAF200 was required for Tat activation of the LTR [52]. We and other laboratories have found synergism between the acetyltransferase p300 and SWI/SNF in LTR activation. This activation was found to be dependent on Tat acetylation [36,38]. Our novel data indicating derepression of LTR transcription in response to depletion of BAF-specific subunits suggested that the BAF complex is recruited to the HIV promoter independently of Tat. We therefore sought to determine which SWI/SNF complex, PBAF or BAF, is specifically recruited and required by Tat to activate transcription, and whether Tat acetylation is necessary for this interaction. We performed immunoprecipitation experiments in the J-Lat A2 cells, which express no Tat under basal conditions and epitope-tagged Tat (Tat-FLAG) after reactivation of HIV by PMA (Figure 4A). Using this system we have shown previously that Tat coimmunoprecipitated with INI-1, BRG1, and b-actin, three core subunits shared between BAF and PBAF complexes [38]. Importantly, Tat co-immunoprecipitated with BAF180, but not with BAF250a or the unrelated protein kinase D (Figure 4A). Thus, Tat interacts specifically with the BAF180-containing PBAF complex.

To determine whether the interaction of Tat with PBAF is modulated by acetylation, we cotransfected 293T cells with wildtype or K50R/K51R mutant Tat in the presence or absence of a p300 expression vector (Figure 4B). To prevent Tat deacetylation, cells were treated with nicotinamide and trichostatin A, inhibitors of class III and class I and II histone deacetylases, respectively. We found that Tat association with BAF180 increased in the presence of p300 as shown by co-immunoprecipitation of BAF180 with Tat (Figure 4B). The same treatment markedly increased Tat acetylation (Figure 4B). Importantly, the Tat (K50R/K51R) mutant did not display increased affinity for BAF180 in response to p300 (Figure 4B). Mutation of Tat residues Lys^{50} and Lys^{51} to arginine decreased Tat acetylation significantly, but not completely, consistent with the existence of other Tat acetylation sites [53]. These results support a model in which p300 acetylated Tat specifically recruits the PBAF complex to the HIV LTR. Our data suggested that the distinct SWI/SNF complexes BAF and PBAF might play temporally distinct roles in HIV transcription: Tat-independent basal repression of promoter activity by BAF and Tat-dependent activation of promoter activity by PBAF. To test this model, we examined the effect of selective knockdown of BAF250a and BAF180, unique to each complex, on Tat-independent and Tat-dependent HIV transcription. Jurkat 1G5 cells contain an integrated LTR-luciferase reporter construct and allow convenient monitoring of HIV promoter activity [54]. Cells were first transfected with siRNAs against either BAF180 or BAF250a leading to their efficient depletion (Figure 4C). To probe the effect of BAF/PBAF depletion on Tat-dependent

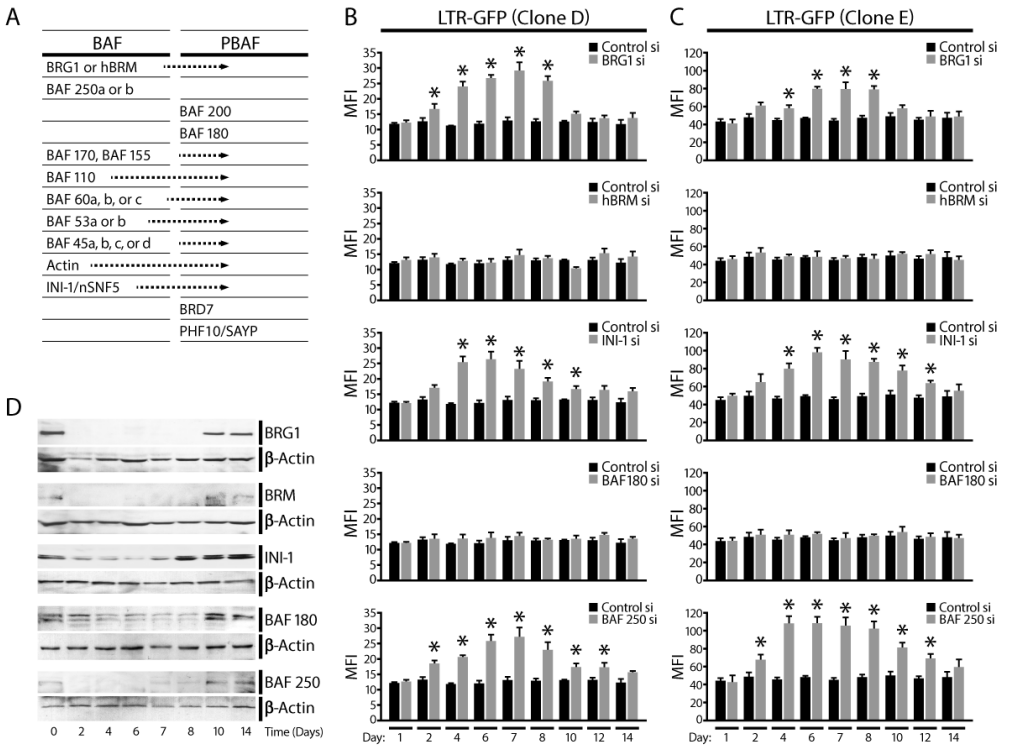

Figure 2. The BAF complex represses basal transcription at the HIV promoter. (A) Table of subunit composition of the two distinct SWI/SNF complexes, BAF and PBAF, in mammals. (B) GFP expression was monitored by flow cytometry at indicated times after siRNA transfection to measure HIV promoter activity. Results are presented as MFI for cells treated with a control siRNA or siRNAs specific for SWI/SNF subunits. (C) Same experiments as shown in (B) for clone D, with clone E, another Jurkat cell line containing an integrated LTR-GFP virus. Error bars represent the SEM of five independent experiments. * p,0.05. (D) Jurkat cells containing an integrated LTR-GFP virus (clone D) were transfected with control siRNA or siRNAs targeting various SWI/SNF complex subunits as indicated. Western blot analysis shows expression of each SWI/SNF subunit after its specific depletion 0, 2, 4, 6, 7, 8, 10, and 14 d after siRNA transfection with each specific antibody and β-actin loading control as indicated

LTR transcription, we introduced Tat exogenously by re-transfecting the cells with an expression vector for Tat or an empty control vector. Nucleofection of siRNA or control vectors had no nonspecific effect on LTR-driven luciferase expression (Figure S4B). As seen before (Figures 2 and 3), depletion of BAF250a and not BAF180 resulted in an increase in basal promoter activity, confirming the repressive role of BAF250a in Tat-independent LTR-driven, luciferase expression in 1G5 cells (Figure 4D). In the presence of Tat, depletion of BAF250a resulted in a significant increase in Tat-mediated activation, suggesting a synergistic effect between Tat expression and loss of the repressive BAF complex on LTR activity. In contrast, depletion of BAF180 suppressed Tatdependent HIV promoter activity at both concentrations of Tat tested (Figure 4D). CMV-driven Tat expression, driven by the CMV

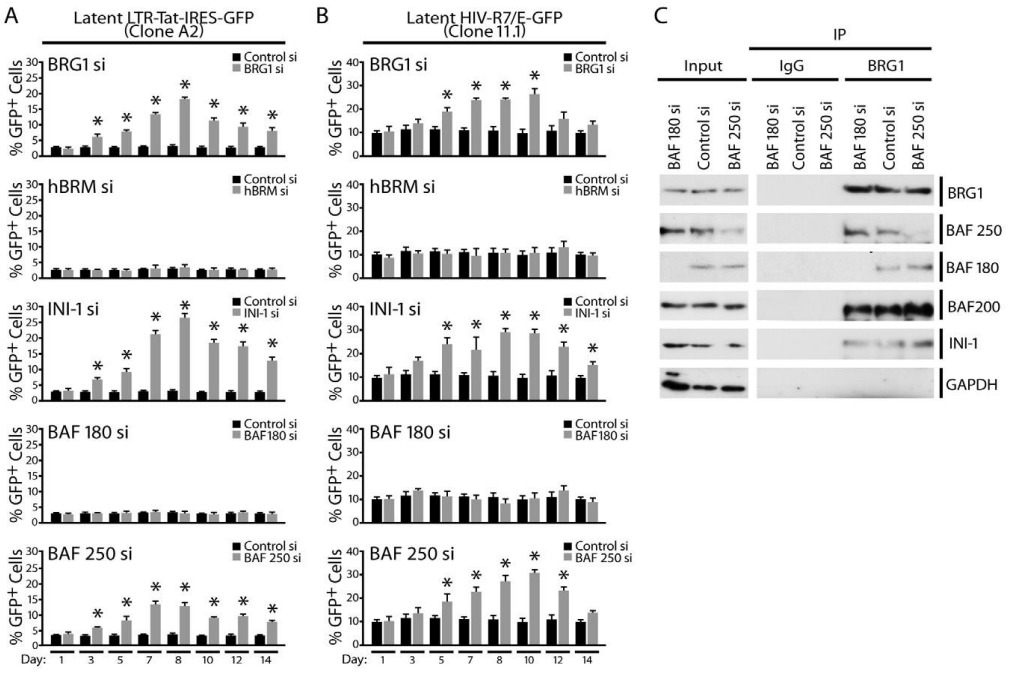

Figure 3. Reactivation of latent HIV mediated by knockdown of BAF subunits. (A) J-Lat A2 cells latently infected with LTR-Tat-IRES GFP virus were transfected with either control siRNA or siRNAs targeting various SWI/SNF subunits as indicated. GFP expression was monitored by FACS at the times indicated after transfection and is expressed as % GFP-positive cells. (B) J-Lat 11.1, clone of Jurkat cells containing a latent integrated full-length HIV virus harboring GFP in place of Nef, was transfected with either control siRNA or siRNAs targeting various SWI/SNF subunits as indicated. GFP expression was monitored by FACS at the times indicated after transfection and is expressed as % GFP-positive cells. Error bars represent the SEM of five independent experiments. * p,0.05. (C) The BRG-1 complex remains intact following depletion of BAF or PBAF-specific subunits. BRG-1 was immunoprecipitated from J-Lat A2 cells transfected with control siRNA or siRNA targeting BAF250 or BAF180, and its associated proteins were examined by SDS-PAGE and Western blotting with antibodies against BRG-1 itself, the BAF or PBAF-specific subunits BAF250a, BAF180 and BAF200, the core subunit INI-1 and GAPDH as control.

Promoter, was not affected by depletion of BAF180 or BAF250a (Figure S4A). We also compared the effect of both wild type and K50,51R mutant Tat in 1G5 cells containing or depleted of BAF180 or BAF250a (Figure S4B). While depletion of BAF180 suppressed optimal LTR activation by wild type Tat, it had no significant suppressive effect on LTR activation by K50,51R mutant Tat, supporting the notion that acetylated Tat recruits PBAF to facilitate LTR activation. Altogether, these results indicate that PBAF is specifically required for optimal Tat mediated transactivation of the HIV promoter.

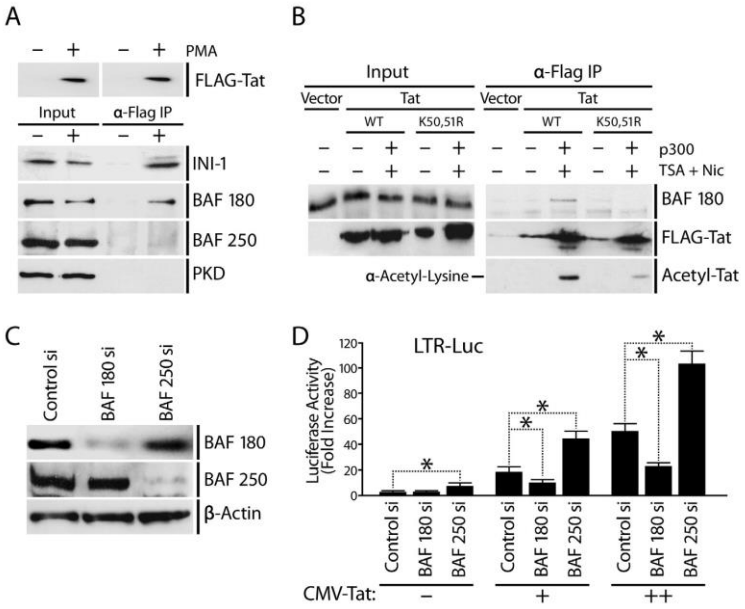

Figure 4. PBAF, recruited by K50K51 acetylated Tat, is a co-factor for Tat activation of the HIV promoter. (A) J-Lat A2 cells containing an integrated LTR-Tat-FLAG-GFP were stimulated with PMA to induce expression of Tat-FLAG. Tat was immunoprecipitated from untreated or PMAstimulated cell lysates and its associated proteins were examined by SDS-PAGE and Western blotting with antibodies against the BAF- or PBAFspecific subunits BAF250a and BAF180, and protein kinase D-1 and 14-3-3 as controls. (B) Tat co-immunoprecipitation with BAF180 is modulated by Tat acetylation. Tat (wild-type or K50R/L51R) was immunoprecipitated using anti-FLAG antibody and analyzed by Western blotting using antibody specific for BAF180. Tat acetylation levels were assessed using an anti-acetyl lysine antibody. All proteins were expressed at similar levels under the different experimental conditions as shown by the Inputs. (C) 1G5 Jurkat cells containing integrated LTR-Luciferase (LTR-Luc) were nucleofected with siRNAs against BAF180, BAF250, or with a control siRNA pool. Expression of BAF180, BAF250, and b-actin was analyzed by Western blotting after depletion of either BAF180 or BAF250. (D) Transactivation of the HIV promoter by Tat is reduced in the absence of BAF180. 48 h after siRNA depletion of BAF180 or BAF250, cells were re-transfected with either a control or Tat-expression vector (CMV-driven), and luciferase assay performed after 24 h. Error bars represent the SEM of three independent experiments. * p,0.05. (D).

Direct Binding of Distinct SWI/SNF Complexes to the HIV Promoter Before and After Transcriptional Activation

Our results suggested that distinct subunits of the SWI/SNF complexes are recruited to the HIV promoter in the absence and presence of Tat. We used a combinatorial approach based on formaldehyde crosslinking to determine the nucleosome density and DNA accessibility at the LTR, and to demonstrate direct interaction of SWI/SNF subunits with the HIV promoter (Figure 5A). Chromatin from J-Lat 11.1, containing a latent integrated full-length HIV virus (Figure 5) [46] or J-Lat A2 (Figure S5), was prepared from cells at 0, 1, and 12 h after addition of PMA to culture supernatants. Following formaldehyde crosslinking, we fragmented

chromatin by sonication, because it allows the isolation of both nucleosome-bound DNA and nucleosomefree DNA. To be able to distinguish between the nuc-0, DHS1, nuc-1, and nuc-2 regions within the LTR by qPCR, we sonicated the chromatin extensively to obtain small fragments of approximately 100–250 base pairs in size. To determine nucleosome occupancy, we performed chromatin immunoprecipitation experiments (ChIPs) using antibodies against histones H2B and H3 (Figure 5A and 5B). To independently map regions within the HIV LTR that are depleted of nucleosomes, we used FAIRE (Formaldehyde Assisted Isolation of Regulatory Elements) (Figure 5A and 5C). FAIRE relies on a phenol-chloroform extraction to isolate ''nucleosome-free'' DNA fragments that are not cross-linked to histones, and thus provides a complementary approach independent of antibodies, to examine chromatin structure. To examine recruitment of SWI/SNF complexes to the LTR, we subjected the sonicated chromatin to immunoprecipitation with BRG1, the BAF-specific BAF250a, and the PBAFspecific BAF200 and BAF180 antibodies (Figure 5A and 5D). The immunoprecipitated or phenol:chloroform extracted DNA was analyzed by qPCR with primer pairs specific for the nuc-0, DHS1, and nuc-1 regions of the HIV promoter (Figure 5A).

As shown in Figure 5C, PMA stimulation caused a dramatic increase in DNA accessibility, observed over the positioned repressive nuc-1 and encompassing nuc-0, DHS1, and nuc-2 albeit to a lesser extent. This observed increase in DNA accessibility in response to PMA stimulation is accompanied by a loss of histones bound to nuc-1 as determined by H2B and H3 ChIPs (Figure 5B). The ChIP results using antibodies directed against either histone H3 or H2B correlated well with each other. In agreement with the functional data discussed above and the proposed repressive role of BAF on the HIV promoter, we detected the BAF-specific subunit BAF250 and BRG1 bound to the HIV promoter nuc-1 under basal conditions. A remarkable switch in specific SWI/SNF subunits occurred in response to PMA: the BAF-specific subunit BAF250 was lost from the HIV promoter, while the PBAF-specific subunits, BAF180 and BAF200, were recruited during the transcriptional activation of the HIV promoter (Figure 5D). Compared to the control locus, BRG1 was enriched on the HIV promoter in its repressed state and slightly enriched in response to PMA stimulation. Thus, the PBAF complex is absent from the HIV promoter under basal conditions but recruited to the HIV promoter in response to PMA stimulation, while BAF is directly associated with the HIV LTR and required to maintain repression of the HIV promoter in the absence of Tat.

BAF Is Essential for Positioning the Repressive nuc-1 of HIV LTR

We next investigated the mechanism by which the BAF complex represses HIV LTR activity. One intriguing possibility was that nuc-1 positioning downstream of the transcription start site might be an active process driven in part by BAF activity, ATP hydrolysis. Our observation that nuc-1 becomes remodeled upon ATP depletion (Figure S1) was consistent with this

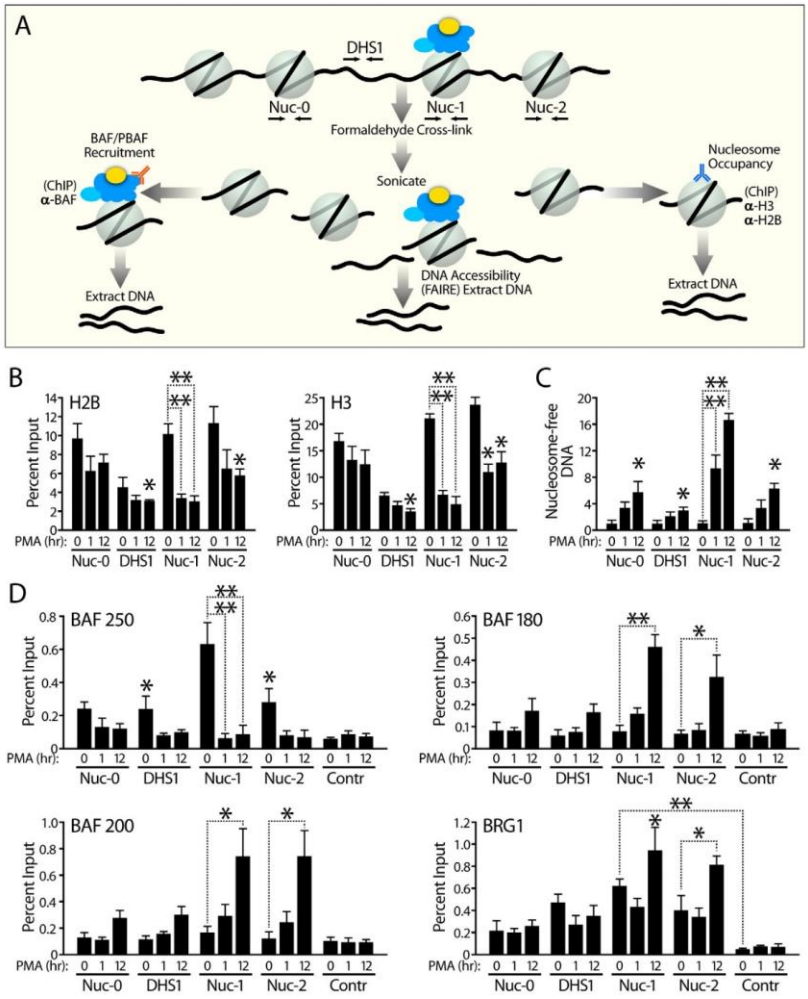

Figure 5. Direct binding of distinct SWI/SNF complexes to the HIV promoter before and after transcriptional activation. (A) Schematic representation of strategy to explore nucleosome position changes and enrichment of SWI/SNF at the HIV LTR in its repressed state and after PMA stimulation. J-Lat 11.1 cells at 0, 1, and 12 h after PMA addition were crosslinked and sonicated to yield fragments of approximately 150 bp. DNA accessibility was monitored by FAIRE while nucleosome occupancy was determined by histone H3 and H2B ChIPs. To determine enrichment of SWI/ SNF complexes, we performed ChIPs with antibodies specific for BAF250, BAF180, BAF200, and BRG1. (B) PMA stimulation causes a reduction in histone density over HIV nuc-1 as determined by H3 and H2B ChIPs. Histone ChIPs are presented as percent immunoprecipitated DNA over input. (C) PMA stimulation is accompanied by an increase in DNA accessibility over the positioned nuc-1 of the HIV LTR. FAIRE results are presented as fold change respective to the unstimulated value (normalized to 1) for each primer pair. (D) BAF250a-specific BAF complex directly binds to nuc-1 in its repressed state, while BAF180 and BAF200-specific PBAF is recruited to nuc-1 upon PMA stimulation. SWI/SNF subunit ChIPs are presented as percent immunoprecipitated DNA over input. Immunoprecipitated DNA from ChIPs and phenol:chloroform extracted DNA from FAIRE were analyzed by qPCR using primer pairs specific for the HIV LTR nuc-0, DHS1, nuc-1, and nuc-2 regions, and control region amplifying upstream of the Axin2 gene. For all ChIP and FAIRE experiments, error bars represent the SEM of at least three independent experiments. * p,0.05, ** p,0.01. We depict results for J-Lat11.1. Similar results were obtained for J-Lat A2(Figure S5).

Model. First we examined the propensity for nucleosome formation of the DNA sequence encompassing the first 1,800 base pairs of the HIV LTR including the positioned nucleosomes nuc-0, nuc-1, nuc-2, and the DNase hypersensitive sites (DHS1 and DHS2) in between (Figure 6A). We determined histone binding affinity score (nucleosome score) as the log likelihood ratio for the given region to be a nucleosome versus a linker using NuPoP software tool [55]. Similar results were obtained with an alternative algorithm (Figure S6A) [56,57]. The mean value for histone affinity score as shown by the blue line and standard deviation shown in red are indicated as reference to the known preferred genomic sites of HIV integration [58]. Comparison of the predicted affinities to the known in vivo positioning of nucleosomes within the HIV LTR [5,6] demonstrated a remarkable opposite correlation (Figure 6A). The region with the highest predicted propensity for nucleosome formation encompasses the DHS1, the nucleosome-free region found between the positioned nucleosomes nuc-0 and nuc-1. Conversely, sequences encompassing nuc0 and nuc-1 have a lower nucleosome score than the DHS1. The positioned nuc-2 and DHS2 separating it from nuc-1 also appear to be the mirror image of the nucleosome score predicted by their DNA sequences (Figure 6A). The negative correlation between predicted and actual nucleosome positioning in the HIV LTR together with the presence and functional requirement of BAF on nuc-1 in the repressed state suggested that BAF may counteract DNA sequence effects to place the repressive nuc-1 in a thermodynamically suboptimal position.

We next sought to probe the role of BAF in nucleosome positioning within the LTR using a siRNA approach to selectively deplete BAF and PBAF subunits. To examine the impact of depletion of BAF on local chromatin structure at the HIV LTR, we first performed FAIRE experiments (Figure 6B and S6B). Using FAIRE we assessed changes in DNA accessibility at the HIV LTR following depletion of BAF and PBAF subunits (Figure 6B). Strikingly, there was a sharp peak in DNA accessibility at nuc-1 following loss of BAF250 and BRG1, accompanied by a decrease in DNA accessibility over DHS1 (Figure 6B and S6B). In contrast, knock-down of BAF180 or BAF200 had no significant effect on DNA accessibility at the HIV LTR (Figure 6B and S6B). We next examined nucleosome density by ChIP-qPCR using antibodies specific for H2B and H3 combined with RNAimediated protein depletion. Complementing the FAIRE data, depletion of BAF250 and BRG1, but not BAF200 or BAF180, caused a strong loss of histone H2B and H3 at nuc-1, which is concomitant with increased DNA accessibility at this position (Figure 6C and S6C). Interestingly, loss of histone density at nuc-1 was accompanied by an increase in histone density within the DHS1 region (Figure 6C and S6C).

To examine if knock-down of individual subunits affects recruitment of the complexes to the LTR, we performed ChIPs to monitor BRG-1 enrichment at the LTR in BAF180 or BAF250depleted J-Lat 11.1 cells in the latent versus PMA stimulated states (Figure 6D). We found that depletion of BAF250 abrogated complex recruitment to the LTR in the latent state but had no effect on recruitment of PBAF to nuc-1 in response to PMA stimulation. Vice versa,

BAF180 depletion abrogated PMA dependent recruitment of the BRG-1 complex to nuc-1, but had no effect on the enrichment of BAF at the LTR nuc-1 in the latent state (Figure 6D). Thus, while BAF- and PBAF-specific factors appear to be targeting subunits required for complex recruitment to the LTR, they do not affect the recruitment of the other functionally distinct complex to the LTR. Together our data indicate that while PBAF is recruited by Tat to the HIV LTR and required for Tat-mediated activation, the BAF complex is essential for repression of basal LTR activity by countering histone-DNA sequence preferences at the LTR and positioning the repressive nuc-1 over less optimal sequences immediately downstream of the transcription start site.

High Resolution Nucleosomal Mapping Reveals a Dramatically Altered LTR Chromatin Structure After Depletion of BAF250

Our data thus far demonstrated a critical role for the BAF250a containing BAF chromatin remodeling complex in actively maintaining a repressive nucleosomal structure at the HIV LTR. To obtain a higher resolution picture of the dynamic changes in LTR nucleosomal structure in the presence or absence of BAF250, we performed high resolution MNase nucleosomal mapping, as previously described [59,60]. We made slight modifications to this protocol to be able to reproducibly visualize in vivo changes in the LTR chromatin structure in the lower numbers of cells obtainable after depletion of BAF250 by siRNA nucelofection. After formaldehyde cross-linking, the isolated chromatin was divided into undigested and MNase digested samples. Digested and undigested DNA was then probed with 20 separate overlapping primer sets, amplifying approximately 100 bp regions along the HIV LTR (Figure 7A and Table S1). MNase cleaves nucleosomefree and linker DNA connecting two nucleosomes, while DNA within nucleosomes is at least partially protected and resistant to digestion. Therefore, the amount of DNA remaining between two primers after MNase digestion, which determines the ability of a primer pair to amplify that region by real-time qPCR, can be used to show the amount of digestion in a particular amplicon at the HIV LTR. We calculated the relative ratio of the amount of digested DNA to the undigested control for each overlapping primer pair scanning the length of the HIV LTR (Figure 7A and 7B). We examined first the LTR nucleosomal profile in J-lat 11.1 cells which were either unstimulated or treated with PMA for 1 or 12 h (Figure 7B red line). As shown previously [5–7], we find that under unstimulated conditions, the HIV LTR contains two distinct chromatin regions: a nucleosome positioned immediately after the TSS (Nuc-1), and a second nucleosome (Nuc-0) at the 59 end of the LTR. 39 to the LTR, there is another strictly positioned nucleosome (Nuc-2) as well as an intervening MNase hypersensitive DNA region between the positioned Nuc-1 and Nuc-2. Surprisingly, we found that DHS1 (nucleotides 200–452) connecting Nuc-0 and Nuc-1 was not devoid of nucleosomes, but rather contained poorly positioned nucleosomes as determined by partial protection from MNase digestion (Figure 7B and 7C). This area,

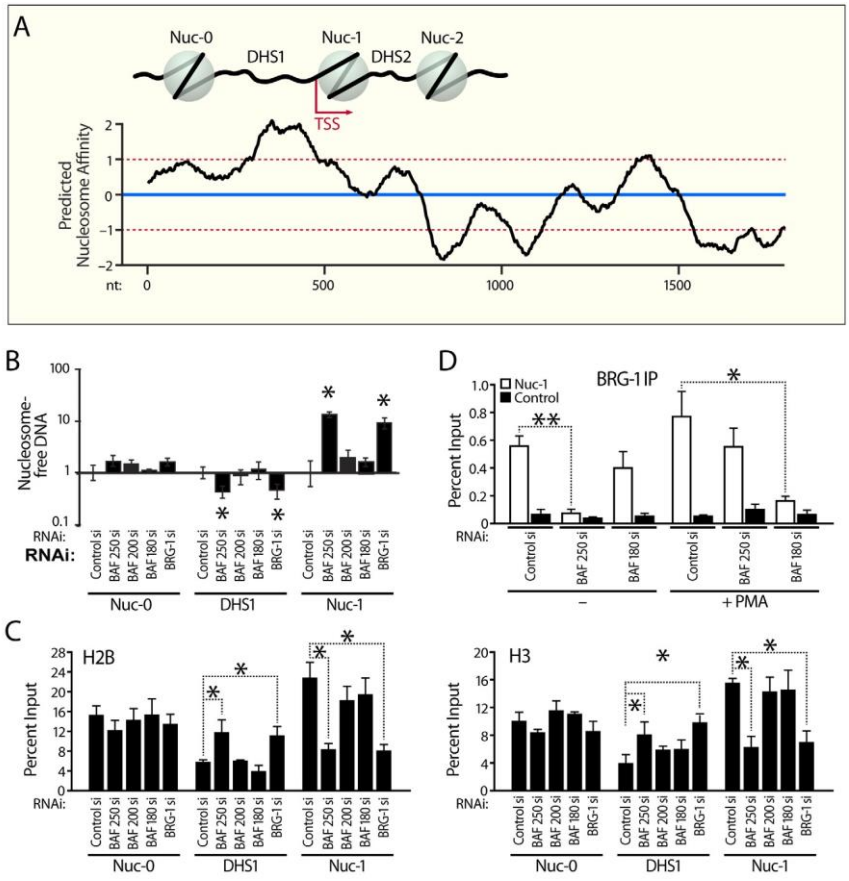

Figure 6. BAF represses HIV transcription by positioning nuc-1 of the HIV LTR. (A) Location of the strictly positioned nucleosomes correlates negatively with the predicted histone binding affinity score (nucleosome score) of the DNA sequence encompassing the HIV LTR. Predicted nucleosome affinity for HIV nucleotide sequence 1–1800 was determined using the algorithm described [55]. Similar results were obtained with an alternative algorithm described (Figure S6) [56,57]. Means and standard deviations for nucleosome score at insertion sites are indicated by blue (mean) and red lines (mean 6 SD) and give reference to known genomic sites of HIV integration [58]. (B) Depletion BAF250 and BRG1 results in a peak in DNA accessibility over positioned nuc-1 and decreased accessibility over DHS1 of the HIV LTR. J-Lat 11.1 cells were nucleofected with either nontargeting siRNA or siRNAs targeting individual SWI/SNF subunits as indicated and subjected to FAIRE. FAIRE results are presented as fold change respective to value obtained for control siRNA transfected cells (normalized to 1) for each primer pair. (C) Depletion of BAF results in a reduction in histone density over the positioned LTR nuc-1 as determined by H3 and H2B ChIPs. (D) BAF180 and BAF250 are distinctly required for targeting of the BRG1 complex to the activated and silenced LTR nuc-1, respectively. Depletion of BAF250 abrogates targeting of the repressive BRG-1 complex while BAF180 depletion interferes with recruitment of BRG1 to nuc-1 in response to PMA stimulation. J-Lat 11.1 cells were nucleofected with either nontargeting siRNA or siRNAs targeting BAF250 or BAF180 as indicated. Depleted cells were then subjected to ChIPs using an antibody specific for BRG1. BRG1 ChIPs were analyzed by qPCR using primer pairs specific for the LTR nuc-1 and control region amplifying upstream of the Axin2 gene and are presented as percent immunoprecipitated DNA over Input. For all ChIP and FAIRE experiments, error bars represent the SEM of at least three independent experiments. * p,0.05, ** p,0.01. We depict results for J-Lat 11.1. Similar results were obtained for J-Lat A2 (Figure S6).

previously demonstrated to be hypersensitive to nuclease digestion, contains consensus binding sites for a range of host cell transcription factors critical for LTR activity. PMA stimulation at 1 h caused a striking decrease in DNA protection between the positioned Nuc-0 and Nuc-2, indicating a loss of nucleosomes within this region including the positioned Nuc-1 (Figure 7B, light green line). At 12 h of PMA stimulation, a more significant and broader loss of nucleosomal DNA protection occurred downstream of the positioned Nuc-0, which now included the positioned Nuc-2 (Figure 7B, dark green line).

We next examined what effect depletion of BAF250a has on the observed high resolution LTR nucleosomal structure. We depleted BAF250 from J-Lat 11.1 cells using siRNA transfection and compared the resulting LTR nucleosomal profile to that of cells transfected with control siRNA (Figure 7D). As expected, nucleofection of cells with control siRNA had no effect on the LTR nucleosomal structure (Figure 7D, red and brown lines). However, siRNA depletion of BAF250 resulted in a dramatically altered LTR chromatin structure (Figure 7D, blue line). Confirming our results from FAIRE experiments, depletion of BAF250 coincided with a loss of the strictly positioned Nuc-1. Interestingly, the sequences within the previously described DHS1 (between Nuc-0 and Nuc-1) and DHS2 (between Nuc1 and Nuc-2) showed significantly more resistance to MNase digestion in the BAF250-depleted samples. Thus, the nucleosomal landscape of BAF250a-depleted J-Lat cells more closely resembles the predicted nucleosome positioning within the LTR (Figure 6A), which is determined by intrinsic histone-DNA sequence preferences. Thus, confirming and extending our data obtained from FAIRE and histone ChIP experiments (Figures 6 and S6), the observed changes in the high resolution MNase chromatin profile of the LTR in response to BAF250a depletion demonstrates a loss of histones within Nuc-1 concomitant with an increase in histone density over the DHS1.

Depletion of BAF Decreases the Incidence of Latent HIV Infections in Jurkat and SupT1 Cells

The de-repression of latent infections observed in J-LatA2 and J-Lat 11.1 cells in response to depletion of the BAF complex subunits BAF250, INI-1, and BRG1 indicated that the BAF complex is necessary to maintain silencing at the LTR during a latent infection (Figure 3). Since depletion of BAF resulted in the loss of the positioned repressive nuc-1 (Figure 6), we wondered whether BAF contributes to the establishment of latent HIV infections by positioning nuc-1 and repressing basal HIV transcription. We therefore tested whether depletion of BAF would decrease the establishment of latent infections. We used siRNAs to deplete BAF250, or the core subunits BRG1 or INI-1, or the PBAF-specific subunits BAF200 and BAF180 from Jurkat cells (Figure 8A). In parallel we examined the effect of BAF/PBAF depletion in latency establishment in another CD4+T cell line, SupT1 cells (Figure S7). Using a strategy we and others described previously [46,61], we then compared the percentage of

latent HIV infections established in the presence or absence of BAF/ PBAF subunits (Figures 8B and S7A). Briefly, we infected Jurkat or SupT1 cells at low multiplicity of infection with an

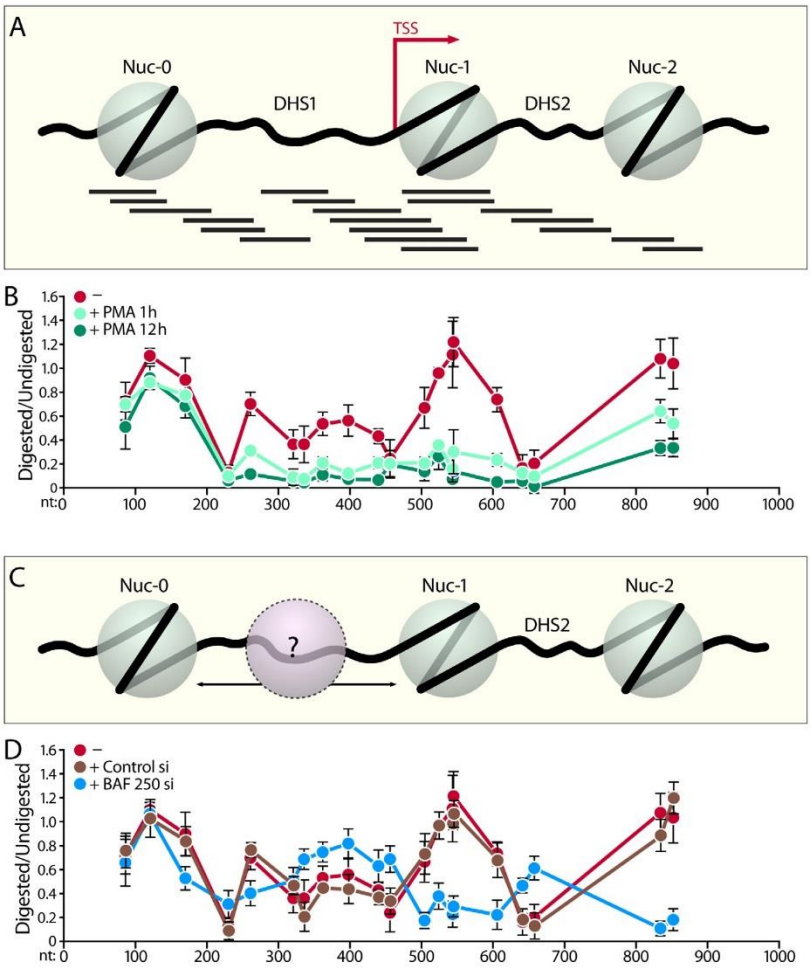

Figure 7. BAF250 depletion induces change in chromatin structure at the HIV LTR as detected by High Resolution MNase nucleosomal mapping. (A) Diagram showing the PCR amplicons used at the HIV LTR covering nucleotides 40–902 corresponding to Nuc0, DHS1, Nuc-1, DHS2, and Nuc-2. PCR products are 100610 bp in size and are spaced approximately 30 bp apart. (B) Change in chromatin structure of the HIV LTR in J-Lat 11.1 cells upon PMA stimulation. The chromatin profile of the HIV LTR was determined at 0 (red line), 1 h (light green line), and 12 h (dark green line) post-PMA stimulation by normalizing the amount of the MNase digested PCR product to that of the undigested product using the DC(t) method (y-axis), which is plotted against the midpoint of the corresponding PCR amplicon (x-axis). The x-axis represents base pair units with 0 as the start of LTR Nuc-0. Error bars represent the average of three independent experiments. (C) A loosely positioned nucleosome between the positioned Nuc-0 and nuc-1 is indicated in pink. (D) Depletion of BAF250 induces restructuring of the HIV LTR chromatin profile. The chromatin profile of the HIV LTR was determined in J-Lat 11.1 cells nucleofected with either control siRNA (brown line) or siRNA targeting BAF250 (blue line). The chromatin profile of untransfected cells are also provided for comparison (red line). Error bars represent the SEM of three independent experiments.

HIV-1 derived virus containing a GFP reporter, LTR-Tat-IRES-GFP. Percent productive infections were scored as percent GFP positive cells 72 h after infection with virus (Figures 8B, 8C, S7A and S7B). Using Flow Cytometry (FACS) we sorted the GFP negative population, which presumably contained uninfected as well as latently infected cells. Treatment of the GFP negative population with PMA led to activation and GFP expression of the latently infected population, which was analyzed and quantitated by FACS (Figures 8B, 8D, S7A and S7C). As shown in Figures 8C and S7B, the percentage of productive infections was slightly decreased in the absence of BAF/PBAF subunits. However, depletion of the core subunits INI-1 or BRG1 or the BAF-specific subunit BAF250 resulted in a significant, greater than 50% lower incidence of latent infections in both Jurkat and SupT1 cells, while depletion of PBAF-specific subunits had no significant effect on latency establishment (Figures 8D and S7C). We also tested whether similar to BAF, depletion of CHD3 also decreases the incidence of latent infections (Figure S8). Using siRNAs we depleted CHD3, BAF250, and BRG-1 either individually or simultaneously in both Jurkat and SupT1 cells (Figure S8). Similar to BAF250 and BRG-1 depletion, depletion of CHD3 resulted in decreased incidence of latent infections. However, we found no synergistic effect on latency establishment when both BAF250 and CHD3 or BRG1 and CHD3 were simultaneously depleted by siRNA transfection. These results suggest that the BAF complex contributes to the establishment of latent infections and points to the ATP-dependent enzyme BRG1 as a putative therapeutic target to deplete the latent HIV-infected reservoir in infected patients.

Discussion

Our results suggest a novel model for the regulation of HIV transcription and the role of the SWI/SNF chromatin-remodeling complex (Figure 9). We find that, on the HIV LTR, active chromatin remodeling is required for the generation of a chromatin conformation that is repressive to transcription; the BAF complex strictly positions nuc-1. Thus, in the absence of Tat, BAF is bound to the HIV promoter where it represses transcription by counteracting intrinsic nucleosome-DNA sequence preferences and positioning nuc-1 in a less energetically favorable position immediately downstream of the TSS. Upon activation, BAF is removed from the LTR, allowing the formation of nucleosomes according to their intrinsic histone-DNA sequence preferences. Upon expression, Tat first recruits acetyltransferases resulting in the acetylation of promoter histones as well as Tat itself [62–66]. Intriguingly, another SWI/SNF complex, PBAF, specifically interacts with acetylated Tat and is recruited to the HIV promoter by Tat in vivo. Thus, the biochemically distinct chromatin-remodeling complexes BAF and PBAF display functional specificity on the HIV promoter, one repressive and the other participating in the transcriptional activation of the HIV promoter by Tat. This model presents a considerably more complex picture of the role of SWI/SNF proteins in the transcriptional regulation of HIV expression than had previously been anticipated.

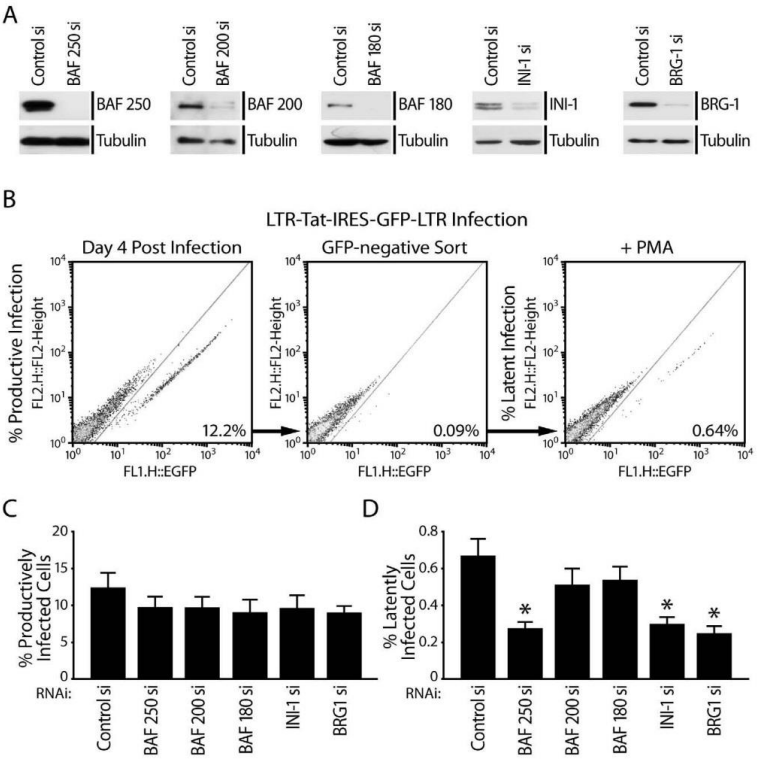

Figure 8. BAF promotes the establishment of latent HIV infections. (A) Western blot analysis demonstrates depletion of BAF/PBAF subunits as indicated 96 h after siRNA transfection. (B) Schematic representation of protocol for comparison of productive and latent infections in Jurkat cells containing or depleted of remodeling complex subunits. Jurkat cells were first nucleofected with control siRNA or siRNA targeting individual SWI/SNF subunits as indicated. After 48 h, cells were infected with retroviral particles containing the vector LTR-Tat-IRES-GFP. The percentage of productive infections (GFP-positive cells obtained after infection (12.2%) (left panel)), FACS-sorted GFP-negative cells (middle panel), or percent latent infections (GFP-positive cells obtained after PMA treatment of sorted GFP-negative cells (0.64%) (right panel)) are shown for Jurkat cells nucleofected with control siRNA. (C) Depletion of BAF/PBAF subunits as indicated does not significantly affect the percentage of productive HIV infections. (D) Depletion of the core SWI/SNF subunits BRG1 and INI-1 and the BAF-specific subunit BAF250 significantly decreases the incidence of latent HIV infections (the percent GFP positive cells obtained after PMA stimulation of GFP negative cell population) while depletion of PBAF-specific subunits BAF200 or BAF180 had no significant effect on latency establishment.

The involvement of the BAF complex in repressing basal HIV transcription raises a number of novel questions. Intriguingly, HIV-1 integrase interacts with the SWI/SNF protein INI-1 in vitro and in vivo [41]. During HIV infection, incoming retroviral pre-integration complexes trigger the cytoplasmic export of the SWI/SNF component INI1 and of the nuclear body constituent PML [50]. The HIV genome associates with these proteins before nuclear migration. In the presence of arsenic, PML is sequestered in the nucleus and the INI-1/preintegration complex interaction is disrupted [50]. Under these conditions, the efficiency of HIVmediated transduction is markedly increased. This observation could be explained in

part by our observations that the BAF complex represses basal HIV transcription by positioning the repressive nuc-1.

Why would the HIV virus position a repressive nucleosome immediately downstream of its transcriptional start site? An attractive possibility is that BAF may remove or pull the thermodynamically favored nucleosome away from the promoter (DHS1) to allow for binding of host cell transcription factors whose binding sites are present within the DHS1. In particular, the NFKB and Sp1 consensus sites within the DHS1 have been shown to be critical for basal HIV LTR promoter activity [67]. In this context, positioning of nuc-1 by BAF downstream of the TSS would make consensus sites within the DHS1 accessible for binding by sequence-specific transcription factors, and allow for the assembly of the initiation complex. Surprisingly, our highresolution MNase nucleosomal mapping of the LTR demonstrated that DHS1 is not devoid of nucleosomes as previously implied. The DNA within this region displayed partial protection against MNase digestion, indicating the presence of loosely positioned nucleosomes, which disappeared upon PMA stimulation. Interestingly, a previous study by Workman and colleagues argued for the presence of a nucleosome over the HIV promoter (DHS1) and the formation of a ternary complex consisting of the transcription factors, histones, and DNA [68]. It is important to note that in this study the HIV promoter/DHS1 sequence was reconstituted into mono-nucleosomes or placed within an array of positioned nucleosomes in vitro and outside the context of the adjacent nuc-0 and nuc-1 sequences [68]. Our modeling data, depicting the high predicted affinity (nucelosome score) of the DHS1 DNA sequence (Figure 6A), are in agreement with these observations implying the presence of a nucleosome over the DHS1 region in vitro.

Our high-resolution MNase nucleosomal mapping data provide a detailed picture of the dynamic nucleosomal landscape of the HIV LTR, comparing the latent, PMA activated, and BAFdepleted de-repressed LTR states. Loss of BAF250a caused a dramatic re-positioning of the nucleosomes according to their intrinsic DNA-histone sequence preference; DNA encompassing nuc-1 became hypersensitive and susceptible to digestion by MNase while the DHS sites were rendered less accessible and protected from MNase, more closely resembling the predicted LTR nucleosomal structure (Figure 6A). These results support a nucleosome repositioning model upon BAF250a depletion, which contrasts with the observed eviction of nucleosomes downstream of the positioned Nuc-0, which occurs after PMA stimulation.

An important question remaining to be resolved is how BAF is recruited to the LTR to position nuc-1. SWI/SNF complexes have been shown to function gene-specifically, recruited by sequencespecific transcription factors to regulatory regions of target genes. The HIV LTR contains many binding sites for multiple sequencespecific host transcription factors, including SP1, NFkb, YY-1, NFAT, LBP1, etc. One possible mechanism is the recruitment of BAF by a repressive sequence-specific transcription factor bound to nuc-1. A number of transcriptional repressors contain binding sites within the region occupied by nuc-1, including LSF-1 and YY-1

[69]. Indeed, the transcriptional repressor YY-1 was bound to the HIV promoter under basal conditions and was displaced in response to Tat expression [38]. Thus, YY-1 is a candidate transcription factor, which may recruit BAF to the HIV LTR to position the repressive nuc-1. In support of this possibility, pleiohomeotic (PHO), the Drosophila homologue of YY-1, has been shown to directly recruit the SWI/SNF complex to target genes [70]. Conversely, the BAF complex, which itself may be recruited by another sequence-specific transcription factor, may allow for YY-1 binding, leading to de-acetylation of histones at the LTR, and the repression of HIV transcription under basal conditions.

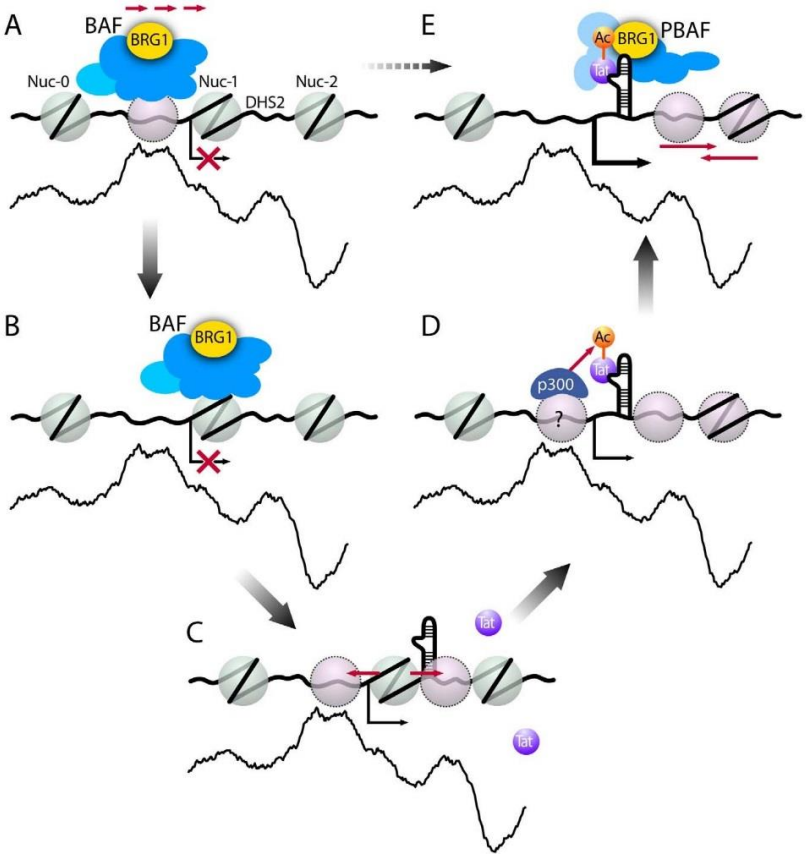

Figure 9. Model for SWI/SNF regulation of HIV LTR transcription. BAF uses energy from ATP hydrolysis to actively counteract intrinsic histone-DNA sequence preferences within HIV LTR. (A–B) BAF pulls/pushes a preferred nucleosome over DHS1 onto DNA sequences less favorable for nucleosome formation immediately downstream of the transcription start site (TSS), leading to positioning of nuc-1 and transcriptional repression. (C) Upon activation, BAF dissociates from the LTR resulting in re-positioning of the nucleosomes to thermodynamically more favorable positions leading to de-repression of HIV transcription. (D) Upon Tat expression, p300, recruited to the LTR, acetylates Tat. (E) p300-acetylated Tat then selectively recruits the PBAF complex, which uses energy from ATP hydrolysis to actively re-position nucleosomes formed downstream of TSS enabling efficient transcription elongation.

Our results indicate that BAF-remodeling activity is necessary to position nuc-1 downstream of the transcriptional start site. The fact that nuc-1 becomes remodeled upon ATP depletion is consistent with this model and, within the limitations of this Complementing the ChIPs, we used FAIRE to assess changes in DNA accessibility within nuc-0, DHS1, and nuc-1 regions of the HIV LTR in response to specific depletion of BAF or PBAF. The mapping of accessible DNA within the LTR by FAIRE negatively correlated with histone H3 and H2B occupancy. Our data indicate that the specific depletion of BAF causes nucleosome repositioning in accordance with intrinsic histone-DNA sequence preferences; in the absence of BAF, the DHS1 region, whose sequence displays a higher propensity for nucleosome formation, displays higher histone density and lower accessibility, while the opposite profile is observed for the DNA sequence encompassing nuc-1. These observations suggest that the energetic cost of positioning the repressive nuc-1 downstream of the TSS is provided and driven by ATP hydrolysis. BAF positions nuc-1 by either pushing the nucleosome from its optimal sequence over DHS1 or by pulling it onto sub-optimal sequences encompassing nuc-1. Thus, the BAF complex counteracts and overrules the DNA sequence effects and intrinsically favored nucleosome position over the DHS1 region of the HIV LTR.

The dramatic increase in DNA accessibility over nuc-1 concomitant with de-repression of LTR activity observed upon depletion of the BAF complex begs the following question: why is recruitment of PBAF by Tat necessary to drive transcription at the LTR? In such a context, the recruitment by Tat of an alternative complex, PBAF, may be necessary for remodeling of the nucleosomes, formed in the absence of BAF according to their preferred DNA sequences, downstream of the TSS, leading to efficient transcription elongation (Figure 9). Indeed, BRG1 was recently shown to be recruited to and facilitate RNA Pol II to overcome nucleosomal barriers during transcription elongation in vivo [71].

The balance between activating and repressive cofactors at the LTR is believed to determine the level of basal transcription from the LTR in the immediate early, Tat-independent phase of HIV transcription. The local availability of positive and negatively acting cofactors at the LTR therefore determines the likelihood of transcriptional silencing. Our data demonstrated a critical role for BAF in positioning nuc-1 and maintaining HIV LTR silencing. We found that depletion of BAF led to a de-repression of latent HIV in the J-Lat system reflecting HIV latency. Despite the effectiveness of modern HAART regimens, latent HIV-infected cells persist in patients, providing the main impediment to cure from HIV infection. Recently, the need for the development of new strategies to treat HIV-infected patients has been discussed [72], highlighting the necessity to deplete the latent HIV-infected reservoirs. As the catalytic subunit of the BAF complex, the enzyme BRG1 may present an attractive candidate for drug targeting to purge the latent HIV-infected reservoir in the treatment of HIV.

Materials and Methods

Cell Lines and Plasmids

We used the following cell lines: Jurkat clones D and E (containing integrated LTR-GFP), J-Lat A2 (integrated latent LTR-Tat-IRES GFP), J-Lat 11.1 (integrated latent full-length HIV genome containing a mutation in the env gene and GFP in place of the nef gene) [45,46], and Jurkat 1G5 cells containing integrated LTR-Luciferase [54]. The HIV LTR-luciferase reporter construct (pEV229), the CMV-driven expression vectors for FLAG-tagged wild-type Tat (pEV280), FLAG-tagged mutant Tat(K50R/K51R) (pEV538), and p300 have been described [62]. Plasmids used to generate HIV-derived virus particles, vesicular stomatitis virus envelope (VSVG), the NL4-3 packaging vector (R8.91), and the retroviral vector LTR-Tat-IRES-EGFP (pEV731), have been previously described [45].

Sodium Azide Treatment and Chromatin-Remodeling Assay

Exponentially growing Jurkat cell line D or J-Lat A2 were treated with increasing concentrations of sodium azide overnight. Restriction enzyme accessibility with AflII was performed on intact nuclei followed by Southern blotting as previously described [45]. Briefly, cells were harvested by centrifugation and washed with ice-cold PBS. The subsequent steps were performed on ice with precooled buffers. Cells (10^7) were resuspended in 400 ml of buffer A (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.3 M sucrose) and incubated on ice for 10 min. An equal volume of buffer A/0.2% NP-40 was added, and cells were incubated for a further 10 min. Nuclei were pelleted at 2406g for 10 min, resuspended in 50 ml of buffer B (10 mM Tris, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, 100 mg/ml BSA, 0.1 mM phenylmethylsulfonyl fluoride), and digested for 20 min with AflII (0.5 U/ml) at 37uC. Digestion reactions were placed on ice, and genomic DNA was purified with DNeasy Tissue kit (Qiagen). The same amounts of DNA from each sample were digested to completion with NcoI. The extent of AflII cleavage was detected by southern blotting. Hybridization was performed with a P^{32}-labeled PCR probe corresponding to a fragment internal to the 5'-LTR generated from pRRL GFP vector by PCR. Primer sequences are provided in Table S1.

Antibodies, Coimmunoprecipitation, and Western Blot Analysis

Anti-BRM, anti-SMARCB1 and anti-SMARCA5/hISWI (Abcam), anti-SMARCA4/BRG1, anti-ARID1a/BAF250a and anti14-3-3 (Santa Cruz Biotechnology), anti-BAF200 (kind gift from C.P. Verrijzer), and anti-BAF180 (abcam and kind gift from W. Wang and D. Murray) were used in Western blot and immunoprecipitation experiments. For immunoprecipitations, Jlat A2 cells were treated with 10 nM phorbal 12 myristate 13-acetate (PMA) for 12–16 h to produce Tat-FLAG. Cells were lysed in IP buffer (25 mM HEPES, pH 7.9, 150 mM KCl, 1 mM EDTA, 5 mM MgCl2, 5% glycerol, 1% NP40, 0.5 mM dithiothreitol, 1 uM TSA, 1 mM nicotinamide, and a protease inhibitor (PI) cocktail (Sigma)) for 20 min on ice and passed through a 26-gauge needle twice. Lysates were centrifuged, and 2 mg of whole-cell protein lysate was incubated with 20 ul M2 agarose beads (Sigma) in immunoprecipitation (IP) buffer overnight at 4uC on a

rotator. After five washes with IP buffer, beads were resuspended in SDS loading buffer, and co-immunoprecipitated proteins were separated on an SDS-PAGE gel and identified by Western blotting. For Tat immunoprecipitation in 293T cells, cells were transfected with empty pcDNA3.1 or N-terminally FLAG-tagged wild-type or mutant K50R/K51R Tat (pEV280 or pEV537) in presence or absence of expression vector for p300; 36 h after transfection, cells were stimulated with 1 mm TSA and 5 mm nicotinamide for 6 h, harvested, and lysed in buffer (phosphate-buffered saline, 2 mm EDTA, 1% Triton X-100, 0.5 mm dithiothreitol, 1 mm TSA, 5 mm nicotinamide and PI cocktail). 5 mg protein lysate was incubated overnight with 40 ml M2-agarose beads at 4uC on a rotator. Beads were washed extensively with lysis buffer, resuspended in SDS loading buffer and co-immunoprecipitaed proteins identified by Western blot analysis using the indicated antibodies.

Amaxa Nucleofection and siRNA Depletion

Nucleofection of Jurkat cells, SupT1 cells, and Jurkat cell clones D, E, J-Lat A2, 11.1, and 1G5 cells was conducted as previously described [38]. Cells were split to 3610^5 cells/ml 24 h before Amaxa nucleofection. Five million cells were centrifuged at 1,000 rpm for 10 min at room temperature, resuspended in 100 ml of solution R, and nucleofected with 20 nM siRNA or 2 mg of expression plasmid using program O28. Nucleofected cells were resuspended in 500 ml of prewarmed, serum-free RPMI lacking antibiotics and allowed to recover at 37uC in a 5% CO_2 incubator for 15 min. Prewarmed complete RPMI (4 ml) was then added to the cells. Dharmacon siRNA control and on-target smartpools targeting transcripts of the human SMARCB1, SMARCA4, SMARCA2, SMARCA5, CHD3, INO80, PB1, ARID2, ARID1a, and ARID1b genes were used to knockdown the expression of respective genes in Jurkat and SupT1 cells, Jurkat clones D, E, JLat A2, 11.1, and 1G5 cells. Protein levels were examined by Western blot analysis 0, 2, 4, 6, 7, 8, 10, and 14 d after nucleofection.

Flow Cytometry

Samples were analyzed on a FACS Calibur flow cytometer with Cell Quest software (Becton Dickinson). The live population was defined by forward versus side scatter profiles. Cells were further gated by using forward scatter versus FL1 to differentiate between GFP-positive and -negative cells. GFP expression in the J-Lat cell lines was analyzed by FACS at 0, 2, 4, 6, 7, 8, 10, 12, and 14 d after siRNA nucleofection.

Chromatin Immunoprecipitation, FAIRE, and Quantitative PCR (qPCR)

J-Lat A2 and 11.1 cells were fixed by adding formaldehyde to a final concentration of 1% for 10 min for histone IPs and FAIRE and 30 min for BAF/PBAF subunit IPs at RT. The reaction was quenched with 125 mM glycine, cells washed with buffer B (0.25% Triton-X 100, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6), buffer C (150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6), and resuspended in ChIP incubation buffer (0.3% SDS, 1% Triton-X 100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6). Chromatin was sheared by

sonication to an apparent length of ,200–400 bp (corresponding to ,100–200 bp of free DNA) using a BioRuptor sonicator (Cosmo Bio Co., Ltd) with 22 45-s pulses at maximum setting. More than 20 million cells were used per IP, and 5 mg of the indicated antibody was incubated with the chromatin and BSA-blocked protein G beads overnight at 4uC. IPs were washed twice with each buffer 1 (0.1% SDS, 0.1% deoxycholate, 1% Triton-X 100, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6), buffer 2 (0.1% SDS, 0.1% deoxycholate, 1% Triton-X 100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6), buffer 3 (250 mM LiCl, 0.5% deoxycholate, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6), and buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6). Immunoprecipitated complexes were eluted in elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 20 min at RT, and decrosslinked overnight at 65uC in presence of 200 mM $NaCl_2$. DNA was phenol:chloroform extracted, chroloform: isoamylalcohol extracted, ethanol precipitated, and resuspended in 100 ml H_2O by shaking at 37uC. Input and immunoprecipitated DNA (5 ml) were subjected to Sybergreen Q PCR cycles with specific primers. For FAIRE, cells were subjected to formaldehyde crosslinking for 10 min. 20 mg of cross-linked chromatin was diluted 96 with buffer D (150 mM NaCl, 20 mM Tris-HCl pH 8.1, 2 mM EDTA pH8.0, 1% Triton-X100 and PI cocktail) and phenol-chloroform extracted. Isolated DNA was subjected to Sybergreen Q PCR cycles with specific primers.

High- Resolution MNase Nucleosomal Mapping

High resolution MNase mapping protocol [59,60] of the HIV LTR was slightly modified to be amenable to the lower cell numbers obtainable after siRNA depletion of specific factors. Briefly, cells were cross-linked according to the ChIP protocol described above. After one wash in cold PBS, 1.5610^7 crosslinked cells were resuspended in 1 ml hypotonic buffer A (300 mM sucrose, 2 mM Mg acetate, 3 mM Cacl2, 10 mM Tris pH 8.0, 0.1% Triton X-100, 0.5 mM DTT), incubated on ice for 5 min, and dounced 20 times with 2 ml dounce homogenizer (tight pestle, Wheaton). Nuclei were collected by centrifuging at 4uC for 5 min at 7206g. Pellets were resuspended in 1 ml buffer D (25% glycerol, 5 mM Mg acetate, 50 mM Tris pH 8.0, 0.1 mM EDTA, 5 mM DTT) at 1.5610^7 nuclei/ml. Chromatin was collected by centrifuging at 4uC for 5 min at 7206g. The pellets were resuspended in 1 ml buffer MN (60 mM KCl, 15 mM NaCl, 15 mM Tris pH 7.4, 0.5 mM DTT, 0.25 mM sucrose, 1.0 mM
CaCl2) at 2.5610^7 nuclei/ml. The equivalent of 2.5610^6 nuclei were used per MNase reaction. MNase (USB), diluted in buffer MN, was added so that 0, 0.5, 5, 20, 50, and 500 total units were used per 150 ul reaction and digested for 30 min at room temperature. Reactions were stopped with the addition of EDTA and SDS to final concentrations of 12.5 mM and 0.5% respectively. After 4 hours of proteinase K digestion at 37uC, each reaction was processed similar to ChIP samples from the point of elution from the beads.

Real-Time qPCR Analysis

ChIP, FAIRE, and MNase digested samples were analyzed by quantitative PCR in an iCycler iQ real-time PCR detection system (BioRad) using iQ Sybergreen Supermix (BioRad). ChIP values

were normalized as a percentage of input. Sequences of qPCR primer pairs used to amplify distinct regions within the HIV-1 LTR are provided in Table S1. For MNase digests a fold difference was calculated using the DCT method between MNase treated and untreated samples. All values used were collected from the linear range of amplification.

Analysis of HIV LTR Sequence for Nucleosome Propensity

The NuPoP algorithm has been described [55]. An alternative algorithm was also used to predict the nucleosome affinity for HIV nucleotide sequence 1–1800 [56,57]. Estimates of the genomic sites of HIV integration were derived from [58].

Latency Establishment Experiment

HIV-derived virus particles were generated as described [45]. Briefly, 293T cells were transfected with VSVG, the NL4-3 packaging vector, and the retroviral vector LTR-Tat-IRES-EGFP (pEV731). Virus was harvested every 12 h starting at 24 h after transfection. Jurkat or SupT1 cells containing or depleted of BRG1, BAF250, BAF200, BAF180, INI-1, or CHD3, by siRNA transfection, were infected with the LTR-Tat-IRES-EGFP virus at low MOI such that less than 20% of cells were infected. 96 h after infection, the GFP negative cell population harboring uninfected as well as presumably latently infected cells were sorted (once or twice depending on the purity of the GFP negative population) by Flow Cytometry Activated Cell sorting (FACS). GFP negative cells were then treated with PMA and analyzed by FACS after 24 h to determine the percent GFP positive (latent) infections.

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References

1. Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, et al. (1997) Presence ofan inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci U S A 94: 13193–13197.
2. Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, et al. (1997) Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science 278: 1295–1300.
3. Wong JK, Hezareh M, Gunthard HF, Havlir DV, Ignacio CC, et al. (1997) Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 278: 1291–1295.
4. Siliciano JD, Siliciano RF (2006) The latent reservoir for HIV-1 in restingCD4+T cells: a barrier to cure. Curr Opin HIV AIDS 1: 121–128.
5. Verdin E, Paras P, Jr., Van Lint C (1993) Chromatin disruption in the promoterof human immunodeficiency virus type 1 during transcriptional activation. EMBO J 12: 3249–3259.
6. Verdin E (1991) DNase I-hypersensitive sites are associated with both longterminal repeats and with the intragenic enhancer of integrated human immunodeficiency virus type 1. J Virol 65: 6790–6799.
7. Van Lint C, Emiliani S, Ott M, Verdin E (1996) Transcriptional activation andchromatin remodeling of the HIV-1 promoter in response to histone acetylation. EMBO J 15: 1112–1120.
8. Demarchi F, D'Agaro P, Falaschi A, Giacca M (1993) In vivo footprintinganalysis of constitutive and inducible protein-DNA interactions at the long terminal repeat of human immunodeficiency virus type 1. J Virol 67: 7450–7460.
9. Coull JJ, Romerio F, Sun JM, Volker JL, Galvin KM, et al. (2000) The humanfactors YY1 and LSF repress the human immunodeficiency virus type 1 long terminal repeat via recruitment of histone deacetylase 1. J Virol 74: 6790–6799.
10. Williams SA, Chen LF, Kwon H, Ruiz-Jarabo CM, Verdin E, et al. (2006) NFkappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. EMBO J 25: 139–149.
11. Jiang G, Espeseth A, Hazuda DJ, Margolis DM (2007) c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter. J Virol 81: 10914–10923.
12. Sheridan PL, Mayall TP, Verdin E, Jones KA (1997) Histone acetyltransferases regulate HIV-1 enhancer activity in vitro. Genes Dev 11: 3327–3340.
13. Lusic M, Marcello A, Cereseto A, Giacca M (2003) Regulation of HIV-1 gene expression by histone acetylation and factor recruitment at the LTR promoter. EMBO J 22: 6550–6561.
14. Easley R, Van Duyne R, Coley W, Guendel I, Dadgar S, et al. (2010) Chromatin dynamics associated with HIV-1 Tat-activated transcription. Biochim Biophys Acta 1799: 275–285.
15. Karn J (1999) Tackling Tat. J Mol Biol 293: 235–254.
16. Ho L, Crabtree GR (2010) Chromatin remodelling during development. Nature 463: 474–484.
17. Hargreaves DC, Crabtree GR (2011) ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell Res 21: 396–420.
18. de la Serna IL, Ohkawa Y, Imbalzano AN (2006) Chromatin remodelling in mammalian differentiation: lessons from ATP-dependent remodellers. Nat Rev Genet 7: 461–473.
19. Klochendler-Yeivin A, Muchardt C, Yaniv M (2002) SWI/SNF chromatin remodeling and cancer. Curr Opin Genet Dev 12: 73–79.
20. Wang W, Cote J, Xue Y, Zhou S, Khavari PA, et al. (1996) Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. EMBO J 15: 5370–5382.
21. Wang W, Xue Y, Zhou S, Kuo A, Cairns BR, et al. (1996) Diversity and specialization of mammalian SWI/SNF complexes. Genes Dev 10: 2117–2130.
22. Katsani KR, Mahmoudi T, Verrijzer CP (2003) Selective gene regulation by SWI/SNF-related chromatin remodeling factors. Curr Top Microbiol Immunol 274: 113–141.
23. Xue Y, Canman JC, Lee CS, Nie Z, Yang D, et al. (2000) The human SWI/ SNF-B chromatin-remodeling complex is related to yeast rsc and localizes at kinetochores of mitotic chromosomes. Proc Natl Acad Sci U S A 97: 13015–13020.
24. Lemon B, Inouye C, King DS, Tjian R (2001) Selectivity of chromatinremodelling cofactors for ligand-activated transcription. Nature 414: 924–928.
25. Chalkley GE, Moshkin YM, Langenberg K, Bezstarosti K, Blastyak A, et al. (2008) The transcriptional coactivator SAYP is a trithorax group signature subunit of the PBAP chromatin remodeling complex. Mol Cell Biol 28: 2920–2929.

26. Yan Z, Cui K, Murray DM, Ling C, Xue Y, et al. (2005) PBAF chromatinremodeling complex requires a novel specificity subunit, BAF200, to regulate expression of selective interferon-responsive genes. Genes Dev 19: 1662–1667.
27. Kaeser MD, Aslanian A, Dong MQ, Yates JR, 3rd, Emerson BM (2008) BRD7, a novel PBAF-specific SWI/SNF subunit, is required for target gene activation and repression in embryonic stem cells. J Biol Chem 283: 32254–32263.
28. Nie Z, Xue Y, Yang D, Zhou S, Deroo BJ, et al. (2000) A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. Mol Cell Biol 20: 8879–8888.
29. Nagl NG, Jr., Wang X, Patsialou A, Van Scoy M, Moran E (2007) Distinct mammalian SWI/SNF chromatin remodeling complexes with opposing roles in cell-cycle control. EMBO J 26: 752–763.
30. Hsiao PW, Fryer CJ, Trotter KW, Wang W, Archer TK (2003) BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatinremodeling complex for transactivation. Mol Cell Biol 23: 6210–6220.
31. Inoue H, Furukawa T, Giannakopoulos S, Zhou S, King DS, et al. (2002) Largest subunits of the human SWI/SNF chromatin-remodeling complex promote transcriptional activation by steroid hormone receptors. J Biol Chem 277: 41674–41685.
32. Moshkin YM, Mohrmann L, van Ijcken WF, Verrijzer CP (2007) Functional differentiation of SWI/SNF remodelers in transcription and cell cycle control. Mol Cell Biol 27: 651–661.
33. Collins RT, Treisman JE (2000) Osa-containing Brahma chromatin remodeling complexes are required for the repression of wingless target genes. Genes Dev 14: 3140–3152.
34. Mohrmann L, Langenberg K, Krijgsveld J, Kal AJ, Heck AJ, et al. (2004) Differential targeting of two distinct SWI/SNF-related Drosophila chromatinremodeling complexes. Mol Cell Biol 24: 3077–3088.
35. El Kharroubi A, Piras G, Zensen R, Martin MA (1998) Transcriptional activation of the integrated chromatin-associated human immunodeficiency virus type 1 promoter. Mol Cell Biol 18: 2535–2544.
36. Agbottah E, Deng L, Dannenberg LO, Pumfery A, Kashanchi F (2006) Effect of SWI/SNF chromatin remodeling complex on HIV-1 Tat activated transcription. Retrovirology 3: 48.
37. Ariumi Y, Serhan F, Turelli P, Telenti A, Trono D (2006) The integrase interactor 1 (INI1) proteins facilitate Tat-mediated human immunodeficiency virus type 1 transcription. Retrovirology 3: 47.
38. Mahmoudi T, Parra M, Vries RG, Kauder SE, Verrijzer CP, et al. (2006) The SWI/SNF chromatin-remodeling complex is a cofactor for Tat transactivation of the HIV promoter. J Biol Chem 281: 19960–19968.
39. Treand C, du Chene I, Bres V, Kiernan R, Benarous R, et al. (2006) Requirement for SWI/SNF chromatin-remodeling complex in Tat-mediated activation of the HIV-1 promoter. EMBO J 25: 1690–1699.
40. Mizutani T, Ishizaka A, Tomizawa M, Okazaki T, Yamamichi N, et al. (2009) Loss of the Brm-type SWI/SNF chromatin remodeling complex is a strong barrier to the Tat-independent transcriptional elongation of human immunodeficiency virus type 1 transcripts. J Virol 83: 11569–11580.
41. Kalpana GV, Marmon S, Wang W, Crabtree GR, Goff SP (1994) Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. Science 266: 2002–2006.
42. Maroun M, Delelis O, Coadou G, Bader T, Segeral E, et al. (2006) Inhibition of early steps of HIV-1 replication by SNF5/Ini1. J Biol Chem 281: 22736–22743.
43. Boese A, Sommer P, Holzer D, Maier R, Nehrbass U (2009) Integrase interactor 1 (Ini1/hSNF5) is a repressor of basal human immunodeficiency virus type 1 promoter activity. J Gen Virol 90: 2503–2512.
44. Lesbats P, Botbol Y, Chevereau G, Vaillant C, Calmels C, et al. (2011) Functional coupling between HIV-1 integrase and the SWI/SNF chromatin remodeling complex for efficient in vitro integration into stable nucleosomes. PLoS Pathog 7: e1001280. doi:10.1371/journal.ppat.1001280.
45. Jordan A, Defechereux P, Verdin E (2001) The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. EMBO J 20: 1726–1738.
46. Jordan A, Bisgrove D, Verdin E (2003) HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J 22: 1868–1877.
47. Cismasiu VB, Paskaleva E, Suman Daya S, et al. (2008) BCL11B is a general transcriptional repressor of the HIV-1 long terminal repeat in T lymphocytes through recruitment of the NuRD complex. Virology 380: 173–181.
48. Kauder SE, Bosque A, Lindqvist A, Planelles V, Verdin E (2009) Epigenetic regulation of HIV-1 latency by cytosine methylation. PLoS Pathog 5: e1000495. doi:10.1371/journal.ppat.1000495.
49. Vanti M, Gallastegui E, Respaldiza I, Rodriguez-Gil A, Gomez-Herreros F, et al. (2009) Yeast genetic analysis reveals the involvement of chromatin reassembly factors in repressing HIV-1 basal transcription. PLoS Genet 5: e1000339. doi:10.1371/journal.pgen.1000339.
50. Turelli P, Doucas V, Craig E, Mangeat B, Klages N, et al. (2001) Cytoplasmic recruitment of INI1 and PML on incoming HIV preintegration complexes:

interference with early steps of viral replication. Mol Cell 7: 1245–1254.
51. Nie Z, Yan Z, Chen EH, Sechi S, Ling C, et al. (2003) Novel SWI/SNF chromatin-remodeling complexes contain a mixed-lineage leukemia chromosomal translocation partner. Mol Cell Biol 23: 2942–2952.
52. Easley R, Carpio L, Dannenberg L, Choi S, Alani D, et al. (2010) Transcription through the HIV-1 nucleosomes: effects of the PBAF complex in Tat activated transcription. Virology 405: 322–333.
53. Deng L, Wang D, de la Fuente C, Wang L, Li H, et al. (2001) Enhancement of the p300 HAT activity by HIV-1 Tat on chromatin DNA. Virology 289:
312–326.
54. Aguilar-Cordova E, Chinen J, Donehower L, Lewis DE, Belmont JW (1994) A sensitive reporter cell line for HIV-1 tat activity, HIV-1 inhibitors, and T cell activation effects. AIDS Res Hum Retroviruses 10: 295–301.
55. Xi L, Fondufe-Mittendorf Y, Xia L, Flatow J, Widom J, et al. (2010) Predicting nucleosome positioning using a duration Hidden Markov Model. BMC
Bioinformatics 11: 346.
56. Kaplan N, Moore IK, Fondufe-Mittendorf Y, Gossett AJ, Tillo D, et al. (2009) The DNA-encoded nucleosome organization of a eukaryotic genome. Nature 458: 362–366.
57. Segal E, Fondufe-Mittendorf Y, Chen L, Thastrom A, Field Y, et al. (2006) A genomic code for nucleosome positioning. Nature 442: 772–778.
58. Brady T, Agosto LM, Malani N, Berry CC, O'Doherty U, et al. (2009) HIV integration site distributions in resting and activated CD4+T cells infected in culture. AIDS 23: 1461–1471.
59. Sekinger EA, Moqtaderi Z, Struhl K (2005) Intrinsic histone-DNA interactions and low nucleosome density are important for preferential accessibility of promoter regions in yeast. Mol Cell 18: 735–748.
60. Petesch SJ, Lis JT (2008) Rapid, transcription-independent loss of nucleosomes over a large chromatin domain at Hsp70 loci. Cell 134: 74–84.
61. Duverger A, Jones J, May J, Bibollet-Ruche F, Wagner FA, et al. (2009) Determinants of the establishment of human immunodeficiency virus type 1 latency. J Virol 83: 3078–3093.
62. Ott M, Schnolzer M, Garnica J, Fischle W, Emiliani S, et al. (1999) Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity. Curr Biol 9: 1489–1492.
63. Kiernan RE, Vanhulle C, Schiltz L, Adam E, Xiao H, et al. (1999) HIV-1 tat transcriptional activity is regulated by acetylation. EMBO J 18: 6106–6118.
64. Benkirane M, Chun RF, Xiao H, Ogryzko VV, Howard BH, et al. (1998) Activation of integrated provirus requires histone acetyltransferase. p300 and P/ CAF are coactivators for HIV-1 Tat. J Biol Chem 273: 24898–24905.
65. Marzio G, Tyagi M, Gutierrez MI, Giacca M (1998) HIV-1 tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter. Proc Natl Acad Sci U S A 95: 13519–13524.
66. Deng L, de la Fuente C, Fu P, Wang L, Donnelly R, et al. (2000) Acetylation of HIV-1 Tat by CBP/P300 increases transcription of integrated HIV-1 genome and enhances binding to core histones. Virology 277: 278–295.
67. Kim JY, Gonzalez-Scarano F, Zeichner SL (1993) Replication of type 1 human immunodeficiency viruses containing linker substitution mutations in the 2201 to 2130 region of the long terminal repeat. J Virol 67: 1658–1662.
68. Steger DJ, Workman JL (1997) Stable co-occupancy of transcription factors and histones at the HIV-1 enhancer. EMBO J 16: 2463–2472.
69. He G, Margolis DM (2002) Counterregulation of chromatin deacetylation and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) by the HIV-1 repressor YY1 and HIV-1 activator Tat. Mol Cell Biol 22: 2965–2973.
70. Mohd-Sarip A, Venturini F, Chalkley GE, Verrijzer CP (2002) Pleiohomeotic can link polycomb to DNA and mediate transcriptional repression. Mol Cell Biol 22: 7473–7483.
71. Subtil-Rodriguez A, Reyes JC (2010) BRG1 helps RNA polymerase II to overcome a nucleosomal barrier during elongation, in vivo. EMBO Rep 11: 751–757.
72. Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, et al. (2009) The challenge of finding a cure for HIV infection. Science 323: 1304–1307.

Figure S1

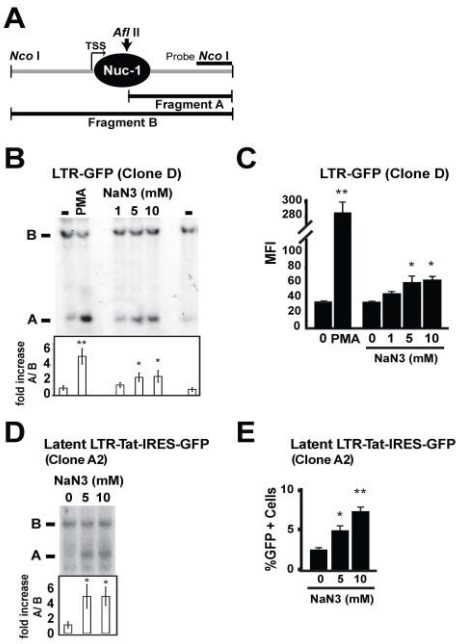

Figure S1. ATP depletion results in nuc-1 remodeling and HIV promoter activation. (A) Schematic representation of the restriction sites and probe used to analyze the remodeling of nuc-1. Nuclei isolated from cells treated either with PMA or sodium azide (NaN_3) were digested in vitro with AflII to probe for accessibility of the DNA encompassing nuc-1. Genomic DNA was subsequently digested with *NcoI* in vitro, and the DNA was analyzed by indirect-end labeling. The *NcoI* genomic fragment (fragment B) and the double *NcoI/AflII* digestion product (fragment A) are shown. (B) Indirect-end labeling after PMA or NaN_3 treatment and (C) corresponding increase in GFP expression in Jurkat clone D containing an integrated LTR-GFP virus. (D) Indirect-end labeling after NaN_3 treatment and (E) corresponding increase in GFP expression in J-Lat A2 containing an integrated latent LTR-Tat-IRES-GFP virus. GFP, measured by flow cytometry, is shown as mean fluorescence intensity (MFI) (C) or increase in percent GFP positive cells (E) 16 h after treatment as detailed above. The intensities of bands from three experiments were quantitated using Odyssey software and used to compare fold increase in ratio of bands A/B in each condition and plotted as mean ± SEM. * p<0.05, ** p<0.01.

Figure S2

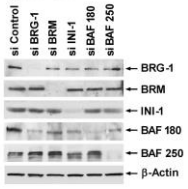

Figure S2. Stability of SWI/SNF complex component protein levels after siRNA depletion of individual subunits. Jurkat cells containing an integrated LTR-GFP virus (clone D) were transfected with either control siRNA or siRNAs targeting various SWI/SNF complex subunits as indicated. Western blot analysis shows expression of SWI/SNF complex subunits in presence of siRNA depletion of distinct subunits as indicated.

Figure S3

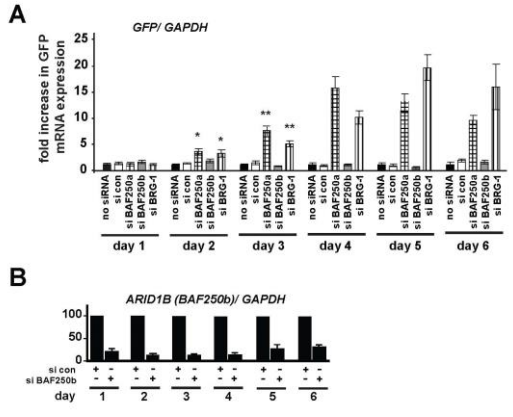

Figure S3. The BAF250a, but not BAF250b-containing BAF complex, is specifically required for maintenance of repression of latent HIV. (A) J-Lat A2 cells latently infected with LTR-Tat-IRES GFP virus were nucleofected with either control siRNA or siRNAs targeting BAF250a, BAF250b, and BRG-1 as indicated. GFP mRNA expression was determined by RT-PCR at the times indicated after transfection, was normalized to GAPDH, and is presented as fold increase over untransfected control. (B) RT-PCR analysis indicated stable depletion of ARID1B/BAF250b mRNA up to 6 d after siRNA transfection. Error bars represent the SEM of three independent experiments. * p<0.05, ** p<0.01.

Figure S4

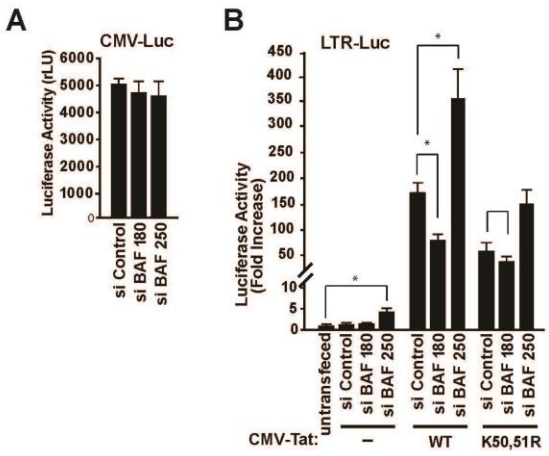

Figure S4. BAF180-facilitated Tat activation of the LTR is dependent on Tat residues K50,51. (A) CMV-driven luciferase activity is not affected by the depletion of either BAF180 or BAF250a. Jurkat cells were nucleofected with siRNAs against BAF180, BAF250, or with a control siRNA pool. Forty-eight hours after siRNA treatment, cells were transfected with a CMV-luciferase vector. (B) Transactivation of the HIV promoter by wild-type but not K50,51 mutant Tat is reduced in the absence of BAF180. Jurkat cells containing integrated LTR-Luciferase (LTR-Luc) were nucleofected with siRNAs against BAF180, BAF250, or with a control siRNA pool. After 48 h, cells were re-transfected with either a control, a CMV-driven wild-type Tat or K50,51R mutant Tat-expression vector. Luciferase was measured after 24 h. Error bars represent the SEM of three independent experiments. * p<0.05.

Figure S5

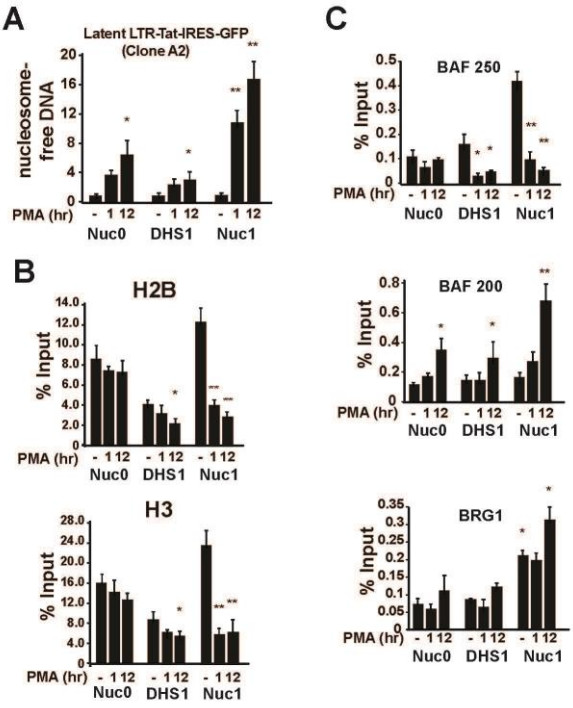

Figure S5. Analysis of chromatin structure and direct binding of distinct SWI/SNF complexes to the HIV promoter before and after PMA stimulation in J-Lat A2. (A) PMA stimulation causes increase in DNA accessibility over the LTR nuc-1. FAIRE results are presented as fold change respective to unstimulated value for each primer pair. (B) PMA stimulation is accompanied by reduction in histone density over LTR nuc-1 as determined by H3 and H2B ChIPs. Histone ChIP results are presented as fold change (histone/mock IP) respective to unstimulated value for each primer pair. (C) BAF directly binds to nuc-1 in its repressed state, while PBAF is recruited to nuc-1 upon PMA stimulation. SWI/SNF subunit ChIPs are presented as ratio of immunoprecipitated DNA over input. Immunoprecipitated DNA from ChIPs and phenol:chloroform extracted DNA from FAIRE were analyzed by qPCR using primer pairs specific for nuc-0, DHS1, and nuc-1 LTR regions. For all ChIP and FAIRE experiments, error bars represent the SEM of at least three independent experiments. * p<0.05, ** p<0.01.

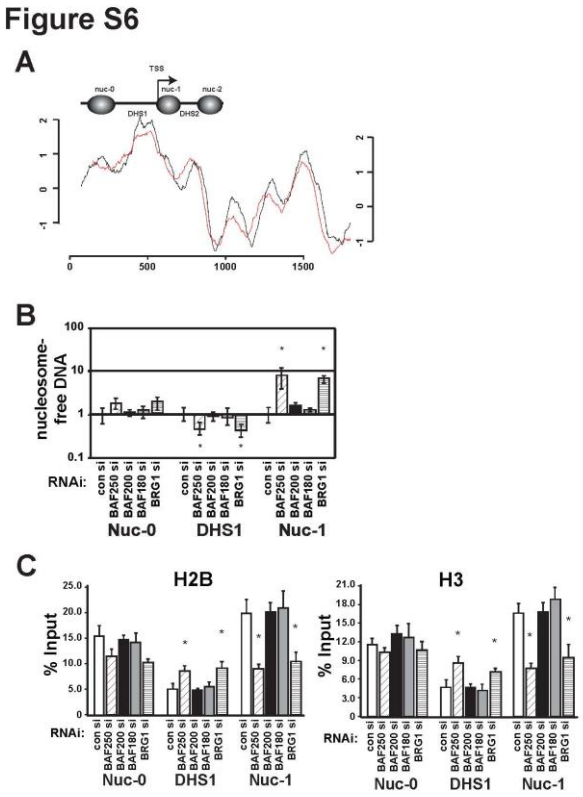

Figure S6. BAF positions nuc-1 of HIV LTR. (A) Location of strictly positioned nucleosomes correlate negatively with the predicted histone binding affinity score (nucleosome score) of the DNA sequence encompassing the HIV LTR. Similarity of the predicted nucleosome affinity for HIV nucleotide sequence 1–1800 determined using the algorithm described in (Xi et al., 2010 [55]) (shown in black) and an alternative algorithm described in (Kaplan et al., 2009 [56]; Segal et al., 2006 [57]) (shown in red). (B) Depletion of BAF250 and BRG1 results in a peak in DNA accessibility over LTR nuc-1. J-Lat A2 cells were nucleofected with either control nontargeting siRNA or siRNAs targeting individual SWI/SNF subunits as indicated and subjected to FAIRE after 72 h. FAIRE results are presented as fold change respective to the value obtained for control siRNA transfected cells given a value of 1 for each primer pair. (C) Depletion of BAF results in reduced histone density over nuc-1 of HIV LTR as determined by H3 and H2B ChIPs. Histone ChIP results are presented as percent immunoprecipitated over Input. For all ChIP and FAIRE experiments error bars represent the SEM of at least three independent experiments. *p<0.05, ** p<0.01.

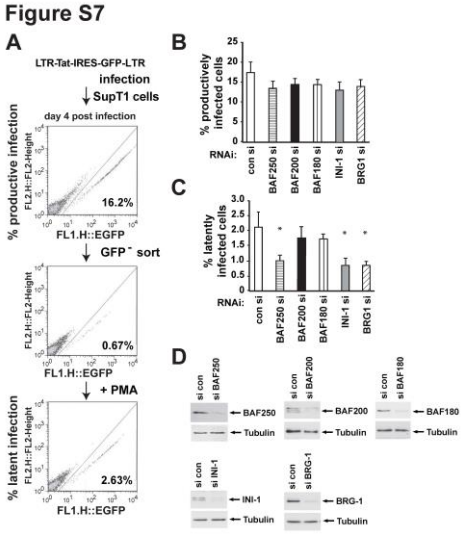

Figure S7. BAF promotes the establishment of latent HIV infections in SupT1 cells. (A) Top panel: schematic representation of protocol for comparison of productive and latent infections in SupT1 cells containing or depleted of remodeling complex subunits. SupT1 cells were first nucleofected with control siRNA or siRNA targeting individual SWI/SNF subunits as indicated. After 48 h, cells were infected with retroviral particles containing the vector LTR-Tat-IRES-GFP. The percentage of productive infections (GFP-positive cells obtained after infection (16.2%) (top panel)), FACS-sorted GFP-negative cells (middle panel), or percent latent infections (GFP-positive cells obtained after PMA treatment of sorted GFP-negative cells (2.63%) (bottom panel)) are shown for SupT1 cells nucleofected with control siRNA. (B) Depletion of BAF/PBAF subunits as indicated does not significantly affect the percentage of

productive HIV infections. (C) Depletion of the core SWI/SNF subunits BRG1 and INI-1 and the BAF-specific subunit BAF250 significantly decreases the incidence of latent HIV infections (the percent GFP positive cells obtained after PMA stimulation of GFP negative cell population) while depletion of PBAF-specific subunits BAF200 or BAF180 had no significant effect on latency establishment. (D) Western blot analysis demonstrates depletion of BAF/PBAF subunits as indicated 96 h after siRNA transfection.

Figure S8

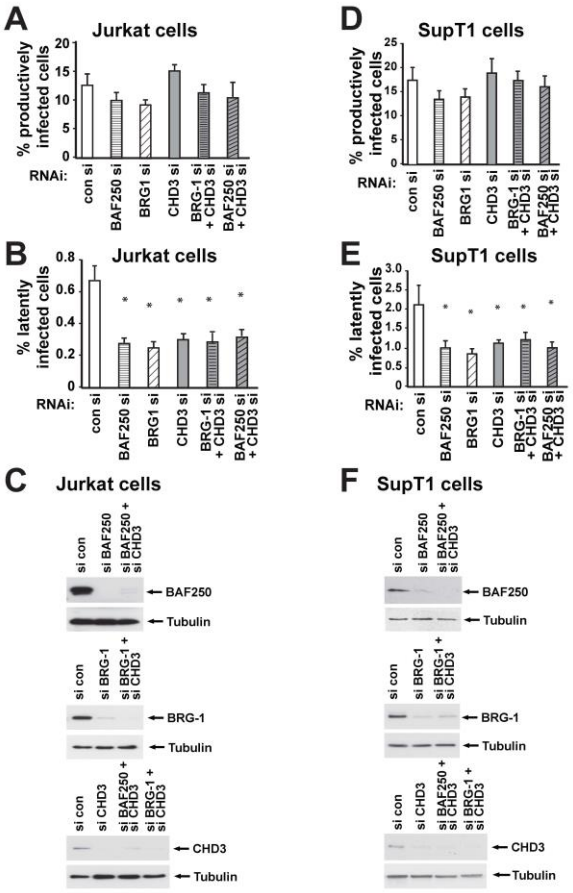

Figure S8. BAF and CHD3 do not synergize in promoting the establishment of latent HIV infections. Jurkat cells (A–C) or SupT1 cells (D–F) were first nucleofected with control siRNA or siRNA targeting BAF250, BRG1, CHD3, BAF250 together with CHD3, or BRG1 together with CHD3. After 48 h, cells were infected with retroviral particles containing the vector LTR-Tat-IRES-GFP. The percentages of productive or latent infections were determined as described in Figures 8 and S7. Depletion of CHD3 and BAF subunits alone or together with CHD3 does not significantly affect the percentage of productive HIV infections in either Jurkat (A) or SupT1 (D) cells. Depletion of BAF subunits BRG1 and BAF250 and the Mi2 catalytic subunit CHD3 significantly decreases the incidence of latent HIV infections. However, simultaneous depletion of BAF and CHD3 does not result in an additive decrease in latency establishment in Jurkat (B) or SupT1 (E) cells. Western blotting analysis indicates depletion of the indicated remodeling subunits in Jurkat (C) and SupT1 (F) cells 96 h post-siRNA transfection.

Chapter 3

Targeting the Wnt pathway for activation activates latent HIV-1 and is synergistically enhanced upon concomitant inhibition of histone deacetylation

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Submitted

Abstract

New pharmaceutical strategies aimed at HIV-1 eradication have focused on molecules able to induce HIV-1 replication from latently infected cells in order to make them susceptible to viral cytopathic effects and immune response. Here we examined the role of the Wnt signaling pathway in activation of transcription of latent HIV-1. We found that induction of the Wnt pathway resulted in activation of latent HIV-1, recruitment of the Wnt molecular effectors LEF1 and β-catenin to the LTR, and drastic restructuring of the latent HIV-1 LTR nucleosomal structure. Lithium, a Wnt agonist in clinical use, activated HIV-1 transcription in latently infected primary $CD4^+$ T cells. Wnt-mediated activation of the latent HIV-1 LTR was synergistically enhanced in the presence of HDAC inhibitors. Thus, targeting the Wnt pathway by small molecules and Wnt agonists may be an attractive strategy in a combinatorial therapy aimed at activation of latent HIV-1.

Key words: HIV-1; latency; β-catenin; LEF-1; Wnt; Lithium

Introduction

The opinion on the pursuit of a cure for HIV-1 has been nuanced in the past decade.
Previously it was advocated to avoid immune activation and treat patients with continued Combination Anti-Retroviral Therapy (c-ART), in an attempt to prevent replication of the virus and deplete infected cells over time. However, decline of the HIV infected pool in the presence of fully suppressive c-ART proved to be far slower than assumed based on earlier mathematical models (Chun et al., 2007; Fraser et al., 2000; Sedaghat et al., 2008). Studies have shown that HIV-1 is able to persist in the presence of c-ART, even in patients exhibiting prolonged suppression of viremia (Chun et al., 2010; Le et al., 2011). This is attributable to the fact that HIV-1 establishes a transcriptionally silent or latent infection in resting memory CD4+ T cells, making them the main functional reservoir of latent provirus (Archin et al., 2012b; Blankson et al., 1999; Finzi et al., 1999; Finzi et al., 1997; Jordan et al., 2003; Siliciano et al., 2003; Siliciano and Greene, 2011). While masked from c-ART, this latent HIV reservoir is transcriptionally competent, and thus retains the capacity to become activated, leading to viral replication upon therapy interruption (Pierson et al., 2000). As a consequence, c-ART is not curative and has to be taken life-long.

Recently, the concept of a cure for HIV-1 has captured a lot of attention (Archin et al., 2009; Hamimi et al., 2013; Katlama et al., 2012; Kent et al., 2013; Lewin and Rouzioux, 2011; Margolis and Hazuda, 2013; Pantaleo and Levy, 2013; Richman et al., 2009; Stone et al., 2013). The use of fully suppressive c-ART has led to important insights into the dynamics of the persisting latent viral pool, and new pharmaceutical strategies aimed at HIV-1 eradication have focused on molecules able to induce HIV-1 replication from latently infected cells in order to make them susceptible to viral cytopathic effects, antiviral immune responses and antiretroviral drugs (Durand et al., 2012; Lehrman et al., 2005; Margolis and Archin, 2007; Margolis and Hazuda, 2013; Xing and Siliciano, 2012). Progress in the HIV-1 transcription field has resulted in new insights into molecular mechanisms that govern the latent state of HIV-1 in patients (Donahue and Wainberg, 2013; Karn, 2011; Van Lint et al., 2013). These studies suggest alternative targets for therapies aimed at eradicating both latent and active HIV-1 (Archin et al., 2009; Banerjee et al., 2012; Boehm et al., 2013). Inhibitors of one of these molecular targets, histone deacetylases, (HDACi's) are currently under clinical evaluation for their potential to activate latent HIV-1 (Archin et al., 2009; Contreras et al., 2009; Edelstein et al., 2009; Ylisastigui et al., 2004). Pertinent to the eradication of HIV-1, therefore, is the identification of molecular targets and development of therapeutics which target the latent HIV-1 infected reservoir for activation followed by its safe elimination.

Expression of the HIV-1 viral genome is driven by the HIV-1 promoter or 5'-LTR and is tightly controlled. Regardless of position of virus integration, the 5'-LTR is organized into precisely positioned nucleosomes in its repressed transcriptional state (Rafati et al., 2011;

Verdin, 1991; Verdin and Van Lint, 1995). In particular, the nucleosome positioned immediately downstream of the transcription start site (TSS), Nuc-1, is highly repressive to transcription and becomes rapidly and specifically disrupted upon activation (Rafati et al., 2011; Van Lint et al., 1996; Verdin, 1991; Verdin et al., 1993). Using a high resolution MNase nucleosomal mapping technique for the HIV-1 LTR, we recently demonstrated that the BAF ATP-dependent chromatin remodeller drives the strict positioning of Nuc-1 in a thermodynamically sub-optimal position, leading to the establishment and maintenance of HIV-1 latency (Rafati et al., 2011). In addition to the contribution of chromatin landscape and organization, basal transcription at the HIV-1 LTR is critically dependent on the activity of the repressive and activating host cell transcription factors and their associated cofactors. Scattered throughout the HIV-1 LTR are consensus binding sites for a number of cellular transcription factors able to induce or repress the HIV-1 promoter (Li et al., 1994; Lusic et al., 2003; Pereira et al., 2000; Perkins et al., 1993; Romerio et al., 1997; Sune and Garcia-Blanco, 1995). Their balance determines whether transcription from the HIV-1 promoter will be silenced, leading to latency, or result in accumulation of the potent viral transactivator TAT and the initiation of a TAT-dependent positive regulatory loop leading to a productive infection (Burnett et al., 2009; Karn, 2011; Mahmoudi, 2012). While HDACi's have shown promise in activating latent HIV-1 (Archin et al., 2012a; Contreras et al., 2009; Edelstein et al., 2009; Lehrman et al., 2005), studies suggest that alone, they may not be sufficient for full activation of the latent cell pool (Archin et al., 2010; Blazkova et al., 2012; Sagot-Lerolle et al., 2008; Steel et al., 2006). Studies on HIV-1 transcription regulation indicate that alternative pathways and transcription complexes function to silence the HIV-1 LTR and might serve as molecular targets for compounds to activate latent HIV-1 (Donahue and Wainberg, 2013; Hakre et al., 2011; Margolis, 2010; Xing and Siliciano, 2012). The identification and characterization of the full repertoire of transcription factors and cofactors involved in silencing the latent HIV-1 LTR is critical to the development of specific therapeutics to deplete HIV-1 latency.

One of the transcription factor binding sequences on the HIV-1 LTR is for LEF1. The founding member of the TCF/LEF family proteins LEF1 (or TCF1) was initially identified as a protein purified from Jurkat T cells that bound to three sites in the HIV-1 LTR (Sheridan et al., 1995; Waterman and Jones, 1990). TCF/LEF transcription factors are the downstream molecular effectors of the Wnt signaling pathway. The Wnt pathway is a highly conserved signalling pathway controlling a variety of biological processes (Clevers and Nusse, 2012). The key regulatory step involves the phosphorylation, ubiquitination, and subsequent proteosomal degradation of the downstream effector protein, β-catenin by a dedicated cytoplasmic destruction complex. The β-catenin destruction complex contains the core proteins AXIN1, adenomatouspolyposis coli (APC) and the kinases glycogen synthasekinase-3 / (GSK-3) and casein kinase-1 (CKI). In the absence of Wnt, cytoplasmic β-catenin is continuously degraded by the β-catenin destruction complex and TCF/LEF factors bind to and

repress Wnt target genes in the absence of β-catenin in the nucleus. Upon Wnt activation, ubiquitination of phosphorylated β-catenin is blocked, leading to saturation of the destruction complex with phosphorylated β-catenin and its functional inactivation (Clevers and Nusse, 2012; Li et al., 2012). Newly synthesized β-catenin then accumulates and is transported to the nucleus where it complexes with TCF/LEF factors to activate Wnt target genes.

Given the well described activating role of Wnt and β-catenin in regulation of Wnt target genes together with the strategic presence of TCF/LEF consensus sites within the HIV-1 LTR, we asked whether HIV-1 would also respond as a Wnt target gene to induction of the Wnt/ β-catenin signaling pathway, and lead to activation of transcription of the latent HIV-1 LTR. We found that activation of the Wnt pathway by natural ligands, chemical inhibitors or exogenous expression of a constitutively active β-catenin activates latent HIV-1. Wnt activation of latent HIV-1 is accompanied by recruitment of the molecular effectors β-catenin and LEF1 to the HIV-1 LTR, concomitant with drastic nucleosome remodelling of the latent HIV-1 LTR as demonstrated by high resolution MNase nucleosomal mapping assays. Wnt-mediated activation of the latent HIV-1 LTR was synergistically enhanced in the presence of HDACi's, a class of drugs currently under clinical investigation for activation of latent HIV-1. Finally therapeutically relevant concentrations of the Wnt agonist Lithium activated latent HIV-1 from *ex-vivo* infected primary $CD4^+$ T cells.

Our data suggest that targeting the Wnt pathway for activation may be an attractive strategy in a combinatorial therapy aimed at activation of latent HIV-1 infected cells followed by their elimination in the presence of c-ART.

Results

TCF1/LEF1 expression is cell type specific and LEF1 binding sites on the HIV-1 LTR are conserved

The transcriptional activity of the HIV-1 promoter or LTR is strictly controlled in its basal state by the combined activity of the numerous cellular transcription factors whose consensus binding sites are scattered throughout this regulatory region. Figure 1A shows a selection of the transcription factors binding site profile on HIV-1 LTR. HIV-1 LTR sequence specific transcription factor binding sites identified by Consite analysis are listed in Table S1. As shown in Figure 1A there are three binding sites for LEF1 on the HIV-1 LTR. LEF1 (TCF1), the founding member of TCF/LEF proteins which was originally identified as a protein binding to three sites on the HIV-1 LTR (Pereira et al., 2000; Sheridan et al., 1995; Waterman and Jones, 1990). We evaluated the degree of conservation of the LEF1 binding sites among HIV-1 sequences

belonging to subtype B. Figure 1A shows the nucleotide sequence for each of the 3 LEF1 binding sites present in 5'-LTR, with the relative letter size indicating the presence and frequency of a nucleotide in a specific position. Our data shows high degree of conservation in LEF1 binding sites between different HIV-1 subtype B isolates.

To evaluate the effect of Wnt signaling and its downstream molecular effectors on activity of latent HIV-1, we first determined which TCF/LEF member proteins are expressed in CD4$^+$ T cells. We compared expression levels of all mammalian TCF/LEF members TCF1, LEF1, TCF3 and TCF4 in the CD4$^+$ T cell lines Jurkat and SupT1 as well as the colorectal cancer cell line Ls174T, HeLa and HEK293T cells by Western Blot analysis (Figure 1B) and RT-PCR (Figure S1). TCF1 and LEF1 but not TCF3 or TCF4 are expressed in Jurkat and SupT1 CD4$^+$ T cells, while TCF3 and TCF4 are the predominant TCF/LEF family members expressed in Ls174T, HeLa and 293T cells (Figure 1B and Figure S1). Moreover, LEF1 and TCF1 but not TCF3 or TCF4 are specifically expressed in primary memory CD4$^+$ T cells, the major latent HIV-1 reservoir in vivo in c-ART suppressed patients (Figure 1C).

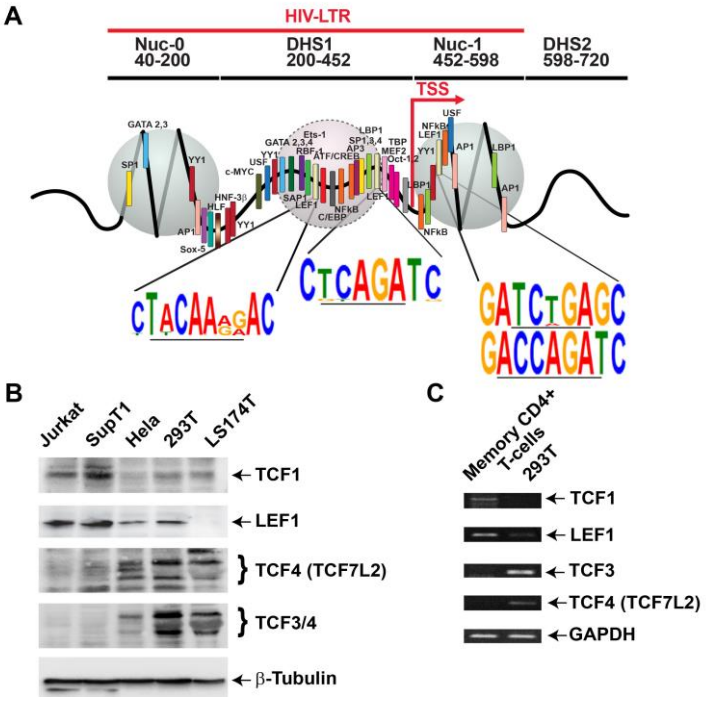

Figure 1. Localization of LEF1 binding sites on the HIV-1 LTR and expression profile of TCF/LEF protein family members among different cell types. A) Schematic representation of HIV-1 LTR showing chromatin organization and the distribution of transcription factor binding sites. Sequence logos illustrate the nucleotide distribution for the putative LEF1 binding sites in HIV-1 subtype B. B) Western blot analysis of TCF/LEF members in Jurkat, SupT1, HeLa, 293T and LsI74T cells. C) RT-PCR analysis of distinct TCF/LEF members in memory CD4+ T cells and 293T cells.

Activation of the Wnt pathway activates latent HIV-1

To investigate the role of the Wnt signaling pathway in regulation of latent HIV-1 transcription, we induced Wnt signaling in latent HIV-1 infected Jurkat (J-Lat) or SupT1 (S-Lat) cell lines using three independent methods: we activated the Wnt pathway by treatment of latently infected cells with the natural ligands Wnt3A and Rspondin, by exogenous expression of the constitutively active S33Y β-catenin mutant, and by treatment of latently infected cells with small molecule inhibitors of GSK3 , a critical kinase in the destruction complex responsible for β-catenin phosphorylation.

We stimulated S-Lat/ J-Lat cells by treatment with Wnt3A ligand (Willert et al., 2003) and Rspondin, the ligand for LGR4/5/6 receptor family proteins, which potentiate Wnt signalling through physical interaction with the Wnt receptors LRP5/6 and Frizzeld in the presence of Wnt ligands (de Lau et al., 2011). LGR 6, the receptor for Rspondin was highly expressed in T cell lines (Figure S2A). We monitored expression of the endogenous Wnt target genes *AXIN2* (Lustig et al., 2002), *ZCCHC12* (Mahmoudi et al., 2009), *TCF1* (Roose and Clevers, 1999), and *MMP9* (Wu et al., 2007a) in response to Wnt3A/R-spondin stimulation. Significant increase in mRNA expression was detected post Wnt3A/R-spondin stimulation by quantitative RT-PCR confirming induction of the Wnt pathway (Figure 2A). A parallel increase in GFP production was observed following Wnt3A/R-spondin treatment in J-Lat 11.1 and J-Lat A2 cells (Figure S2B). Of note, Wnt3A/R-spondin treatment resulted in a concomitant activation of latent HIV-1 as quantitated by increased mRNA expression of HIV-1 *GAG* and the LTR-reporter *GFP* genes, but not the NF-kB target genes *TLR2* and *ICAM1*.

We next examined the effect of exogenous expression of mutant S33Y β-catenin on the HIV-1 LTR in latently infected cells. This point mutation in β-catenin results in its constitutive stabilization and activation of the Wnt pathway mimicking a Wnt "on" system when expressed. S-Lat 30 cells were nucleofected with a control vector or an expression vector for S33Y β-catenin in the presence of either a TCF-reporter TOPFLASH or the mutant FOPFLASH control reporter (van de Wetering et al., 2002). As expected, expression of S33Y β-catenin resulted in activation of TCF/LEF-driven TOPFLASH luciferase activity (Figure 2B, top panel) concomitant with increased mRNA expression of the endogenous Wnt target genes *AXIN2, ZCCHC12, TCF1*, *MMP2 (*Wu et al., 2007b*), MMP9*, but not *TLR2* and *ICAM1* in S-Lat 30 cells. Exogenous expression of S33Y β-catenin also caused a significant increase in expression of HIV-1 *GAG* and the LTR-reporter *GFP*, indicating a S33Y β-catenin-dependent activation of latent HIV-1 (Figure 2 B, bottom panel).

Finally we used the Wnt agonist and GSK3β inhibitor, Lithium, to activate the Wnt pathway in J-Lat/S-Lat cells. S-Lat 30 and J-Lat P32 were untreated or treated with increasing concentrations of Lithium as indicated (Figure 2C). A significant increase in expression of the

LTR-driven *GAG* and *GFP* was detected concomitant with the expected increase in expression of the endogenous Wnt target genes. Similar activation of latent HIV-1 was observed upon treatment of a panel of latently infected cells with CHIRR, a specific GSK3 inhibitor (Figure S2C). Collectively, our data demonstrate that activation of the Wnt signalling pathway by natural ligands, exogenous expression of a constitutively active β-catenin mutant or the GSK3-β inhibitor Lithium, activates the HIV-1 LTR in latently infected S-lat/J-Lat cells.

Our results suggested a direct Wnt-induced molecular effect on the latent HIV-1 LTR. To obtain more insight into the molecular mechanisms involved in Wnt-induced LTR activation, we performed high resolution MNase nucleosomal mapping assays (Rafati et al., 2011) on the latent and Wnt-activated HIV-1 LTR. S-Lat 30 cells were either untreated or treated with increasing concentrations of Lithium. This method relies on the preference of MNase to digest nucleosome– free and linker DNA while nucleosomal DNA is at least partially protected from digestion. Digested and undigested DNA were probed with overlapping primer sets amplifying the HIV-1 LTR. The ratio of the amount of digested DNA to the undigested control for each overlapping primer pair scanning the length of the HIV-1 LTR correlates directly to its nucleosome occupancy (Figure 2D, top panel). Treatment of cells with Lithium caused decrease in DNA protection between the positioned Nuc-0 and Nuc-2, indicating a loss of nucleosomes within this region including the positioned repressive Nuc-1 (Figure 2D, middle and bottom panels).

To examine direct Wnt-induced recruitment of the Wnt molecular effectors to the latent HIV-1 LTR, we performed chromatin immunoprecipitation assays. J-Lat/S-Lat cells were untreated or treated with Lithium or the natural ligands Wnt3A/Rspondin and subjected to ChIP assays using antibodies against the downstream Wnt molecular effectors β-catenin and LEF1, as well as the methyltransferase DOT1L and its active histone mark H3K79 trimethylation, an activity which we showed previously to be a β-catenin-associated co-activator of Wnt signalling (Mahmoudi et al., 2010; Mohan et al., 2010). qPCR analysis of the immunoprecipitated material with primers specific for distinct regions within the HIV-1 LTR indicated that β-catenin was specifically recruited to the LTR DHS-1 and Nuc-1 region. While present on the HIV-1 LTR in the unstimulated condition, LEF1 was significantly enriched on Nuc-1 in response to Wnt activation. The activating H3K79 trimethyl mark was enriched on the HIV-1 LTR transcribed region specifically in response to Wntactivation, mimicking DOT1L enrichment (Figure 2E).

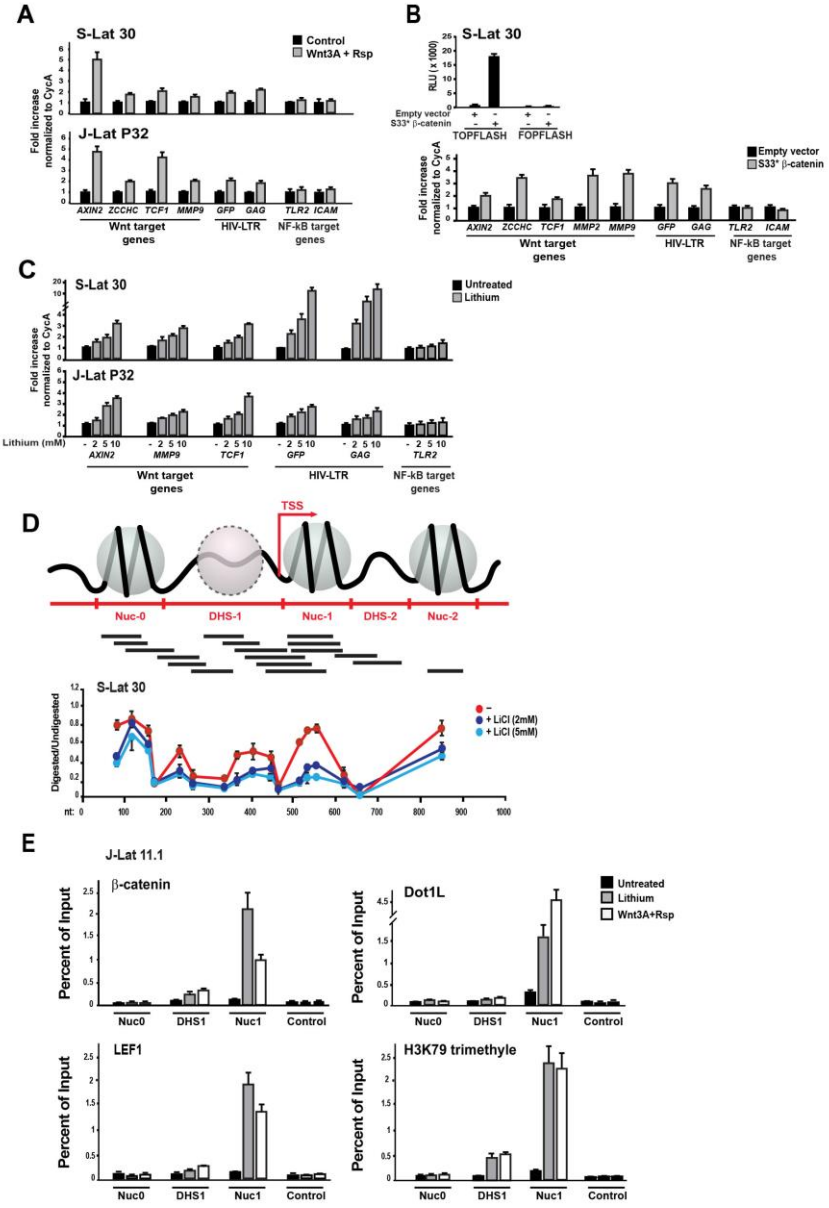

Figure 2. Activation of the Wnt pathway via different strategies activates latent HIV-1. A) Activation of Wnt pathway by natural ligands induce HIV-1 expression. SupT1 derived latent cell line (S-Lat30) and Jurkat derived latent cell line (J-Lat P32) were treated with Wnt3A/Rspondin conditioned media or control conditioned media. Relative mRNA expression levels of Wnt target genes *AXIN2, ZCCHC12, TCF1, MMP9,* LTR-dependent genes *GAG* and *GFP*, and NF-kB target genes *TLR2* and *ICAM* were quantitated by qRT-PCR. B) Exogenous expression of a the constitutively active β-catenin activates HIV-1. Exogenous expression of S33Y mutated β-catenin strongly activates luciferase activity (upper panel) in TOPFLASH transfected cells. Data are expressed as the average RLU+SD from three independent experiments comparing cells transiently transfected with the S33Y mutated β-catenin to cells transfected with empty vector. As a control, cells were transfected with the mutant control FOP-FLASH reporter plasmid. Bar plots (lower panel) show the variation of the relative mRNA expression levels of the Wnt target genes *AXIN2, ZCCHC12, TCF1,*

MMP2, MMP9, and NF-kb target genes *TLR2* and *ICAM1* following exogenous expression of S33Y β-catenin in S-Lat 30 latent cells. C) Small molecule GSK3 inhibitor Lithium activates HIV-1. S-Lat 30 and J-Lat P32 latent cell lines were treated with 0, 2, 5 or 10 mM Lithium. Expression of HIV-1 *GAG* and *GFP*, and Wnt target genes *AXIN2, MMP9*, *TCF1* and NF-kB target gene *TLR2* was quantitated by qRT-PCR. mRNA expression data are presented as fold increase normalized to Cyclophillin A control, bars represent average + SD of three experiments. D) High Resolution MNase nucleosomal mapping analysing Lithium induced modifications of the chromatin structure of the latent HIV-1 LTR. Top panel shows the position of PCR primers spanning Nuc-0, DHS-1, Nuc-1, DHS-2, and Nuc-2 regions (nucleotides 40-902 of HIV-1 clone PNL4.3(AF324493.2)). PCR products are 100 ± 10bp in size and are spaced approximately 30 bp apart. Bottom panels show the chromatin profile of the HIV-1 LTR in unstimulated, and cells stimulated with 2 mM Lithium (dark blue line) and Lithium 5 mM (light blue line). Nucleotide positions are plotted on the X-axis. For each amplicon, relative abundance of PCR target was calculated comparing the MNase digested and undigested samples and plotted against the nucleotide position corresponding to the center of the amplicon. Dots and whiskers represent the average +/- SD of three experiments. E) Activation of Wnt signaling pathway resulted in enrichment of β-catenin, LEF1, DOT1L, and H3K79 trimetylation in the HIV-1 LTR. J-Lat 11.1 latent cell line were stimulated either with Lithium or Wnt3A/Rspondin and subjected to ChIP using antibodies against β-catenin, LEF1, DOT1L, and H3K79 trimetyl. Chromatin immunoprecipitation with the specified antibodies was followed by qPCR using primer pairs spanning the HIV-1 LTR. Control sequences upstream the AXIN2 promoter were used as a not bound control. Results are presented as percentage DNA immunoprecipitated over input. Bars represent the average + SD of three experiments.

Wnt-induced activation of latent HIV-1 is synergistically enhanced in the presence of HDACi's

HDACi's are a promising class of drugs under clinical investigation to activate transcription of latent HIV-1 by targeting HDACs, a class of chromatin modifying enzymes shown to repress the HIV-1 LTR (Coull et al., 2000; Jiang et al., 2007; Shirakawa et al., 2013; Wightman et al., 2012; Williams et al., 2006). We asked whether inhibition of HDACs would enhance the observed Wntinduced activation of latent HIV-1. Therefore we activated the Wnt pathway by treating the J-Lat/S-Lat cells with the natural ligands Wnt3A/Rspondin or Lithium, in the presence or absence of the HDACi's SAHA (Vorinostat) or Valproic acid (VPA). We monitored activation of latent HIV-1 by flow cytometry as increase in the percentage of LTR-driven GFP expressing cells as well as by RT-qPCR analysis of LTR-driven *GAG* and *GFP* mRNA expression. As shown in Figure 3A, while treatment with VPA and Wnt3A/Rspondin alone results in a moderate increase in the percentage GFP positive cells in the concentrations examined, treatment with both compounds indicates strong synergism between Wnt and VPA in activation of latent HIV-1.

We next examined the effect of the GSK3 inhibitor Lithium alone or together with HDACi's in activation of HIV-1 in a panel of HIV-1 infected cell lines. S-Lat 30 and J-Lat 11.1 cells were treated with increasing concentration of Lithium and SAHA alone or in combination and expression of LTR-driven *GFP* and *GAG* genes, and the endogenous Wnt target genes *AXIN2, TCF1*, and *MMP9* was determined by qRT-PCR (Figure 3B). As expected, treatment with Lithium activated expression of the Wnt target genes, *AXIN2*, *TCF1*, and *MMP9*. To exclude the presence of position bias related to the site of integration we evaluated HIV-1

reactivation in presence of Lithium and SAHA by FACS analysis on a larger panel of cell lines harbouring the LTR-TATIRES-GFP cassette (Figure 3C). While treatment with Lithium or SAHA alone resulted in a concentration dependent low activation of latent HIV-1, combination treatment caused enhanced activation of latent HIV-1 that was at least additive or synergistic depending on the cell line used. For some latent cell lines, we could detect a significant increase in GFP even at concentrations of Lithium lower than 2mM. Our data demonstrating synergism between Lithium and HDACi's in latent LTR activation was confirmed using Jurkat 1G5 (Figure 3D), which contain an HIV-1 LTRdriven luciferase reporter. Thus Wnt activators and HDACi's functionally synergize in activation of the latent HIV-1 LTR.

Synergistic and direct enrichment of Wnt molecular effectors β-catenin/LEF1 and histone acetylation on the HIV-1 LTR in response to Lithium and SAHA

To examine whether the functional synergism between Lithium and SAHA on the HIV-1 LTR can be observed at the level of chromatin, we performed ChIP assays to probe both direct recruitment of the molecular effectors of the Wnt pathway, β-catenin and LEF1 to the HIV-1 LTR, as well as to examine its state of histone occupancy and acetylation. J-Lat 11.1 (Figure 4A) and S-Lat 30 (Figure 4B) latent HIV-1 infected cells were subjected to ChIP assays after treatment with Lithium and SAHA as indicated using antibodies specific for LEF1, β-catenin, Histone H3, Acetyl H3 and Acetyl H4. qPCR analysis of the immunoprecipitated material with primers specific for distinct regions within the HIV-1 LTR indicated that LEF1 and β-catenin were specifically recruited to the HIV-1 LTR in response to treatment with Lithium. LEF1/ β-catenin enrichment was specific to the DHS-1 and strongly to the Nuc-1 region of the HIV LTR and not the non-bound upstream *AXIN2* control region. Detection of LEF1 was strongly dependent on the antibody used; a LEF1 antibody raised against the N-terminal β-catenin interaction domain of LEF1 failed to immunoprecipitate LEF1-LTR presumably due to occupancy of the antibody binding site by β-catenin (Figure S3). As expected, treatment with SAHA resulted in an increase in histone acetylation on the HIV-1 LTR as detected by increased enrichment of Acetyl H3 and Acetyl H4 on the LTR DHS-1 and Nuc-1, concomitant with a loss in histone density in this region (H3 enrichment). Interestingly, treatment with Lithium also resulted in an increase in histone acetylation at the LTR Nuc-1. In support of this observation, β-catenin has previously been shown to recruit histone acetyltransferases P300/CBP to Wnt target genes to acetylate histones and activate transcription (Clevers and Nusse, 2012; Li et al., 2007; Yang et al., 2006). Cotreatment of latent cells with Lithium and SAHA resulted in a striking increase in enrichment of LEF1 and β-catenin to the LTR Nuc-1 compared to Lithium treatment alone, demonstrating synergism between these distinct pathways at the molecular level in activation of latent HIV-1. Importantly, the activating Acetyl H3 and Acetyl H4 marks

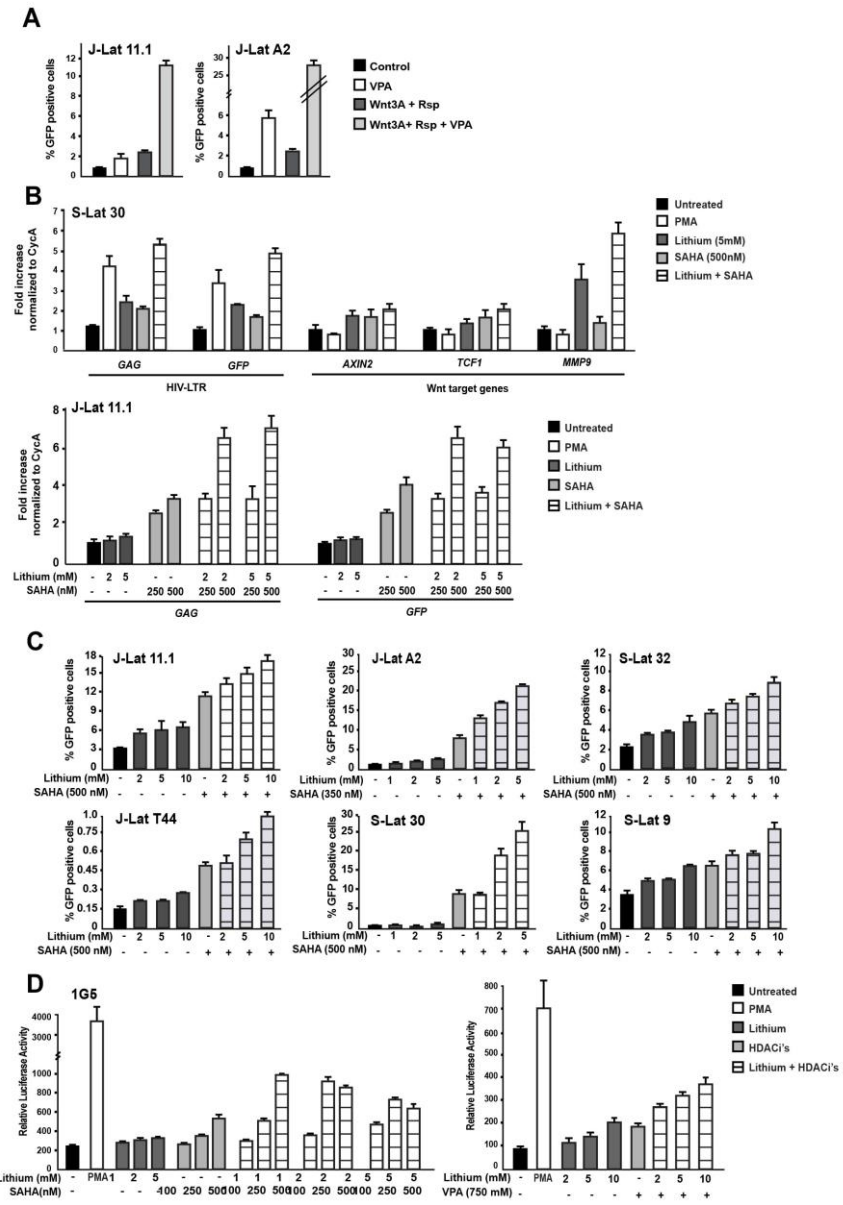

Figure 3. Lithium and SAHA/VPA synergistically activate the HIV-1 LTR in latent cell lines. A) J-Lat 11.1 and J-Lat A2 latent cell lines were treated with VPA and Wnt3A/Rspondin alone or in combination. GFP expression was monitored by FACS analysis. Bars represent the average percentage + SD of GFP positive cells of three experiments performed in duplicate. B) S-Lat 30 and J-Lat 11.1 latent cell lines were either untreated or treated with Lithium, and SAHA alone or in combination. Expression of *GAG, GFP*, and the Wnt target genes *AXIN2, TCF1*, and *MMP9* was monitored by qRT-PCR. mRNA expression are presented as fold increase normalized to Cyclophillin A control. Bars represent average +SD of three experiments. C) J-Lat 11.1, J-Lat A2, S-Lat 32 , J-Lat T44, S-Lat 30 and S-Lat 9 latent cell lines were either untreated or treated with Lithium, SAHA alone or in combination. GFP expression was monitored by FACS analysis. Bars represent the average % of GFP positive cells + SD of three experiments performed in duplicate. D) Jurkat 1G5

cells were either untreated or treated with PMA, increasing concentration of of Lithium and HDAC inhibitors (VPA or SAHA) alone or in combination. Activation of HIV-1 LTR was measured as increase in the luciferase activity, data are represented as average RLU + SD of three experiments performed in duplicate.

were also synergistically enriched on the HIV-1 LTR Nuc-1 region specifically upon Lithium-SAHA co-treatment, concomitant with histone depletion. Together, these results underline the molecular basis for the functional synergism observed between Lithum and SAHA in activation of latent HIV-1.

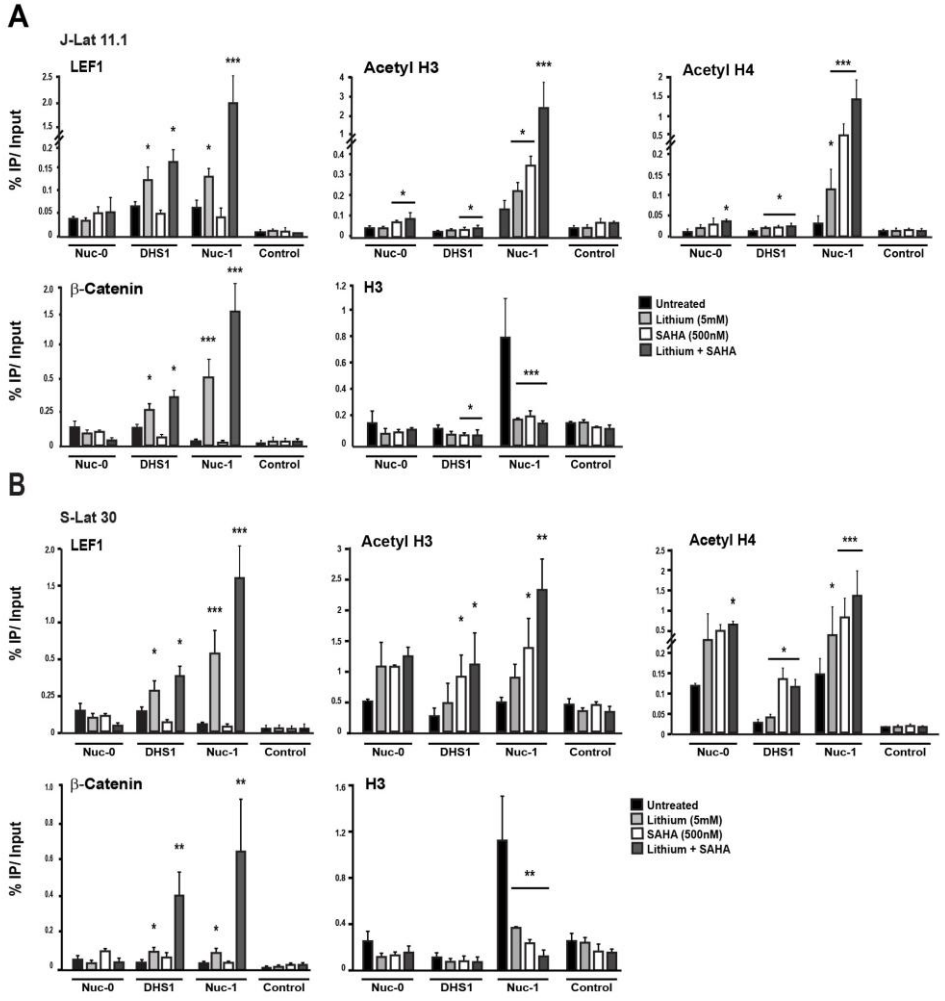

Figure 4. Synergistic and direct enrichment of Wnt molecular effectors β-catenin/LEF1 and histone acetylation on the HIV-1 LTR in response to Lithium and SAHA. J-Lat 11.1 (A) and S-Lat 30 (B) cells were subjected to ChIP using antibodies against β-catenin and LEF1, Acetyl H3, Acetyl H4, and histone H3. Chromatin immunoprecipitation with the specified antibodies was followed by qPCR using primer pairs spanning the HIV-1 LTR. Control sequences upstream the *AXIN2* promoter were used as a not bound control. Results are presented as percentage DNA immunoprecipitated over input, bars represent average + SD of three experiments.

High Resolution Nucleosomal Mapping reveals dramatic alteration to the latent HIV-1 LTR chromatin structure upon Lithium-HDACi combination treatment

Our data thus far demonstrated functional synergism between activation of the Wnt pathway and inhibition of HDACs in activation of latent HIV-1. We found this functional synergism to be accompanied by direct synergistic recruitment of LEF1 and β-catenin to the HIV-1 LTR Nuc-1 as well as by synergistic increase in LTR histone acetylation. To obtain a higher resolution picture of the Wnt-HDACi-induced dynamic changes in LTR nucleosomal structure, we performed high resolution MNase nucleosomal mapping, as previously described (Rafati et al., 2011). We examined the latent LTR nucleosomal profile in S-Lat 30 (Figure 5B) and J-Lat 11.1 (Figure 5C) cells which were untreated or treated with Lithium, the HDACi's SAHA or VPA, alone or in combination. Under unstimulated conditions, the HIV-1 LTR contains two strictly positioned nucleosomes, Nuc-0 and Nuc-1 at the 5' and 3' ends of the LTR, which are separated by a loosely positioned nucleosome over the intervening DHS-1 region (Figure 5A) (Rafati et al., 2011). Treatment with Lithium, SAHA or VPA alone at the low concentrations used caused a moderate decrease in DNA protection between the positioned Nuc-0 and Nuc-2, indicating a partial loss of nucleosomes within this region. However, when Lithium and HDACi's were used in combination, significantly broader loss of nucleosomal DNA protection occurred downstream of the positioned Nuc-0, resulting in complete eviction of the repressive Nuc-1 and included loss of the positioned Nuc-2 (Figure 5B and 5C). Thus, targeting the Wnt pathway for activation in combination with HDAC inhibition causes synergistic alterations of the repressive chromatin structure resulting in eviction of Nuc-1, necessary for optimal activation of the latent HIV-1 promoter.

Combination treatment with Lithium and SAHA synergistically activate latent HIV in *ex-vivo* HIV-1 infected primary $CD4^+$ T cells

Memory $CD4^+$ T cells constitute the main reservoir of HIV-1 in vivo (Chomont et al., 2009). To investigate the expression of Wnt target genes after Lithium treatment we performed qRT-PCR on memory $CD4^+$ T cells treated with increasing concentration of Lithium. Our results showed that Lithium induces upregulation of Wnt target genes *AXIN2*, and *TCF1* in primary memory $CD4^+$ T cells. Expression levels were stable for genes that are not regulated by the Wnt pathway, such as *ICAM* and *TLR2* (Figure 6A). The efficacy of Lithium treatment in actual physiological targets of HIV-1 infection, $CD4^+$ T cells, was tested in a model mimicking HIV-1 latency in primary $CD4^+$ T cells (Figure S4). Briefly, $CD4^+$ T were

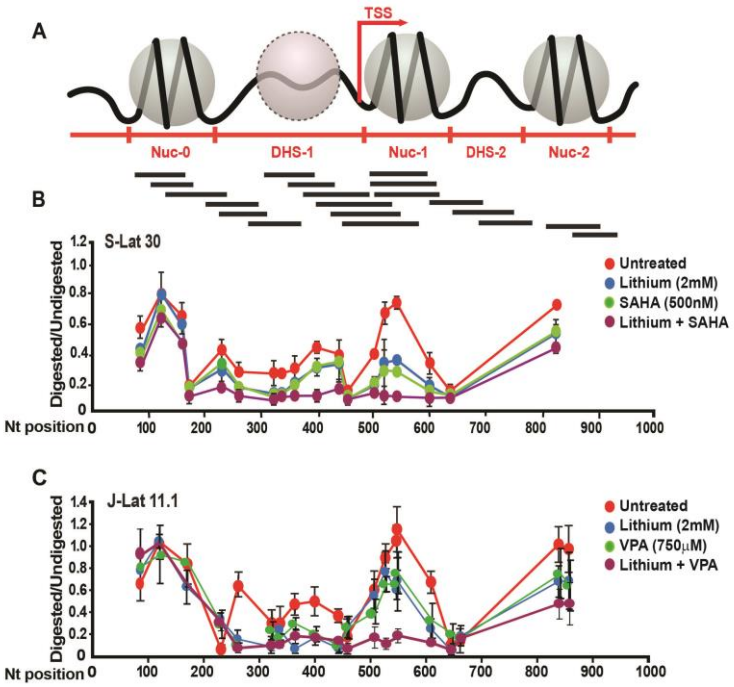

Figure 5. High Resolution Nucleosomal Mapping reveals dramatic alteration to the latent HIV-1 LTR chromatin structure upon Lithium-HDACi combination treatment. A) Chromatin organization of the HIV-1 LTR showing the position of PCR primers spanning Nuc-0, DHS-1, Nuc-1, DHS-2, and Nuc-2 regions used in the MNase assay. MNase mapping shows the synergistic changes in chromatin structure of the HIV-1 LTR induced by the combination of low concentrations of Lithium (2 mM) and HDACi's (SAHA and VPA) . The X-axis represents base pair units with 0 corresponding to the start of LTR Nuc-0. For each amplicon, relative abundance of PCR product was calculated comparing the MNase digested and undigested sample and plotted against the midpoint of the amplicon. Panel B represents the chromatin profile of the HIV-1 LTR in S-Lat 30 cells in absence of stimulation (red line), or after treatment with 2mM Lithium (blue line), 500 nM SAHA (light green line), and a combination of Lithium (2mM) and SAHA (500nM) (purple line). Panel C shows the chromatin profile of the HIV-1 LTR in J-Lat 11.1 cells in absence of stimulation (red line), or after treatment with 2mM Lithium (blue line), 750 μM VPA (green line), and a combination of Lithium (2mM) and VPA (750μM) (purple line). Dots and whiskers represent the average +/- SD of three experiments.

stimulated for 3 days with antiCD3/CD28 coated beads in presence of TGF-β1, anti IL-4, and anti IL-12 antibodies, and then cultured in presence of IL-2 for additional 4 days to allow the cells to acquire the Non Polarized (NP) phenotype. NP cells are closely similar to Central Memory T cells (T_{CM}), that represent the main compartment for HIV-1 latency in vivo (Bosque and Planelles, 2009; Chomont et al., 2009). Flow cytometric analysis demonstrated that this system produced a mixed population of memory cells (CD4+ CD45RO+), the majority of which (>60%) are phenotypically similar to T_{CM} cells (CCR7+/CD27+). Cells were infected with PNL4.3Luc.R-E- or LTR-TAT-IRES-GFP retroviral vector. 6 days post infection cells infected with

PNL4.3Luc.R-E were stimulated with Lithium (0.5, 1 mM), PMA/Ionomycin or anti-CD3/CD28 and luciferase activity was evaluated 24h after stimulation. Lithium treatment was kept in the range of therapeutic concentrations observed in patients treated for psychotic disorders. Even though these concentrations were only partially effective in reactivating HIV-1 from latently infected cells lines, we observed a 3 fold induction in luciferase activity in primary $CD4^+$ T cells (Figure 6B). Similarly, in cells infected with LTR-Tat-IRES-GFP retroviral vector Lithium induced a significant increase in the percentage of cells expressing GFP (Figure 6C). These results were further confirmed at mRNA level, indeed RT-PCR showed an increase in *GAG* gene expression in response to Lithium treatment (Figure 6D). Therefore these results demonstrated that therapeutic concentration of Lithium are effective in activating HIV-1 in physiologically relevant primary targets of HIV-1 persistence.

Discussion

New pharmaceutical strategies aimed at HIV-1 eradication have focused on molecules able to induce HIV-1 replication from latently infected cells in order to render them susceptible to viral cytopathic effects, antiviral immune responses and antiretroviral drugs (Chan and Greene, 2011; Hakre et al., 2011; Josefsson et al., 2010; Margolis and Hazuda, 2013; Massanella et al., 2013; Rasmussen et al., 2013) . Progress in the HIV-1 field has provided new insight into mechanisms that may be targeted to deplete latent HIV-1 from patients. Particularly, histone deacetylase inhibitors (HDACi's) are currently under clinical evaluation. Recently, a well-tolerated dose of the HDACi vorinostat (SAHA) was shown to disrupt the quiescent state of latently infected cells in a pilot clinical study (Archin et al., 2012a). Importantly, alternative pathways and transcription complexes have been described to silence the HIV LTR (Barton and Margolis, 2013; Boehm et al., 2013; Donahue and Wainberg, 2013; Friedman et al., 2011; Hakre et al., 2011; Rafati et al., 2011; Romerio et al., 1997; Williams et al., 2006), suggesting that a combinatorial approach targeting multiple pathways will be likely the most effective in inducing viral replication (Burnett et al., 2010).

Here we identified Wnt agonists and activators of the LEF1/β-catenin/Wnt signaling pathway as an attractive source of molecules for activating transcription from the latent HIV-1 LTR. We activated Wnt pathway using three different strategies: treatment with natural Wnt ligands, exogenous expression of a constitutively active form of β-catenin as well as by treatment with the GSK3β inhibitor Lithium. The Wnt pathway could be specifically activated in all latent HIV-1 infected T cell model systems via all three methods, as demonstrated by increase in the expression of endogenous Wnt target gene. Parallel to Wnt target gene activation we observed a specific activation of latent HIV-1 both transcriptionally and at the protein level.

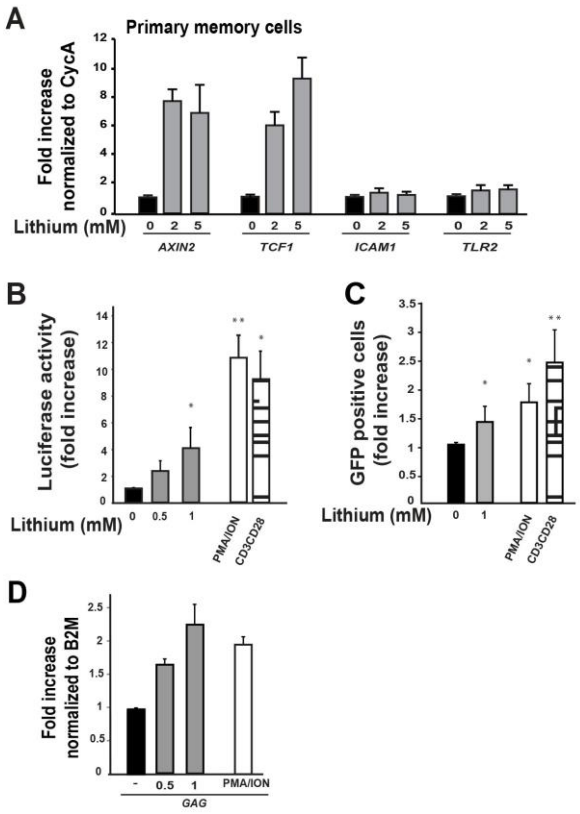

Figure 6. Therapeutically relevant concentrations of Lithium activate Wnt pathway and latent HIV-1 in primary CD4$^+$ T cells. A) Memory CD4$^+$ T cells purified by magnetic separation were treated with increasing concentration of Lithium. mRNA expression of Wnt target genes *AXIN2* and *TCF1*, as well as NFkB target genes *TLR2* and *ICAM1* was monitored by qRT-PCR. Expression levels were normalized to Cyclophillin A, bars represent the average + SD of three independent experiments. B) Primary CD4$^+$ T cells were purified from buffy coats from healthy donors and infected *ex-vivo* with HIV-1 PNL4.3LucR-E- or LTR-Tat-IRES-GFP retroviral vectors. Reactivation following PMA/Ionomycin, α-CD3/CD28 beads, and Lithium treatment was evaluated by luciferase assay (B), FACS analysis (C) and *GAG* RNA quantification (D). Increase in luciferase activity and in the percentage of GFP positive cells are shown as fold increase relative to the control. *GAG* RNA levels were normalized to β-2-microglobulin expression. Data represented as mean + SD of at least three experiments performed on cells obtained from different donors.

Several studies indicate a relationship between LEF1/β-catenin/Wnt and HIV-1 replication. LEF1 was among the first transcription factors identified as regulators of HIV-1 expression in early studies on the HIV-1 promoter (Jones and Peterlin, 1994; Sheridan et al., 1995; Waterman and Jones, 1990). In particular, LEF1 was shown to activate the HIV-1 enhancer in Jukat cells and counteract nucleosomal repression *in vitro* reconstituted LTR chromatin (Sheridan et al., 1995). Further evidence of a role for LEF1/β-catenin/Wnt signaling pathway in the regulation of HIV-1 viral cycle comes from the fact that the HIV-1 accessory proteins

Nef and Vpu have been reported to directly interact with β-catenin. Indeed it has been recently demonstrated that Nef can bind to β-catenin and downregulate its activity in 293T cells. The motif involved in the binding is conserved across HIV-1 subtype B strains, suggesting that this interaction is relevant for HIV-1 replication in the host (Weiser K, 2013). A second HIV-1 accessory protein, Vpu is also able to interact with β-catenin inhibiting its degradation and inducing its accumulation in the cytoplasm and in the nucleus (Besnard-Guerin et al., 2004). An siRNA screen for host cell factors involved in HIV-1 activation identified AXIN1, the core scaffold component of the β-catenin destruction complex, as a suppressor of HIV-1 replication (Kameoka et al., 2007). Inactivation of the β-catenin destruction complex by siRNA mediated depletion of AXIN1 activated HIV-1, demonstrating an activating role for LEF1/β-catenin/Wnt signaling in HIV-1 LTR regulation (Kameoka et al., 2009). In contrast, several studies have described a repressive role for LEF1/β-catenin/Wnt on HIV-1 transcription and replication in astrocytes (Carroll-Anzinger et al., 2007; Henderson et al., 2012a; Henderson et al., 2012b; Narasipura et al., 2012; Wortman et al., 2002). β-catenin signaling was also found to inhibit HIV-1 replication in PBMCs (Kumar et al., 2008). The ambiguity in defining a role for β-catenin/Wnt signalling in HIV-1 regulation may be explained by the distinct cell types used in the various studies and the different phases of the HIV-1 life cycle examined. The four distinct TCF/LEF transcription factor family members are expressed in a tissue specific manner and have been shown to be functionally distinct (Mao and Byers, 2011). While in astrocytes, the main model system in which a negative role has been described for Wnt signaling on HIV-1 replication, TCF4 has been predominantly studied (Narasipura et al., 2012), in T cells, TCF1 and LEF1 but not TCF4 are specifically expressed. TCF1 and LEF1, the two TCF/LEF member proteins co-expressed in T cells have been shown display critical roles in T cell development (Staal and Sen, 2008; Xue and Zhao, 2012). Interestingly, in T cells, TCF1 and LEF1 have cooperative but also opposing roles; TCF1 was found to function as a tumor suppressor while aberrant LEF1 expression was linked to malignant transformation of thymocytes (Staal and Clevers, 2012; Yu et al., 2012). In this study we evaluated the expression of the four TCF/LEF members in $CD4^+$ T cell lines and primary memory $CD4^+$ T cells which constitute the main latent HIV-1 infected cell reservoir. Western blot and mRNA expression analysis confirmed the specific expression of TCF1 and LEF1, whereas TCF3 and TCF4 could not be detected. Moreover, chromatin immunoprecipitation experiments, demonstrated that LEF1, but not TCF1 binds to the HIV-1 LTR Nuc-1 in response to Wnt stimulation and activates transcription from the latent HIV-1 LTR. Finally, epidemiological studies carried out on HIV-1 infected Long Term Non Progressor (LNTP) patients suggested an association between Wnt pathway activation and disease control. Wnt signaling is upregulated in $CD4^+$ and $CD8^+$ T cells from LNTP compared to successfully treated patients and its protective role is suggested to be related to the Wnt mediated induction of Bcl-2 and Bcl-xL, which would result in increased survival of immune cells and increased antiviral response (Fonseca et al., 2011; Wu et al., 2011). It is intriguing to speculate that in these patients Wnt activation may also induce HIV-1 expression, further

improving the ability of LNTP patients to control disease by exposing infected cells to cytotoxic immune responses.

Among Wnt activators, we focused on Lithium since this molecule is already in use in clinical practice in treatment of bipolar disorder and thus has known pharmaco-kinetic properties and safety and toxicity profiles (McKnight et al., 2012). Treatment with Lithium induced significant HIV-1 reactivation, as demonstrated by increase both in *GAG* mRNA levels and in the expression of LTR reporter genes in latently infected T cell lines. For its immuno-stimulatory properties, Lithium was proposed and used as a treatment for immune depletion in AIDS patients in the late 1980s (Parenti et al., 1988; Sztein et al., 1987), and its use for the treatment of HIV-1 related dementia is under clinical investigation (Harvey et al., 2002; Schifitto et al., 2009). Of note, an early study performed on naïve patients indicates that the levels of HIV-1 viremia were increased following treatment, suggesting a stimulatory effect of Lithium on HIV-1 replication (Parenti et al., 1988). Clinical studies performed on the HIV-1 positive bipolar patients show that the concentration of Lithium in the plasma ranges from 0.4mM to 1.2mM (Capetti et al., 2006; Letendre et al., 2006; Schifitto et al., 2009). We observed that Lithium in the same physiologically relevant concentration range (0.5-1mM) was able to stimulate Wnt signalling in memory $CD4^+$ cells, as demonstrated by increase in endogenous Wnt target gene expression. The same concentrations of Lithium induced a concomitant activation of HIV-1 in latently infected primary $CD4^+$ T cells. A recent evaluation of the effects of Lithium administration on the size of the latent HIV-1 reservoir reported no significant changes after 12 weeks of Lithium therapy in 9 HAART treated patients (Puertas et al, abstract presented at 6th International Workshop on HIV-1 Persistence During Therapy, Miami 3-6 December 2013). This finding may reflect the inability of chronic HIV-1 patients' immune system to effectively eliminate HIV-1 after activation (Shan et al., 2012). In addition, the limited HIV-1 activation following Lithium treatment alone is likely not sufficient to activate the heterogeneous pool of latently infected cells *in vivo*. Indeed, in patients, latency is a complex phenomenon resulting from multiple genetic and epigenetic mechanisms taking place in different cell types and body compartments. Taken together, these data further point out the importance of introducing the concept of combinatorial therapy in the context of *shock and kill* strategies in order to achieve a complete activation of latent HIV-1 in patients. Importantly, examination of the regulation of HIV-1 expression reveals that various cellular pathways and transcription complexes participate to regulate activity of the HIV-1 LTR and might serve as molecular targets for compounds to activate latent HIV-1 (Van Lint et al., 2013). We report functional synergism between Wnt activators and HDAC inhibitors in activation of latent HIV-1. At the molecular level, the observed Wnt effect and synergism with HDACi's was demonstrated by synergistic recruitment of the downstream effectors of Wnt, LEF1 and β-catenin to the LTR Nuc-1 as well as enrichment of acetylated histones at the LTR Nuc-1 region. In addition, Lithium treatment synergized with HDACi's to cause dramatic

remodeling of the latent HIV-1 LTR leading to complete eviction of the repressive Nuc-1 as shown by high resolution MNase nucleosomal mapping studies.

Latent integrated HIV-1 proviruses are subject to varying degrees of chromatin repression dependent on sites of integration and chromatin environment, and their LTRs are characterized by hypoacetylated histones, generated by the enzymatic activity of HDACs. This hypoacetylated histone profile results in a compact chromatin structure incompatible with gene expression (Pearson et al., 2008; Tyagi et al., 2010). Our data is consistent with a two-step model for synergistic activation of the latent HIV-1 LTR in response to combinatorial treatment with HDACi's and Wnt pathway activators (Figure 7): as a first step (figure 7A), inhibition of HDACs by HDACi's prevents deacetylation of histones resulting in increased accumulation of basal acetylation. This limited chromatin remodeling at the HIV-1 LTR generates a more relaxed chromatin structure allowing DNA access to sequence specific transcription factors and converts it into a transcriptionally permissive state. As a result, LEF1, which in the latent state binds to its recognition sequences buried within hypoacetylated chromatin with limited ability, now gains access to its consensus sequences. As a second step (figure 7B), in presence of Wnt signalling, β-catenin complexes with LEF1, increasing its affinity for DNA and recruits a number of transcriptional co-activators including the acetyl transferases P300/CBP to the HIV-1 LTR resulting in synergistic LTR histone acetylation and chromatin remodelling as demonstrated by removal of the repressive Nuc-1, and synergistic transcriptional activation.

Whereas HIV-1 treatment itself has profited immensely from the concept of combination therapy, now also spreading to cancer treatment, clinical studies probing activation of latent HIV-1 have remained the domain of single drug treatments. Potential limitations for use of single drugs in activating latent HIV-1 include their toxicity, and lack of specificity, as many of these drugs including HDACi's target cellular proteins with important pleiotropic functions. To increase the target specificity and efficiency of drugs in a future purge and kill therapeutic strategy, it will be essential to target pathways, which when targeted combinatorially would confer specificity for the HIV-1 promoter. Our work identifies a positive role for Wnt pathway agonists and in particular for Lithium in activation of latent HIV-1. Moreover, our data showed that Lithium and HDACi's are able to synergistically activate HIV-1, via a synergistic mechanism that is detected strongly at the molecular level. The combination of low concentrations of LTR de-repressors and activators, potentially including HDACi's and Wnt agonists displaying synergism and specificity on the HIV-1 LTR represent an attractive strategy in efficient activation of latent HIV-1. More studies are needed to clarify the complex interplay between the Wnt pathway, immune cells and HIV-1. Importantly, future studies will determine the effect of Lithium and other Wnt agonists alone and in combination with HDACi's and other putative LTR activators in activation of latent HIV-1 in HIV-1 infected patients.

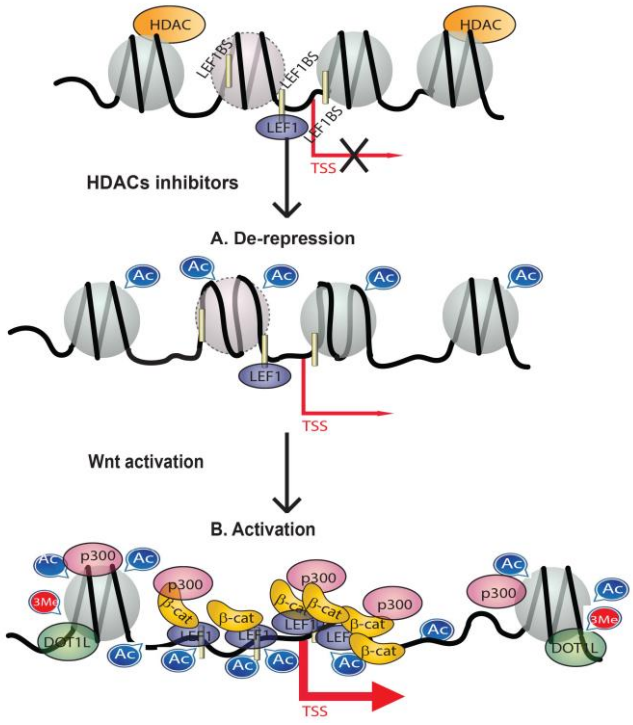

Figure 7. Two-step model depicting molecular synergism between HDAC inhibition and Wnt activation on the HIV-1 LTR 1. De-repression: inhibition of HDACs by HDACi's results in increased accumulation of basal acetylation and limited chromatin remodelling at the HIV-1 LTR converting it to a transcriptionally permissive state. As a result, LEF1, which in the latent state binds to its recognition sequences (LEF1BS) buried within hypoacetylated chromatin with limited ability, now gains access to its consensus sequences. 2: Activation: in presence of Wnt signalling, β-catenin complexes with LEF1 increasing its affinity for LEF1 consensus binding sites and recruits a number of transcriptional co-activators including P300/CBP resulting in synergistic LTR histone acetylation and chromatin remodelling as demonstrated by eviction of the repressive Nuc-1 leading to transcriptional activation.

Materials and Methods

Cell Lines, Plasmids, Chemical, and Reagents

Jurkat 1G5 (containing an integrated LTR-luciferase cassette) (Aguilar-Cordova et al., 1994), , J-Lat A2 cells (containing a latent integrated LTR-Tat-IRES-GFP cassette) (Jordan et al., 2001) and J-Lat 11.1 (containing a latent integrated defective copy of the HIV-1 genome harboring *GFP* in place of *Nef* and a frameshift mutation in the E*nv* gene) (Jordan et al., 2003), have been previously described as models of HIV-1 latency. We also generated additional clonal latently infected Jurkat (J-Lat) and SupT1 (S-Lat) cell lines (J-Lat P32, J-Lat P20, J-lat T44, S-Lat 30, S-Lat 9, S-Lat 8) containing one integrated copy of the LTR-Tat-IRES-GFP retroviral vector as previously described (Jordan et al., 2003).

Primary memory $CD4^+$ cells were isolated from buffy coats from healthy donors by Ficoll gradient followed by magnetic separation using EasySep Human Memory $CD4^+$ T Cell Enrichment Kit (StemCells Technologies).

TOPFLASH (optimal) and FOPFLASH (mutated) TCF-driven luciferase-reporter constructs (kind gift of H. Clevers) were described previously (van de Wetering et al., 2002). We used constitutively active β-catenin and control empty expression vectors which generated previously (van de Wetering et al., 2002).

Lithium (Fluka), Valproic acid (Sigma), SAHA/ vorinostat (sellekchem), PMA (Sigma), Ionomycin (Sigma), α- CD3/CD28 coated beads (Dynabeads), IL-2 (Sigma), and TGF-β (Sigma) were used to treat cells as indicated.

Wnt 3A and control L-cell conditioned media was collected as supernatant from mouse L cells (ATCC CRL-2647 (control) and ATCC CRL-2648 (producing Wnt3A protein) as previously described (Willert et al., 2003).

Antibodies, and Western Blot Analysis

Antibodies recognizing β-catenin (Santa Cruz), LEF1 (Santa Cruz), TCF1 (Upstate), TCF3/4 (Upstate), TCF4 (Santa Cruz), DOT1L (Abgent), H3K79 trimethylation (Abcam), Acetyl-Histone H3 (Abcam), Acetyl-Histone H4 (Millipore), H2B (Abcam), and H3 (Millipore) were used in chromatin immunoprecipitation (ChIP) or Western blot analysis.

Jurkat, SupT1, LS174T, Hela, and 293T cells were lysed in protein lysis buffer (1% Triton X-100, 2mM EDTA, 1mM DTT, 5% glycerol in PBS), subjected to SDS-PAGE and western blot analysis and probed with α-TCF1, LEF1, TCF3/4, and TCF4 antibodies as indicated.

Amaxa Nucleofection and Luciferase Assays

Nucleofection of S-Lat 30 latent cell line was done as previously described (Mahmoudi et al., 2006; Rafati et al., 2011). Briefly, cells were split to $3x10^5$ cells/ml 24 h prior to Amaxa nucleofection. Five million cells were centrifuged at 1,000 rpm for 10 min at room temperature, resuspended in 100 µl of solution R, and nucleofected with 2 µg of expression plasmid using program O28. Nucleofected cells were resuspended in 500 µl of pre-warmed, serum-free RPMI lacking antibiotics and allowed to recover at 37˚C in a 5% CO2 incubator for 15 min. Pre-warmed complete RPMI (4 ml) was then added to the cells.

S-Lat 30 cells were nucleofected with TOPFLASH/FOPFLASH reporter vectors in presence or absence of β-catenin or control expression vectors as indicated. After 24-48 h, cells were lysed in Luciferase Lysis Buffer (Promega), and luciferase activity was measured in a luminometer using the Dual-Luciferase Reporter Assay System (Promega).

Chromatin Immunoprecipitation (ChIP)

J-Lat 11.1 and S-Lat 30 cells were subjected to ChIP assays as previously described (Rafati et al., 2011). Briefly, cells were crosslinked by addition of formaldehyde to a final concentration of 1% for 30 min (for immunoprecipitation (IP) of transcription factors and co-factors) or 10 min (for IP of total or modified histones) at room temperature. The crosslinking reaction was quenched with 125 mM glycine, cells were washed with buffer B (0.25%Triton-X 100, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6) and buffer C (150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6), and resuspended in ChIP incubation buffer (1% SDS, 1% Triton-X 100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6). Chromatin was sheared by sonication to an apparent length of 150-350 nt using a Bio Ruptor sonicator (Cosmo Bio Co., Ltd) with 20 X 1 min pulses at maximum setting. Approximately 20 or 10 million cells were used per transcription factor/cofactor or histone IP respectively. 2-5 µg of the indicated antibody was used in IP reaction with the chromatin and BSA-blocked protein A+G beads overnight at 4˚C. IPs were washed twice with each buffer 1 (0.1% SDS, 0.1% deoxycholate, 1% Triton-X 100, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6), buffer 2 (0.1% SDS, 0.1% deoxycholate,1% Triton-X 100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA,20 mM Hepes pH 7.6), buffer 3 (250 mM Lithium, 0.5% deoxycholate,0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6), and buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6). Immunoprecipitated complexes were eluted in elution buffer (1% SDS, 0.1 M NaHCO3) for 30 min at RT and decrosslinked overnight at 65˚C in presence of 200 mM $NaCl_2$. DNA was phenol:chloroform extracted, chloroform:isoamylalcohol extracted, ethanol precipitated, and resuspended in 100 µl H2O by shaking at 37˚C. Input and immunoprecipitated DNA (5 µl) were subjected to qPCR cycles with specific primers.

High Resolution MNase Nucleosomal Mapping

High resolution MNase mapping protocol for the HIV-1 LTR was performed as previously described (Rafati et al., 2011). Briefly, cells were cross-linked according to the ChIP protocol described above. After one wash in cold PBS, $1.5x10^7$crosslinked cells were resuspended in 1 ml hypotonic buffer A (300 mM sucrose, 2 mM Mg acetate, 3 mM Cacl2, 10 mMTris pH 8.0,0.1% Triton X-100, 0.5 mM DTT), incubated on ice for 5 min, and dounced 20 times with 2 ml dounce homogenizer (tight pestle, Wheaton). Nuclei were collected by centrifugation at 4˚C for 5 min at 720xg. Pellets were resuspended in 1 ml buffer D (25% glycerol, 5 mM Mg acetate, 50 mM Tris pH 8.0, 0.1 mM EDTA, 5 mM DTT) at $1.5X10^7$ nuclei/ml. Chromatin was collected by centrifuging at 4˚C for 5 min at 720xg. The pellets were resuspended in 1 ml buffer MN (60 mM KCl, 15 mM NaCl, 15 mM Tris pH 7.4, 0.5 mM DTT, 0.25 mM sucrose, 1.0 mM CaCl2) at 2.5x107 nuclei/ml. The equivalent of $2.5x10^6$ nuclei were used per MNase reaction. MNase (USB), diluted in buffer MN, was added so that 0, 0.5, 5, 20, 50, and 500 total units were used per 150 µl reaction and digested for 30 min at room temperature. Reactions

were stopped with the addition of EDTA and SDS to final concentrations of 12.5 mM and 0.5% respectively. After 4 hours of proteinase K digestion at 37˚C, each reaction was processed similar to ChIP samples from the point of elution from the beads.

Real-Time qPCR Analysis

ChIP and MNase digested samples were analyzed by quantitative PCR in CFX manager real-time PCR detection system (BioRad) using Go Taq qPCR master mix 2x (Promega). ChIP values were normalized as a percentage of input. For MNase digests fold difference was calculated using the DCT method between MNase treated and untreated samples. Sequences of qPCR primer pairs used to amplify distinct regions within the HIV-1 LTR were described previously (Rafati et al., 2011). The primer sequences used for qRT-PCR are provided in Table S2. RNA was purified using ReliaPrep RNA Cell Miniprep System (Promega) and cDNA was made using Superscript II Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. Expression data are presented as fold increase normalized to Cyclophillin A control.

Isolation and Latency Establishment Experiment in Primary $CD4^+$ T Cells

An *ex vivo* model of HIV-1 latency was set up following the method developed by Bosque and Planelles (Bosque and Planelles, 2009) with some modifications. PBMCs were isolated from buffy coats obtained from healthy donors by Ficol gradient density sedimentation. Purified PBMCs were directly subjected to the $CD4^+$ T-Cell enrichment kit (Stem cell technologies). $CD4^+$ T cells were purified according to manufacturer instructions and cultured in RPMI 1640 containing 10% FCS and pen/strep in presence of TGF-β (10 ng/ml), α- IL-4 (1ug/ml), α- IL-12 (2ug/ml), and α-CD3/CD28 coated beads in 1:1 ratio for 3 days. After 3 days α-CD3/CD28 coated beads were removed and cells were cultured in RPMI 1640 containing 10% FCS and IL-2 (5 ng/ml) for 4 days. To phenotype the cells, $1x10^6$ cells were stained with PB–anti-CD4 (Becton Dickinson), FITC–anti-CD45RO (Dako), APC–anti-CD27 (Becton Dickinson), PE-anti-CCR7 (eBioscience), and analysed in a FACS Fortessa flow cytometer (Becton Dickinson, MountainView, CA).

On day 7 post stimulation cells were infected with HIV-derived virus particles. Viral pseudotypes were produced essentially as described (Jordan et al., 2001) by co-transfecting HXB2 Env or VSVG expression vector together with either HIV-1 backbone (pNL43.Luc.R-E-) or the HIV-derived 731 vector (LTR-Tat-IRES-GFP) in the presence of packaging vector R8.91, into 293T cells using the FuGENE 6 Transfection Reagent (Promega). Twenty four, 48 and 72 hours post-transfection, the pseudovirus-containing supernatant was collected, filtered through a 0.45μm filter, aliquoted, and stored at -80˚C. HIV-1 molecular clone pNL4-3.Luc.R-E- and HIV

HXB2-Env expression vector were obtained from the Centre for AIDS Reagents, NIBSC and were donated by Dr. Nathaniel Landau and Drs Kathleen Page and Dan Littman, respectively.

Primary $CD4^+$ T cells, were infected with the PNL4.3LUC virus by spinoculation (90 minutes at 1200g), washed in PBS and cultured in RPMI 1640 containing 10% FCS and IL-2. 6 days after infection cells were either untreated or treated with PMA, α-CD3/CD28 coated beads, and increasing concentrations (0.5-1 mM) of Lithium. Cells were harvested at 6h post treatment to evaluate gene expression in response to stimulation. 24h after stimulation luciferase activity was evaluated using Luciferase Assay System (Promega). Infection with the LTR-Tat-IRES-GFP retroviral vector were performed as follows: primary $CD4^+$ T cells were infected with the virus at low MOI so that less than 5% of cells were infected. 5 days after infection, the GFP negative cell population harbouring uninfected as well as presumably latently infected cells was sorted by Flow Cytometry Activated Cell sorting (FACS). GFP negative cells were then either untreated or treated with PMA, α-CD3/CD28 coated beads, increasing concentrations (1, 2 mM) of Lithium. 24 after treatment % GFP positive cells was monitored by FACS.

Computational and Statistical Analysis

TFconsite tool (Sandelin et al., 2004) was used for determination of different transcription factor binding sites on HIV-1 LTR in pNL4-3 HIV-1.

Position weighted matrices were generated from the alignment for each LEF1 binding site (Pereira et al., 2000) and sequence logos were generated A representative set of HIV-1 subtype B sequences was downloaded from the HIV database (http://hiv.lan.org) and aligned using Bioedit. using TINYRAY weblogo application (http://demo.tinyray.com/weblogo).

The data are shown as the average ± S.D of two or more independent experiments. Statistical significance for multiple comparisons was calculated using Student's *t* test, *, ** and *** indicate the level of significance at $p<0.05$, 0.01 and 0.001, respectively.

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References

Aguilar-Cordova, E., Chinen, J., Donehower, L., Lewis, D.E., and Belmont, J.W. (1994). A sensitive reporter cell line for HIV-1 tat activity, HIV-1 inhibitors, and T cell activation effects. AIDS Res Hum Retroviruses *10*, 295-301.

Archin, N.M., Cheema, M., Parker, D., Wiegand, A., Bosch, R.J., Coffin, J.M., Eron, J., Cohen, M., and Margolis, D.M. (2010). Antiretroviral intensification and valproic acid lack sustained effect on residual HIV-1 viremia or resting CD4+ cell infection. PLoS One *5*, e9390.

Archin, N.M., Espeseth, A., Parker, D., Cheema, M., Hazuda, D., and Margolis, D.M. (2009). Expression of latent HIV induced by the potent HDAC inhibitor suberoylanilide hydroxamic acid. AIDS Res Hum Retroviruses *25*, 207-212.

Archin, N.M., Liberty, A.L., Kashuba, A.D., Choudhary, S.K., Kuruc, J.D., Crooks, A.M., Parker, D.C., Anderson, E.M., Kearney, M.F., Strain, M.C*., et al.* (2012a). Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. Nature *487*, 482-485.

Archin, N.M., Vaidya, N.K., Kuruc, J.D., Liberty, A.L., Wiegand, A., Kearney, M.F., Cohen, M.S., Coffin, J.M., Bosch, R.J., Gay, C.L*., et al.* (2012b). Immediate antiviral therapy appears to restrict resting CD4+ cell HIV-1 infection without accelerating the decay of latent infection. Proc Natl Acad Sci U S A *109*, 9523-9528.

Banerjee, C., Archin, N., Michaels, D., Belkina, A.C., Denis, G.V., Bradner, J., Sebastiani, P., Margolis, D.M., and Montano, M. (2012). BET bromodomain inhibition as a novel strategy for reactivation of HIV-1. J Leukoc Biol *92*, 1147-1154.

Barton, K., and Margolis, D. (2013). Selective targeting of the repressive transcription factors YY1 and cMyc to disrupt quiescent human immunodeficiency viruses. AIDS Res Hum Retroviruses *29*, 289-298.

Besnard-Guerin, C., Belaidouni, N., Lassot, I., Segeral, E., Jobart, A., Marchal, C., and Benarous, R. (2004). HIV-1 Vpu sequesters beta-transducin repeat-containing protein (betaTrCP) in the cytoplasm and provokes the accumulation of beta-catenin and other SCFbetaTrCP substrates. J Biol Chem *279*, 788-795.

Blankson, J., Persaud, D., and Siliciano, R.F. (1999). Latent reservoirs for HIV-1. Curr Opin Infect Dis *12*, 5-11.

Blazkova, J., Chun, T.W., Belay, B.W., Murray, D., Justement, J.S., Funk, E.K., Nelson, A., Hallahan, C.W., Moir, S., Wender, P.A*., et al.* (2012). Effect of histone deacetylase inhibitors on HIV production in latently infected, resting CD4(+) T cells from infected individuals receiving effective antiretroviral therapy. J Infect Dis *206*, 765-769.

Boehm, D., Calvanese, V., Dar, R.D., Xing, S., Schroeder, S., Martins, L., Aull, K., Li, P.C., Planelles, V., Bradner, J.E*., et al.* (2013). BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism. Cell Cycle *12*, 452-462.

Bosque, A., and Planelles, V. (2009). Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. Blood *113*, 58-65.

Burnett, J.C., Lim, K.I., Calafi, A., Rossi, J.J., Schaffer, D.V., and Arkin, A.P. (2010). Combinatorial latency reactivation for HIV-1 subtypes and variants. J Virol *84*, 5958-5974.

Burnett, J.C., Miller-Jensen, K., Shah, P.S., Arkin, A.P., and Schaffer, D.V. (2009). Control of stochastic gene expression by host factors at the HIV promoter. PLoS Pathog *5*, e1000260.

Capetti, A., Landonio, S., Faggion, I., and Rizzardini, G. (2006). Drop in plasma lithium levels in 2 patients on concomitant boosted protease inhibitor regimens. J Int Assoc Physicians AIDS Care (Chic) *5*, 119-120.

Carroll-Anzinger, D., Kumar, A., Adarichev, V., Kashanchi, F., and Al-Harthi, L. (2007). Human immunodeficiency virus-restricted replication in astrocytes and the ability of gamma interferon to modulate this restriction are regulated by a downstream effector of the Wnt signaling pathway. J Virol *81*, 5864-5871.

Chan, J.K., and Greene, W.C. (2011). NF-kappaB/Rel: agonist and antagonist roles in HIV-1 latency. Curr Opin HIV AIDS *6*, 12-18.

Chomont, N., El-Far, M., Ancuta, P., Trautmann, L., Procopio, F.A., Yassine-Diab, B., Boucher, G., Boulassel, M.R., Ghattas, G., Brenchley, J.M*., et al.* (2009). HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat Med *15*, 893-900.

Chun, T.W., Justement, J.S., Moir, S., Hallahan, C.W., Maenza, J., Mullins, J.I., Collier, A.C., Corey, L., and Fauci, A.S. (2007). Decay of the HIV reservoir in patients receiving antiretroviral therapy for extended periods: implications for eradication of virus. J Infect Dis *195*, 1762-1764.

Chun, T.W., Justement, J.S., Murray, D., Hallahan, C.W., Maenza, J., Collier, A.C., Sheth, P.M., Kaul, R., Ostrowski, M., Moir, S*., et al.* (2010). Rebound of plasma viremia following cessation of antiretroviral therapy despite profoundly low levels of HIV reservoir: implications for eradication. AIDS *24*, 2803-2808.

Clevers, H., and Nusse, R. (2012). Wnt/beta-catenin signaling and disease. Cell *149*, 1192-1205.

Contreras, X., Schweneker, M., Chen, C.S., McCune, J.M., Deeks, S.G., Martin, J., and Peterlin, B.M. (2009). Suberoylanilide hydroxamic acid reactivates HIV from latently infected cells. J Biol Chem *284*, 6782-6789.

Coull, J.J., Romerio, F., Sun, J.M., Volker, J.L., Galvin, K.M., Davie, J.R., Shi, Y., Hansen, U., and Margolis, D.M. (2000). The human factors YY1 and LSF repress the human immunodeficiency virus type 1 long terminal repeat via recruitment of histone deacetylase 1. J Virol *74*, 6790-6799.
de Lau, W., Barker, N., Low, T.Y., Koo, B.K., Li, V.S., Teunissen, H., Kujala, P., Haegebarth, A., Peters, P.J., van de Wetering, M.*, et al.* (2011). Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature *476*, 293-297.
Donahue, D.A., and Wainberg, M.A. (2013). Cellular and molecular mechanisms involved in the establishment of HIV-1 latency. Retrovirology *10*, 11.
Durand, C.M., Blankson, J.N., and Siliciano, R.F. (2012). Developing strategies for HIV-1 eradication. Trends Immunol *33*, 554-562.
Edelstein, L.C., Micheva-Viteva, S., Phelan, B.D., and Dougherty, J.P. (2009). Short communication: activation of latent HIV type 1 gene expression by suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor approved for use to treat cutaneous T cell lymphoma. AIDS Res Hum Retroviruses *25*, 883-887.
Finzi, D., Blankson, J., Siliciano, J.D., Margolick, J.B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Lori, F., Flexner, C.*, et al.* (1999). Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med *5*, 512-517.
Finzi, D., Hermankova, M., Pierson, T., Carruth, L.M., Buck, C., Chaisson, R.E., Quinn, T.C., Chadwick, K., Margolick, J., Brookmeyer, R.*, et al.* (1997). Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science *278*, 1295-1300.
Fonseca, S.G., Procopio, F.A., Goulet, J.P., Yassine-Diab, B., Ancuta, P., and Sekaly, R.P. (2011). Unique features of memory T cells in HIV elite controllers: a systems biology perspective. Curr Opin HIV AIDS *6*, 188-196.
Fraser, C., Ferguson, N.M., Ghani, A.C., Prins, J.M., Lange, J.M., Goudsmit, J., Anderson, R.M., and de Wolf, F. (2000). Reduction of the HIV-1-infected T-cell reservoir by immune activation treatment is dose-dependent and restricted by the potency of antiretroviral drugs. AIDS *14*, 659-669.
Friedman, J., Cho, W.K., Chu, C.K., Keedy, K.S., Archin, N.M., Margolis, D.M., and Karn, J. (2011). Epigenetic silencing of HIV-1 by the histone H3 lysine 27 methyltransferase enhancer of Zeste 2. J Virol *85*, 9078-9089.
Hakre, S., Chavez, L., Shirakawa, K., and Verdin, E. (2011). Epigenetic regulation of HIV latency. Curr Opin HIV AIDS *6*, 19-24.
Hamimi, C., Pancino, G., Barre-Sinoussi, F., and Saez-Cirion, A. (2013). Will it be possible to live without antiretroviral therapy? Curr Opin HIV AIDS *8*, 196-203.
Harvey, B.H., Meyer, C.L., Gallichio, V.S., and Manji, H.K. (2002). Lithium salts in AIDS and AIDS-related dementia. Psychopharmacol Bull *36*, 5-26.
Henderson, L.J., Narasipura, S.D., Adarichev, V., Kashanchi, F., and Al-Harthi, L. (2012a). Identification of novel T cell factor 4 (TCF-4) binding sites on the HIV long terminal repeat which associate with TCF-4, beta-catenin, and SMAR1 to repress HIV transcription. J Virol *86*, 9495-9503.
Henderson, L.J., Sharma, A., Monaco, M.C., Major, E.O., and Al-Harthi, L. (2012b). Human immunodeficiency virus type 1 (HIV-1) transactivator of transcription through its intact core and cysteine-rich domains inhibits Wnt/beta-catenin signaling in astrocytes: relevance to HIV neuropathogenesis. J Neurosci *32*, 16306-16313.
Jiang, G., Espeseth, A., Hazuda, D.J., and Margolis, D.M. (2007). c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter. J Virol *81*, 10914-10923.
Jones, K.A., and Peterlin, B.M. (1994). Control of RNA initiation and elongation at the HIV-1 promoter. Annu Rev Biochem *63*, 717-743.
Jordan, A., Bisgrove, D., and Verdin, E. (2003). HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J *22*, 1868-1877.
Jordan, A., Defechereux, P., and Verdin, E. (2001). The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. EMBO J *20*, 1726-1738.
Josefsson, L., Dahl, V., and Palmer, S. (2010). Can HIV infection be eradicated through use of potent antiviral agents? Curr Opin Infect Dis *23*, 628-632.
Kameoka, M., Kameoka, Y., Utachee, P., Kurosu (2009). Short communication: RNA interference directed against Axin1 upregulates human immunodeficiency virus type 1 gene expression by activating the Wnt signaling pathway in HeLa-derived J111 cells. AIDS Res Hum Retroviruses *25*, 1005-1011.
Kameoka, M., Kitagawa, Y., Utachee, P., Jinnopat, P., Dhepakson, P., Isarangkura-na-ayuthaya, P., Tokunaga, K., Sato, H., Komano, J., Yamamoto, N.*, et al.* (2007). Identification of the suppressive factors for human immunodeficiency virus type-1 replication using the siRNA mini-library directed against host cellular genes. Biochem Biophys Res Commun *359*, 729-734.
Karn, J. (2011). The molecular biology of HIV latency: breaking and restoring the Tat-dependent transcriptional circuit. Curr Opin HIV AIDS *6*, 4-11.

Katlama, C., Deeks, S.G., Autran, B., Berkhout, B., Benkirane, M., Cairns, S., Chomont, N., Chun, T.W., Churchill, M., Di Mascio, M., *et al.* (2012). Towards an HIV cure: a global scientific strategy. Nat Rev Immunol *12*, 607-614.
Kent, S.J., Reece, J.C., Petravic, J., Martyushev, A., Kramski, M., De Rose, R., Cooper, D.A., Kelleher, A.D., Emery, S., Cameron, P.U., *et al.* (2013). The search for an HIV cure: tackling latent infection. Lancet Infect Dis *13*, 614-621.
Kumar, A., Zloza, A., Moon, R.T., Watts, J., Tenorio, A.R., and Al-Harthi, L. (2008). Active beta-catenin signaling is an inhibitory pathway for human immunodeficiency virus replication in peripheral blood mononuclear cells. J Virol *82*, 2813-2820.
Le, T., Farrar, J., and Shikuma, C. (2011). Rebound of plasma viremia following cessation of antiretroviral therapy despite profoundly low levels of HIV reservoir: implications for eradication. AIDS *25*, 871-872; author reply 872-873.
Lehrman, G., Hogue, I.B., Palmer, S., Jennings, C., Spina, C.A., Wiegand, A., Landay, A.L., Coombs, R.W., Richman, D.D., Mellors, J.W., *et al.* (2005). Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. Lancet *366*, 549-555.
Letendre, S.L., Woods, S.P., Ellis, R.J., Atkinson, J.H., Masliah, E., van den Brande, G., Durelle, J., Grant, I., Everall, I., and Group, H. (2006). Lithium improves HIV-associated neurocognitive impairment. AIDS *20*, 1885-1888.
Lewin, S.R., and Rouzioux, C. (2011). HIV cure and eradication: how will we get from the laboratory to effective clinical trials? AIDS *25*, 885-897.
Li, J., Sutter, C., Parker, D.S., Blauwkamp, T., Fang, M., and Cadigan, K.M. (2007). CBP/p300 are bimodal regulators of Wnt signaling. EMBO J *26*, 2284-2294.
Li, V.S., Ng, S.S., Boersema, P.J., Low, T.Y., Karthaus, W.R., Gerlach, J.P., Mohammed, S., Heck, A.J., Maurice, M.M., Mahmoudi, T., *et al.* (2012). Wnt signaling through inhibition of beta-catenin degradation in an intact Axin1 complex. Cell *149*, 1245-1256.
Li, Y., Mak, G., and Franza, B.R., Jr. (1994). In vitro study of functional involvement of Sp1, NF-kappa B/Rel, and AP1 in phorbol 12-myristate 13-acetate-mediated HIV-1 long terminal repeat activation. J Biol Chem *269*, 30616-30619.
Lusic, M., Marcello, A., Cereseto, A., and Giacca, M. (2003). Regulation of HIV-1 gene expression by histone acetylation and factor recruitment at the LTR promoter. EMBO J *22*, 6550-6561.
Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P.M., Birchmeier, W., *et al.* (2002). Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol *22*, 1184-1193.
Mahmoudi, T. (2012). The BAF complex and HIV latency. Transcription *3*, 171-176.
Mahmoudi, T., Boj, S.F., Hatzis, P., Li, V.S., Taouatas, N., Vries, R.G., Teunissen, H., Begthel, H., Korving, J., Mohammed, S., *et al.* (2010). The leukemia-associated Mllt10/Af10-Dot1l are Tcf4/beta-catenin coactivators essential for intestinal homeostasis. PLoS Biol *8*, e1000539.
Mahmoudi, T., Li, V.S., Ng, S.S., Taouatas, N., Vries, R.G., Mohammed, S., Heck, A.J., and Clevers, H. (2009). The kinase TNIK is an essential activator of Wnt target genes. EMBO J *28*, 3329-3340.
Mahmoudi, T., Parra, M., Vries, R.G., Kauder, S.E., Verrijzer, C.P., Ott, M., and Verdin, E. (2006). The SWI/SNF chromatin-remodeling complex is a cofactor for Tat transactivation of the HIV promoter. J Biol Chem *281*, 19960-19968.
Mao, C.D., and Byers, S.W. (2011). Cell-context dependent TCF/LEF expression and function: alternative tales of repression, de-repression and activation potentials. Crit Rev Eukaryot Gene Expr *21*, 207-236.
Margolis, D.M. (2010). Mechanisms of HIV latency: an emerging picture of complexity. Curr HIV/AIDS Rep *7*, 37-43.
Margolis, D.M., and Archin, N.M. (2007). Eliminating persistent HIV infection: getting to the end of the rainbow. J Infect Dis *195*, 1734-1736.
Margolis, D.M., and Hazuda, D.J. (2013). Combined approaches for HIV cure. Curr Opin HIV AIDS *8*, 230-235.
Massanella, M., Martinez-Picado, J., and Blanco, J. (2013). Attacking the HIV reservoir from the immune and viral perspective. Curr HIV/AIDS Rep *10*, 33-41.
Mohan, M., Herz, H.M., Takahashi, Y.H., Lin, C., Lai, K.C., Zhang, Y., Washburn, M.P., Florens, L., and Shilatifard, A. (2010). Linking H3K79 trimethylation to Wnt signaling through a novel Dot1-containing complex (DotCom). Genes Dev *24*, 574-589.
Narasipura, S.D., Henderson, L.J., Fu, S.W., Chen, L., Kashanchi, F., and Al-Harthi, L. (2012). Role of beta-catenin and TCF/LEF family members in transcriptional activity of HIV in astrocytes. J Virol *86*, 1911-1921.
Pantaleo, G., and Levy, Y. (2013). Vaccine and immunotherapeutic interventions. Curr Opin HIV AIDS *8*, 236-242.
Parenti, D.M., Simon, G.L., Scheib, R.G., Meyer, W.A., 3rd, Sztein, M.B., Paxton, H., DiGioia, R.A., and Schulof, R.S. (1988). Effect of lithium carbonate in HIV-infected patients with immune dysfunction. J Acquir Immune Defic Syndr *1*, 119-124.
Pearson, R., Kim, Y.K., Hokello, J., Lassen, K., Friedman, J., Tyagi, M., and Karn, J. (2008). Epigenetic silencing of human immunodeficiency virus (HIV) transcription by formation of restrictive chromatin structures at the viral long terminal repeat drives the progressive entry of HIV into latency. J Virol *82*, 12291-12303.
Pereira, L.A., Bentley, K., Peeters, A., Churchill, M.J., and Deacon, N.J. (2000). A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. Nucleic Acids Res *28*, 663-668.

Perkins, N.D., Edwards, N.L., Duckett, C.S., Agranoff, A.B., Schmid, R.M., and Nabel, G.J. (1993). A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. EMBO J *12*, 3551-3558.
Pierson, T., McArthur, J., and Siliciano, R.F. (2000). Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. Annu Rev Immunol *18*, 665-708.
Rafati, H., Parra, M., Hakre, S., Moshkin, Y., Verdin, E., and Mahmoudi, T. (2011). Repressive LTR nucleosome positioning by the BAF complex is required for HIV latency. PLoS Biol *9*, e1001206.
Rasmussen, T.A., Tolstrup, M., Winckelmann, A., Ostergaard, L., and Sogaard, O.S. (2013). Eliminating the latent HIV reservoir by reactivation strategies: Advancing to clinical trials. Hum Vaccin Immunother *9*, 790-799.
Richman, D.D., Margolis, D.M., Delaney, M., Greene, W.C., Hazuda, D., and Pomerantz, R.J. (2009). The challenge of finding a cure for HIV infection. Science *323*, 1304-1307.
Romerio, F., Gabriel, M.N., and Margolis, D.M. (1997). Repression of human immunodeficiency virus type 1 through the novel cooperation of human factors YY1 and LSF. J Virol *71*, 9375-9382.
Roose, J., and Clevers, H. (1999). TCF transcription factors: molecular switches in carcinogenesis. Biochim Biophys Acta *1424*, M23-37.
Sagot-Lerolle, N., Lamine, A., Chaix, M.L., Boufassa, F., Aboulker, J.P., Costagliola, D., Goujard, C., Pallier, C., Delfraissy, J.F., Lambotte, O.*, et al.* (2008). Prolonged valproic acid treatment does not reduce the size of latent HIV reservoir. Aids *22*, 1125-1129.
Sandelin, A., Wasserman, W.W., and Lenhard, B. (2004). ConSite: web-based prediction of regulatory elements using cross-species comparison. Nucleic Acids Res *32*, W249-252.
Schifitto, G., Zhong, J., Gill, D., Peterson, D.R., Gaugh, M.D., Zhu, T., Tivarus, M., Cruttenden, K., Maggirwar, S.B., Gendelman, H.E.*, et al.* (2009). Lithium therapy for human immunodeficiency virus type 1-associated neurocognitive impairment. J Neurovirol *15*, 176-186.
Sedaghat, A.R., Siliciano, R.F., and Wilke, C.O. (2008). Low-level HIV-1 replication and the dynamics of the resting CD4+ T cell reservoir for HIV-1 in the setting of HAART. BMC Infect Dis *8*, 2.
Shan, L., Deng, K., Shroff, N.S., Durand, C.M., Rabi, S.A., Yang, H.C., Zhang, H., Margolick, J.B., Blankson, J.N., and Siliciano, R.F. (2012). Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. Immunity *36*, 491-501.
Sheridan, P.L., Sheline, C.T., Cannon, K., Voz, M.L., Pazin, M.J., Kadonaga, J.T., and Jones, K.A. (1995). Activation of the HIV-1 enhancer by the LEF-1 HMG protein on nucleosome-assembled DNA in vitro. Genes Dev *9*, 2090-2104.
Shirakawa, K., Chavez, L., Hakre, S., Calvanese, V., and Verdin, E. (2013). Reactivation of latent HIV by histone deacetylase inhibitors. Trends Microbiol.
Siliciano, J.D., Kajdas, J., Finzi, D., Quinn, T.C., Chadwick, K., Margolick, J.B., Kovacs, C., Gange, S.J., and Siliciano, R.F. (2003). Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. Nat Med *9*, 727-728.
Siliciano, R.F., and Greene, W.C. (2011). HIV latency. Cold Spring Harb Perspect Med *1*, a007096.
Staal, F.J., and Clevers, H. (2012). Tales of the unexpected: Tcf1 functions as a tumor suppressor for leukemias. Immunity *37*, 761-763.
Staal, F.J., and Sen, J.M. (2008). The canonical Wnt signaling pathway plays an important role in lymphopoiesis and hematopoiesis. Eur J Immunol *38*, 1788-1794.
Steel, A., Clark, S., Teo, I., Shaunak, S., Nelson, M., Gazzard, B., and Kelleher, P. (2006). No change to HIV-1 latency with valproate therapy. Aids *20*, 1681-1682.
Stone, D., Kiem, H.P., and Jerome, K.R. (2013). Targeted gene disruption to cure HIV. Curr Opin HIV AIDS *8*, 217-223.
Sune, C., and Garcia-Blanco, M.A. (1995). Sp1 transcription factor is required for in vitro basal and Tat-activated transcription from the human immunodeficiency virus type 1 long terminal repeat. J Virol *69*, 6572-6576.
Sztein, M.B., Simon, G.L., Parenti, D.M., Scheib, R., Goldstein, A.L., Goodman, R., DiGioia, R., Paxton, H., Skotnicki, A.B., and Schulof, R.S. (1987). In vitro effects of thymosin and lithium on lymphoproliferative responses of normal donors and HIV seropositive male homosexuals with AIDS-related complex. Clin Immunol Immunopathol *44*, 51-62.
Tyagi, M., Pearson, R.J., and Karn, J. (2010). Establishment of HIV latency in primary CD4+ cells is due to epigenetic transcriptional silencing and P-TEFb restriction. J Virol *84*, 6425-6437.
van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P.*, et al.* (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241-250.
Van Lint, C., Bouchat, S., and Marcello, A. (2013). HIV-1 transcription and latency: an update. Retrovirology *10*, 67.
Van Lint, C., Emiliani, S., Ott, M., and Verdin, E. (1996). Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. EMBO J *15*, 1112-1120.
Verdin, E. (1991). DNase I-hypersensitive sites are associated with both long terminal repeats and with the intragenic enhancer of integrated human immunodeficiency virus type 1. J Virol *65*, 6790-6799.

Verdin, E., Paras, P., Jr., and Van Lint, C. (1993). Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. EMBO J *12*, 3249-3259.
Verdin, E., and Van Lint, C. (1995). Internal transcriptional regulatory elements in HIV-1 and other retroviruses. Cell Mol Biol (Noisy-le-grand) *41*, 365-369.
Waterman, M.L., and Jones, K.A. (1990). Purification of TCF-1 alpha, a T-cell-specific transcription factor that activates the T-cell receptor C alpha gene enhancer in a context-dependent manner. New Biol *2*, 621-636.
Weiser K, B.M., Gershoony D, Dasgupta R, Cardozo T. (2013). HIV's Nef Interacts with β-Catenin of the Wnt Signaling Pathway in HEK293 Cells. 2013 *8*.
Wightman, F., Ellenberg, P., Churchill, M., and Lewin, S.R. (2012). HDAC inhibitors in HIV. Immunol Cell Biol *90*, 47-54.
Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., 3rd, and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448-452.
Williams, S.A., Chen, L.F., Kwon, H., Ruiz-Jarabo, C.M., Verdin, E., and Greene, W.C. (2006). NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. EMBO J *25*, 139-149.
Wortman, B., Darbinian, N., Sawaya, B.E., Khalili, K., and Amini, S. (2002). Evidence for regulation of long terminal repeat transcription by Wnt transcription factor TCF-4 in human astrocytic cells. J Virol *76*, 11159-11165.
Wu, B., Crampton, S.P., and Hughes, C.C. (2007a). Wnt signaling induces matrix metalloproteinase expression and regulates T cell transmigration. Immunity *26*, 227-239.
Wu, J.Q., Dwyer, D.E., Dyer, W.B., Yang, Y.H., Wang, B., and Saksena, N.K. (2011). Genome-wide analysis of primary CD4+ and CD8+ T cell transcriptomes shows evidence for a network of enriched pathways associated with HIV disease. Retrovirology *8*, 18.
Wu, M., Massaeli, H., Durston, M., and Mesaeli, N. (2007b). Differential expression and activity of matrix metalloproteinase-2 and -9 in the calreticulin deficient cells. Matrix Biol *26*, 463-472.
Xing, S., and Siliciano, R.F. (2012). Targeting HIV latency: pharmacologic strategies toward eradication. Drug Discov Today.
Xue, H.H., and Zhao, D.M. (2012). Regulation of mature T cell responses by the Wnt signaling pathway. Ann N Y Acad Sci *1247*, 16-33.
Yang, C.K., Kim, J.H., Li, H., and Stallcup, M.R. (2006). Differential use of functional domains by coiled-coil coactivator in its synergistic coactivator function with beta-catenin or GRIP1. J Biol Chem *281*, 3389-3397.
Ylisastigui, L., Archin, N.M., Lehrman, G., Bosch, R.J., and Margolis, D.M. (2004). Coaxing HIV-1 from resting CD4 T cells: histone deacetylase inhibition allows latent viral expression. AIDS *18*, 1101-1108.
Yu, S., Zhou, X., Steinke, F.C., Liu, C., Chen, S.C., Zagorodna, O., Jing, X., Yokota, Y., Meyerholz, D.K., Mullighan, C.G., *et al.* (2012). The TCF-1 and LEF-1 transcription factors have cooperative and opposing roles in T cell development and malignancy. Immunity *37*, 813-826.

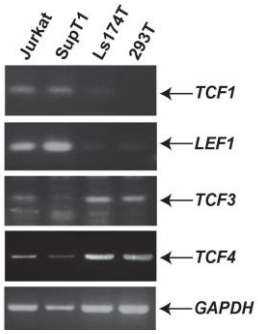

Figure S1. Shows the mRNA levels of the four TCF transcription factors family members in Jurkat, SupT1, Ls174T and 293. This supplemental information refers to figure 1, which shows the levels of protein expression of the TCF family members analysed by WB in the same cell lines.

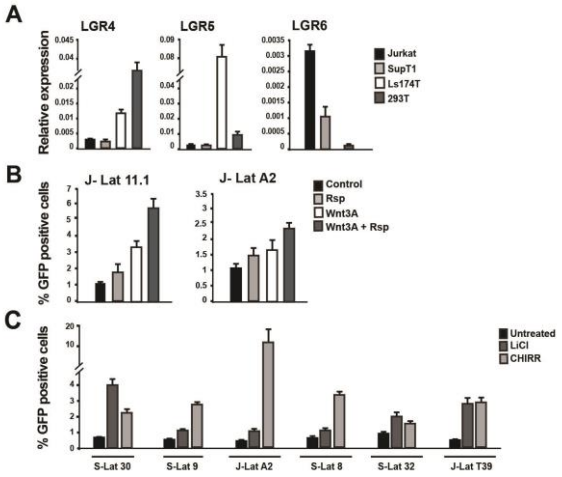

Figure S2. A) shows the expression pattern of LGR proteins in $CD4^+$ T cell lines. Refers to figure 2A, where we show increased Wnt activation after treatment with Wnt3A/Rspondin compared to Wnt3A alone. Expression of LGR is necessary to respond to R-spondin treatment. Panels B) and C) show the increase in GFP expression induced by Wnt activation following treatment with Wnt natural ligands (refers to Figure 2A) or GSK3 inhibitors (refers to Figure 2C).

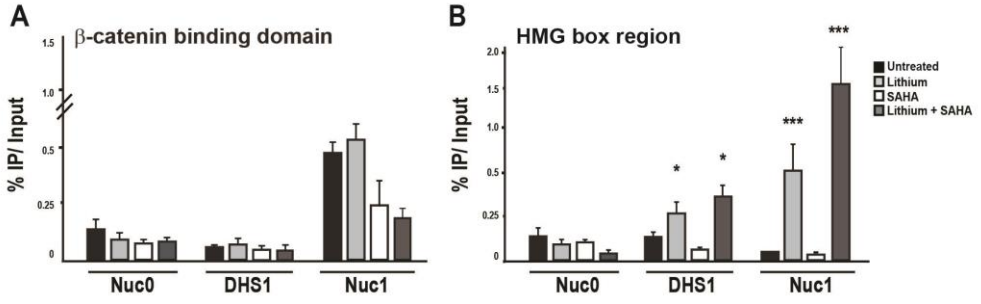

Figure S3. A LEF1 antibody raised against the β-catenin binding domain of LEF1 fails to immunoprecipitate LEF1 at the HIV-1 LTR, while a second LEF1 antibody raised against LEF1- HMG box region reveals a significant enrichment of LEF1 on the HIV-1 LTR following Wnt activation. These data draw caution to the interpretation of ChIP data and the importance of antibody epitopes and recognition domains used in ChIP experiments.

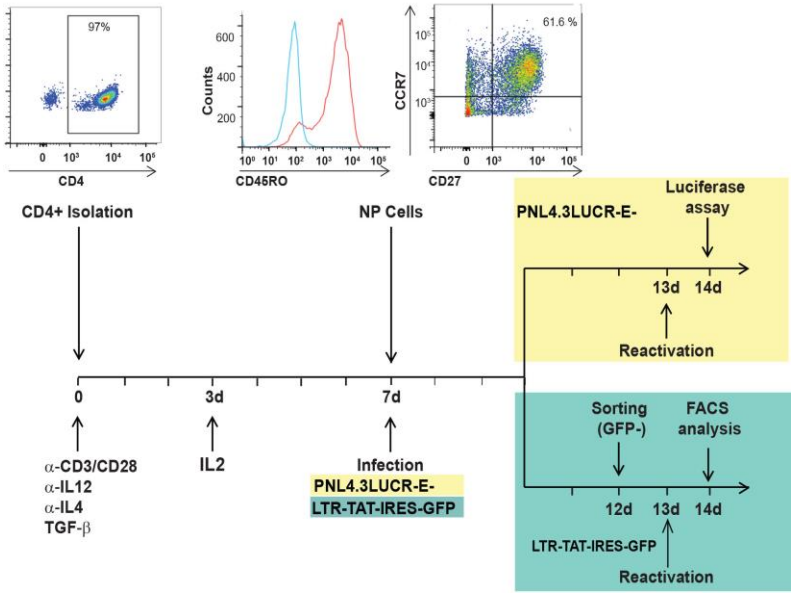

Figure S4. Description of the *ex-vivo* latency model used to evaluate the effects of Lithium in primary $CD4^+$ T cells, refers to figure 6.

Chapter 4

Functional specialization and differential gene regulation of TCF1 and LEF1 in T-ALL SupT1 leukemic cells

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Submitted

Abstract

TCF1 and LEF1, are two members of TCF/LEF protein family which are co-expressed in T cells. TCF1 and LEF1, together with β-catenin are the downstream molecular effector of the Wnt pathway. Deregulations of Wnt signaling has been implicated in formation of T cell leukemia. Studies have shown cooperative but also distinct functions for TCF1 and LEF1 in T cell development and leukemia, however, the mechanism behind this functional specialization is unknown. Here, we used immunoprecipitation coupled to Mass Spectrometry to identify the TCF1 specific and LEF1 specific protein complexes in SupT1 leukemic T in the presence and absence of Wnt stimulation. We also identified differential TCF1-regulated and LEF-regulated target genes in SupT1 cells in the Wnt-induced and uninduced conditions by RNA sequencing analysis. While in the absence of Wnt both LEF1 and TCF1 acted as repressors in the regulation of many target genes, in the presence of Wnt signaling depletion of LEF1 abrogated the Wnt-induced expression of the majority of Wnt targets examined, consistent with a role for LEF1 as an activator. Interestingly, depletion of TCF1 resulted in abrogation of a distinct subset of the Wnt targets, while the expression of other targets remained unchanged. Depletion of several of the novel Mass Spec identified TCF1/LEF1 interactors resulted in a change in the expression of endogenous Wnt target genes in the presence and absence of Wnt signaling, confirming a role for these interactors in Wnt induced transcription regulation. We also examined the recruitment of TCF1, LEF1 and β-catenin to the regulatory region of two novel Wnt targets genes identified by RNA sequencing analysis in response to Wnt signaling. We find that while β-catenin is recruited to *BMP4* and *ROMO1* in a Wnt-dependent manner, TCF1 and LEF1 are enriched differentially at the regulatory regions of these genes. Our results provide mechanistic basis to the *in vivo* observed functional specialization for TCF1 and LEF1 in T cell leukemia.

Key words: T cell factor 1, Lymphoid enhancing factor 1, Wnt signaling, β-catenin, Leukemia

Introduction

TCF1 (T cell factor 1 or TCF7), and LEF1 (Lymphocyte Enhancer binding Factor or TCF7L3) are members of the TCF/LEF family of transcription factors, which together with β-catenin are the downstream molecular effectors of the Wnt signaling pathway (Albano et al., 2013; Bienz, 2005; Cadigan, 2012; Steinke and Xue, 2014; Tiemessen et al., 2012). The Wnt pathway is a highly conserved signaling pathway critical in development, cell proliferation and differentiation, and its deregulation in adult tissues is highly associated with cancer initiation and progression (Clevers and Nusse, 2012; de Boer et al., 2004; Edmaier et al., 2014; Fodde and Brabletz, 2007; Gonzalez and Medici, 2014; Yang et al., 2014). Wnt proteins are secreted ligands that bind to the Frizzled and low-density lipoprotein receptor related proteins LRP-5/6 (Bienz and He, 2012; Willert et al., 2003; Yang et al., 2014). In the absence of Wnt, the cytoplasmic levels of β-catenin are kept low by the β-catenin destruction complex, which contains the core components adenomatous polyposis coli (APC), AXIN1, and kinases glycogen synthase and casein kinase (GSK3βand CK1). The activity of the destruction complex leads to sequential phosphorylation of β-catenin followed by its ubiquitination and proteosomal degradation (Li et al., 2012; Yang et al., 2014). After binding of Wnt ligands to their receptors on the cell surface, a series of events is initiated that leads to inactivation of the β-catenin destruction complex, which no longer ubiquitinates β-catenin (Li et al., 2012), leading to its accumulation in the cytolplasm. As a result, β-catenin translocates to the nucleus where it complexes with members of the TCF/LEF family of transcription factors bound to TCF/LEF consensus DNA elements located in the regulatory regions of Wnt target genes to activate their expression (Steinke and Xue, 2014; Xue and Zhao, 2012; Yang et al., 2014).

All TCF/LEF factors in vertebrates, LEF1, TCF1, TCF3 and TCF4 (TCF7L2) belong to the family of high mobility group (HMG) proteins, and display a high degree of amino acid sequence conservation within their HMG box DNA binding domains (Cadigan, 2012; Kuhnl et al., 2011; Steinke and Xue, 2014; Zhou et al., 2012). Consistent with this, DNA site-selection experiments (Verzi et al., 2010) have defined the core consensus DNA recognition sequence for all TCF/LEF factors to be nearly identical (van de Wetering et al., 1997). TCF/LEF member proteins are expressed in a tissue and cell-type specific manner and within the same cell type, different TCF/LEF isoforms are produced and expressed via alternative splicing and promoter usage (Hoppler and Kavanagh, 2007; Mao and Byers, 2011). Long TCF/LEF isoforms containing the N-terminal β-catenin binding domain are responsive to the Wnt signal (Cadigan and Waterman, 2012; Waterman, 2004), while shorter isoforms lacking this domain will function as dominant negatives and repressors of Wnt target genes (Cadigan, 2012; Hovanes et al., 2000). TCF factors and not LEF also exhibit alternatively spliced C-termi (Hecht and Stemmler, 2003). This region, the E tail is thought to encode a non-sequence specific DNA-binding domain and may confer distinct binding specificity to the isoform (Atcha et al., 2003; Hecht

and Stemmler, 2003; Hoverter et al., 2012; Weise et al., 2010). Functionally, even though all TCF/LEF protein family members show a highly conserved DNA binding domains, genetic evidence indicates that they display distinct functions *in vivo* (Hoppler and Kavanagh, 2007). TCF3 behaves mainly as a repressor (Atlasi et al., 2013; Kuwahara et al., 2014), LEF1 as an activator(Pazin et al., 1996; Sheridan et al., 1995), while TCF1 and TCF4 display dual transcriptional effects depending on the assays and biological systems used (Gradl et al., 2002; Levy et al., 2002; Liu et al., 2005; Mao and Byers, 2011; Nateri et al., 2005; Shulewitz et al., 2006; Struewing et al., 2010; Yi et al., 2011).

Wnt signaling, transcriptionally relayed through the TCF/LEF effectors, plays a critical role in distinct stages of T cell development, proliferation and differentiation (Bigas et al., 2013; Roozen et al., 2012; Staal and Langerak, 2008; Yu et al., 2010). Studies on $Tcf1^{-/-}$ mice compare to the wild type mice have shown a partial block at the transition from $CD8^+$ immature single positive stage to the $CD4^+$ $CD8^+$ double negative stage, pointing to an important role of TCF1 in proliferation and expansion of thymocytes. On the other hand, $Lef1^{-/-}$ mice are normal at T cells development stages, presumably because of redundancy between lef1 and Tcf1 such that mice that are $Tcf1^{-/-}$ and $Lef1^{-/-}$ have a complete block in T cells differentiation at the immature single positive stage (Okamura et al., 1998; Roozen et al., 2012). Deregulations of Wnt signaling, which controls normal T-cell and B cell development, is involved in formation of different leukemias including chronic myeloid leukemia, chronic lymphoblastic leukemia, acute myeloid leukemia and acute lymphoblastic leukemia (Dandekar et al., 2014). In T-ALL cell lines, epigenetic silencing of Wnt inhibitors, abberant nuclear localization of β-catenin and activation of Wnt target genes has been reported (Roman-Gomez et al., 2007). Recently, several studies have shown the cooperative and opposing functions of downstream molecular effectors of Wnt signaling, TCF1 and LEF1, which are co-expressed in T cells (Shuyang Y 2012). TCF1 has been shown to function as a tumor suppressor gene, while LEF1 functions as a proto-oncogene. $Tcf1^{-/-}$ mice develop T-ALL with high frequency, because of high expression levels of Lef1 and active Wnt signaling pathway (Staal and Clevers, 2012; Staal et al., 2007; Tiemessen et al., 2012). Studies on T-ALL $CD4^+$ T cell lines, Jurkat and SupT1 indicate illegitimate partial activation of the Wnt pathway (Derksen et al., 2004; Groen et al., 2008; Roman-Gomez et al., 2007). However, although partially activated, Wnt pathway components are intact in SupT1 and Jurkat T cells and can be induced by Wnt stimulation (Groen et al., 2008; Shi et al., 2006).

Here, using the $CD4^+$ ALL T cell line SupT1, we sought to investigate the mechanistic determinants of the functional specialization between the two TCF/LEF members, TCF1 and LEF1, co-expressed in T cells. We find that, TCF1 and LEF1 are present in distinct isoforms in Jurkat, SupT1 cells as well as $CD4^+$ primary T cells, including both the full length Wnt responsive isoform as well as the dominant negative forms lacking the β-catenin interaction domain. We used immunoprecipitation of TCF1 and LEF1 coupled with mass spectrometry to

identify novel interacting partners for LEF1 and TCF1 in the presence or absence of Wnt signaling in SupT1 leukemic cells. We find the presence of shared but also unique novel cofactors in complex with TCF1 and LEF1. To determine which target genes are regulated by TCF1 and LEF1, we depleted TCF1 or LEF1 using siRNA in SupT1 cells and examined differentially expressed genes in the presence or absence of Wnt signaling by RNA sequencing. Depletion of candidate novel cofactors SMC1, SMC2, MYBBP1a, and BRG1 identified by Mass Spec as TCF1/LEF1 interactors resulted in a change in expression of several Wnt target genes, consistent with their role as co-regulators of Wnt target genes. Focusing on *BMP4* and *ROMO1*, two differentially regulated Wnt target genes identified by RNA sequencing, we performed chromatin immunoprecipitation analysis to examine recruitment of Wnt molecular effectors to their regulatory regions. We found that LEF1 and TCF1 are differentially recruited to the regulatory regions of the LEF1-specific target *BMP4* and the TCF1-specific target gene *ROMO1*, while β-catenin is recruited to the regulatory regions of both genes in response to Wnt stimulation. Our data provide mechanistic evidence for the functional specialization observed in vivo for TCF1 and LEF1.

Results

Wnt-dependent isoforms of TCF1 and LEF1 are selectively expressed in T-ALL and CD4$^+$ T cells

TCF1 and LEF1 are downstream molecular effectors of Wnt signaling co-expressed as different isoforms in leukemic T cells, and thought to display distinct functions in relaying the transcriptional effects of Wnt (Mao and Byers, 2011)(De Mao C 2011). To examine the mechanism of functional specialization between TCF1 and LEF1 in T cells, we first compared the expression profiles of TCF1 and LEF1 in SupT1 and Jurkat leukemic cells and also in primary CD4$^+$ T cells by Western blotting. We used antibodies specific for the β–catenin interaction domains of TCF1 and LEF1 (Figure 1A and B, left panels), as well as antibodies recognizing the region N-terminal to the CRD domains of TCF1 and LEF1, which we here refer to as "N-term-CRD" (Figure 1A and B, right panels). Numbers and arrows indicate the isoforms recognized by each antibody, and the TCF1 or LEF1 epitopes used to generate the distinct antibodies are schematically shown (Figure 1A and B, bottom panels).

The expression profile of LEF1 using the "N-term-CRD" antibody (which recognizes a domain present in both the full length Wnt responsive and the dominant negative isoforms) compared to that obtained using an antibody specific for the LEF1 β–catenin binding domain demonstrates that LEF1 is expressed predominantly in its Wnt responsive form. On the other hand, the expression profile of TCF1, using the "N-term-CRD" antibody (present in both full

length and the dominant negative isoforms) indicates that TCF1 is almost entirely present in the dominant negative form (isoform 3 and 4) in both leukemic cells and in primary CD4$^+$ T cell. Nevertheless, using a TCF1 antibody specific for the β–catenin interaction domain, we were also able to detect the full length (β–catenin interaction domain-containing) isoform of TCF1 indicating that, this Wnt responsive form of TCF1 is also present in leukemic cells albeit in much lower levels.

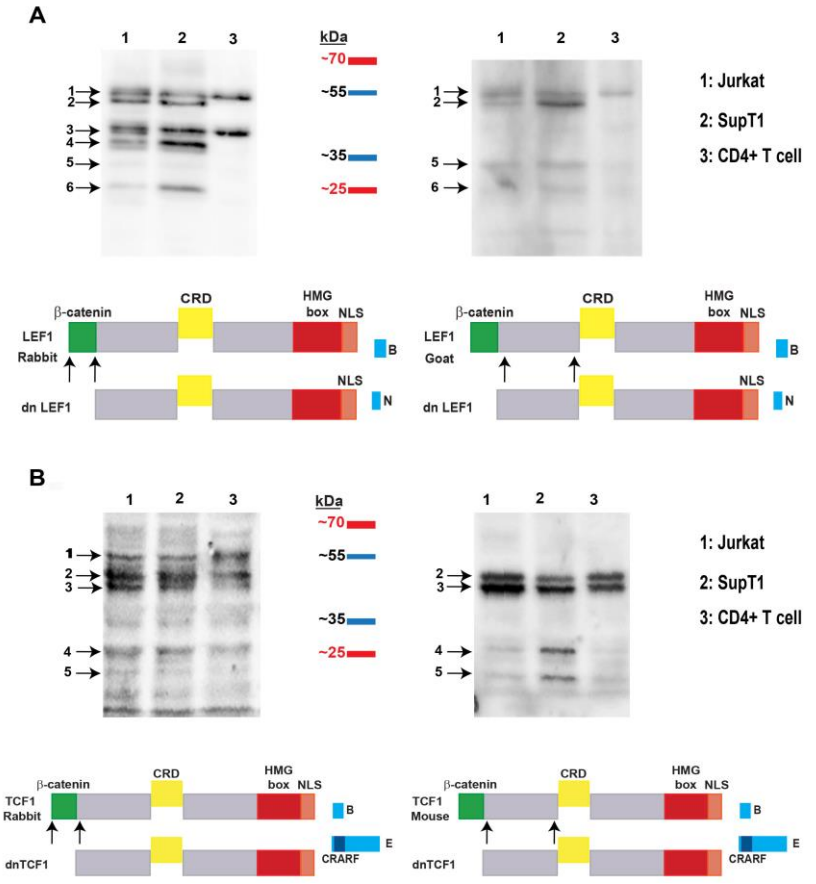

Figure 1. Wnt-dependent isoforms of TCF1 and LEF1 are selectively expressed in T-ALL and CD4$^+$ T cells. A) Western blot analysis of LEF1 protein isoforms expression in Jurkat, SupT1 CD4$^+$ T cell lines, and CD4$^+$ primary T cells respectively. Numbers and arrows comparatively show the isoforms detected with each antibody, which recognizes either the N-terminal β–catenin interaction domain or a region "N-term-CRD". B) Western blot analysis of TCF1 protein isoforms expression in Jurkat, SupT1 CD4$^+$ T cell lines, and CD4$^+$ primary T cells respectively. Numbers and arrows indicate the isoforms recognized by each antibody comparatively. The TCF1 or LEF1 epitopes used to generate the distinct antibodies are schematically shown below.

Identification of the Wnt-dependent complexes of TCF1 and LEF1 in SupT1 T-leukemic cells

One mechanism by which TCF1 and LEF1 display distinct functions in vivo maybe via interaction with functionally distinct co-factors. As an unbiased approach towards the identification of shared and specific co-factors of immunoprecipitation coupled with mass spectrometry analysis. We first stimulated SupT1 cells either with the natural Wnt ligands Wnt3A-Rspondin Conditioned Media (Wnt-Rsp CM) (de Lau et al., 2014; Willert et al., 2003) or Control Conditioned Media (CCM) overnight, and cell lysates were subjected to immunoprecipitation to identify and compare the Wnt-dependent TCF1 and LEF1 complexes. We used an antibody recognizing the N-terminal β–catenin interaction domain of TCF1 and LEF1 (figure 2C and D, top panels). In order to ensure detection of potential interactors, which may have been displaced due to interference from antibody binding to the β–catenin binding domain (figure 2A and B, top panels), we also performed independent immunoprecipitations for TCF1 and LEF1 using the "N-term-CRD" antibodies specific for TCF1 and LEF1 (Fig 2 and S1). The immunoprecipitates were subjected to SDS– PAGE and colloidal blue staining (Figure 2A and B, Figure S1A and B) followed by mass spectrometric identification of interacting proteins.

As expected, TCF1 and LEF1 were readily detected in their respective immunoprecipitates, in lysates both stimulated and unstimulated with Wnt-Rsp (figure 2C and D, figure S1 C and D). Importantly, β–catenin was detected strongly in both TCF1 and LEF1 immunoprecipitation's using antibodies recognizing the β–catenin interacting domain of TCF1 and LEF1 only in the Wnt treated lysates, and not in the absence of Wnt stimulation. This observation confirms that, Wnt signaling and β–catenin nuclear translocation occurs in SupT1 cells only upon exogenous Wnt stimulation. Figure 2C and D, and Figure S1 C and D show a list of proteins identified by mass spectrometry as interactors of TCF1/LEF1 in SupT1 cells in the presence or absence of Wnt signaling as indicated. As further confirmation of the quality of our immunoprecipitation couple to mass spectrometry approach we identified several co-factors previously described to interact with TCF/LEF/β–catenin in different systems (Figure 2 and S1) including CDK2 (Liu et al., 2014), DDX5, SRSF2/3, CARM1, SIN3A (Mao and Byers, 2011), α-catenin (Choi et al., 2013), and WNK2 (Moniz et al., 2007). Among proteins specifically co-precipitating with LEF1 and TCF1 upon Wnt stimulation, several are transcriptional co-regulators, such as MYBBP1a, LDB1, SWI/SNF complex components, CHD4 and CHD5, the FACT complex, BZW1, and RBM4. Interestingly, several enzymatic co-factors were also indentified in the LEF1 and TCF1 complexes including CDK2, CSNK2A, WNK2, Trim 21, Trim 25 and Trim 28 (Figure 2C and S1C). The Cohesin complex components SMC1A and SMC3 were present with high Mascot scores in the LEF1 immunoprecipitation. The condensin complex components SMC2 and SMC4 were found in the TCF1 immunoprecipitation. Among these novel co-factors, many are shared interactors present in both LEF1 and TCF1

complexes. However, a number of novel components were present specifically in the LEF1 or TCF1 immunoprecipitation. Given the distinct *in vivo* functions of LEF1 and TCF1 in leukemic cells, the presence of unique novel components in LEF1 versus TCF1 complexes may help to elucidate the mechanisms mediating their differential functions. These novel co-factors may play a role in distinct binding of TCF1 and LEF1 to Wnt target genes and the functional outcome in differential regulation of target gene expression.

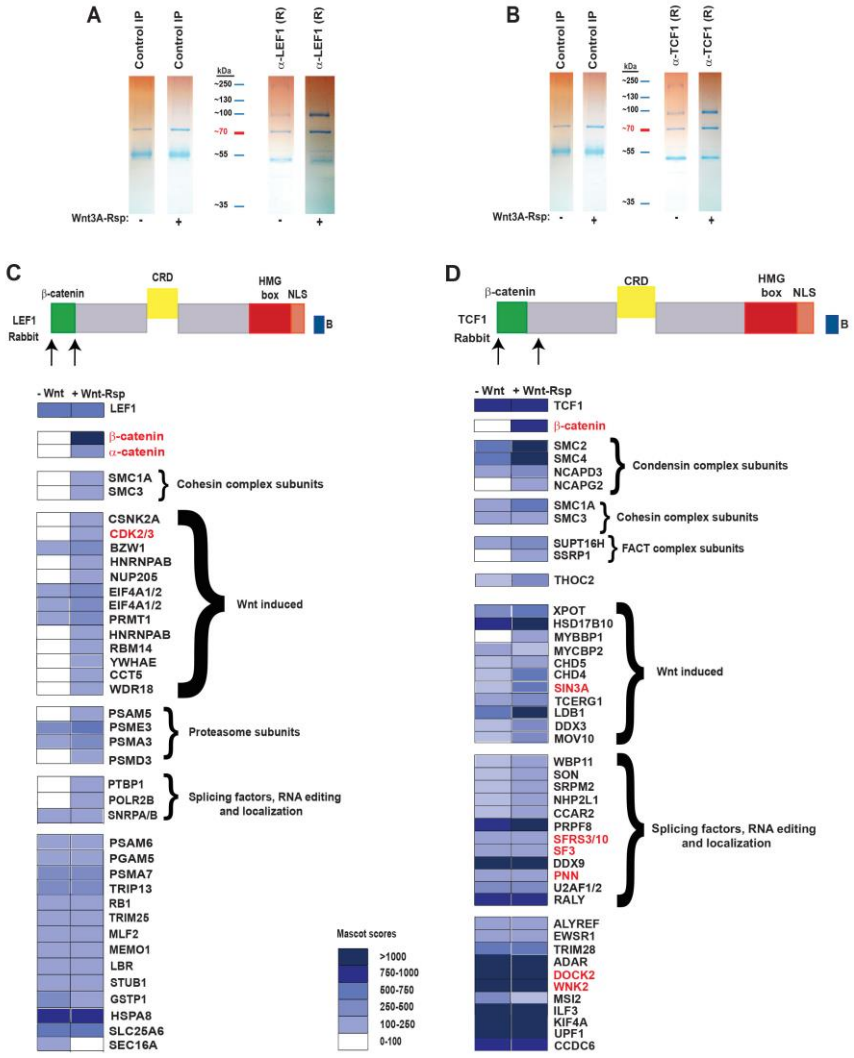

Figure 2. Identification of the Wnt-dependent complexes of TCF1 and LEF1 in SupT1 T-leukemic cells. A) Colloidal blue stained gels of LEF1 containing complexes immunoprecipitated from SupT1 cells using antibodies directed against N-terminal β–catenin interaction domain after either Wnt-Rsp CM stimulation or CCM. B) Colloidal blue stained gels of TCF1 containing complexes immunoprecipitated from SupT1 cells using antibodies directed against the N-terminal β–catenin interaction domain after either Wnt-Rsp CM stimulation or CCM. C) Mass spectrometry analysis of LEF1 co-

factors immunopurified from SupT1 cells either stimulated with Wnt-Rsp CM or CCM overnight. A LEF1 Ab recognizing the N-terminal β-catenin interaction domain of LEF1 (see schematic presentation below) was used to immunoprecipitate the LEF1 complex from pre-cleared SupT1 lysates. A heatmap depicting mascot scores for immunopurified peptides. D) Mass spectrometry analysis of TCF1 co-factors immunopurified from SupT1 cells either stimulated with Wnt-Rsp CM or CCM overnight. A TCF1 Ab recognizing the N-terminal β-catenin interaction domain of TCF1 (see schematic presentation below) was used to immunoprecipitate the TCF1 complex from pre-cleared SupT1 lysates. A heatmap depicting mascot scores for identified peptides.

Dynamics of gene expression after siRNA depletion of TCF1 or LEF1 in SupT1 leukemic cell

We next sought to determine and compare the Wnt target gene profiles differentially regulated by TCF1 and LEF1 in response to Wnt signaling. To determine differential effects of TCF1 and LEF1 on Wnt target genes expression, we used siRNAs specific for TCF1 or LEF1 in order to deplete the proteins (Figure 3A). To identify the Wnt responsive target genes, we compared the gene expression profiles of control, TCF1 depleted or LEF1 depleted SupT1 cells in the presence or absence of Wnt stimulation. To ensure the likelihood of observing direct targets of LEF1 and TCF1, we stimulated the TCF1 or LEF1 depleted cells with Wnt3A-Rspondin for 4 hours and determined the gene expression profile by RNA sequencing analysis (Figure 3).

We first defined the Wnt target gene profile in SupT1 cells by comparing the gene expression profile of cells untreated or treated with Wnt3A-Rsp CM. This comparison led to the identification of 82 differentially expressed genes selected against 1.5-folds change increase after Wnt induction compared to the unstimulated cells (Figure 3B). In the absence of Wnt stimulation, of the genes differentially expressed in the TCF1 or LEF1 depleted condition, the majority were upregulated in both the TCF1 and LEF1 depleted conditions. This argues against redundancy between TCF1 and LEF1 in regulation of these target genes and suggests that both LEF1 and TCF1 are required for their repression, such that removal of either factor results in their de-regulation. In addition, 12 genes were de-regulated specifically in the LEF1 depleted condition but unchanged in the absence of TCF1 while 17 were specifically de-regulated in the TCF1 depleted condition but remained unchanged upon LEF1 knock-down. These results together confirm the transcriptionally repressive role for TCF1 and LEF1 in the absence of Wnt signaling, and suggest that TCF1 and LEF1 regulate the expression of both shared as well as distinct target genes in the absence of Wnt. In the Wnt-induced condition, the absence of LEF1 resulted in decreased expression of the majority of target genes, while expression of 45 genes were not significantly affected. This is consistent with the role of LEF1 as an activator in relaying the Wnt signal. In the TCF1 depleted cells, 32 genes displayed decreased expression while expression of the majority were not significantly affected. Interestingly there was almost no overlap between the genes significantly downregulated in the LEF1 versus TCF1 depleted conditions, suggesting that TCF1 and LEF1 mediate the Wnt-dependent transcription of a distinct subset of Wnt target genes (Figure 3D). Thus, the Wnt-induced expression of distinct Wnt target genes are controlled by and abrogated in the absence of LEF1 and TCF1.

We used qRT-PCR to validate a representative set of genes which are LEF1-specific, or TCF1-specific, or regulated by both LEF1 and TCF1 in the absence or presence of Wnt signaling (Figure 3E). Our data altogether support the proposed opposing and distinct roles played by TCF1 and LEF1, which function both as transcriptional repressors and activators in the absence or presence of Wnt respectively.

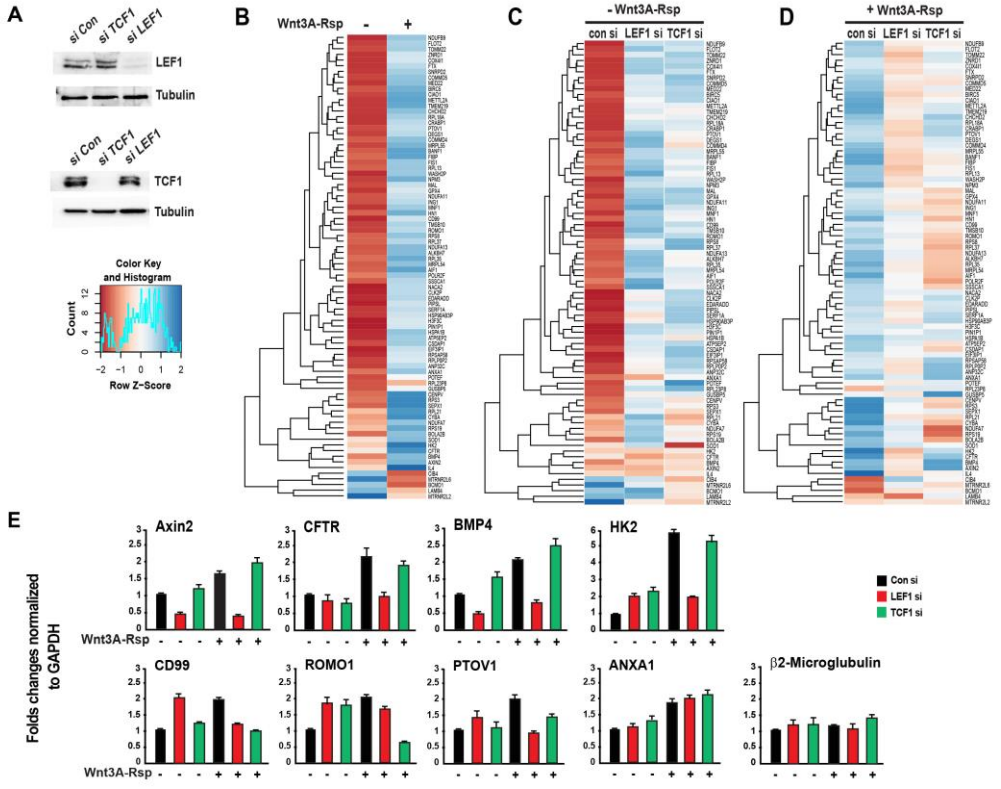

Figure 3. Dynamics of gene expression determined by RNA sequencing analysis after depletion of TCF1 or LEF1 in SupT1 leukemic cells. A) Western blot analysis to demonstrate depletion of TCF1 or LEF1 as indicated 1.5 day after nucleofection. B) Heatmap representation of the 82 selected target genes against 1.5-folds change increase after Wnt induction compared to the unstimulated cells in the absence or presence of Wnt stimulation. The total RNA is collected and extracted after 4 hours Wnt3A-Rsp stimulation and used for RNA sequencing analysis. C) Heat map representation of 82 target genes after TCF1 and LEF1 siRNA depletion in the absence of Wnt stimulation, D) and in the presence of Wnt stimulation. E) Confirmation of RNA seq data by qRT-PCR after 4 hours Wnt3A-Rsp stimulation. Error bars represent the standard deviations.

siRNA depletion of novel TCF1 and/or LEF1 cofactors identified by Mass Spec results in change in expression of Wnt target genes

Our data thus far, identified both novel co-factors in the Wnt responsive TCF1 and LEF1 complexes, as well as distinct TCF1 and LEF1 Wnt target genes by RNA sequencing. We next sought to examine the effect of depletion of some of our novel TCF1/LEF1 interactors on the expression of endogenous Wnt target genes in SupT1 cells.

One of the interacting novel candidates identified in Mass Spectrometry as a Wnt-induced interactor of both LEF1 and TCF1 is MYBBP1a. This factor has been described as a co-factor for different transcription factors in both activation, but also inhibition of transcription (Mori S 2012). To determine the function of MYBBP1a on Wnt target gene regulation in the context of leukemic SupT1 cells, we depleted MYBBP1a by siRNA (Figure 4A) and subjected the cells to Wnt stimulation. We then examined the expression of the Wnt targets *HK2, BMP4, AXIN2, ROMO1,* and *ZnRD1.* Interestingly, depletion of MYBBP1 abrogated Wnt activation of all target genes examined, consistent with a role for MYBBP1 as an essential co-activator for both TCF1 and LEF1 in Wnt target gene regulation (Figure 4B, first panel).

Another interesting co-activating complex identified is the SWI/SWF chromatin remodeling complex. We found the catalytic core subunit of this complex, BRG1 (as well as other complex subunits including BAF60, 170, 250, etc.), to be present in LEF1 immunoprecipitation. SWI/SNF has previously been implicated in Wnt-regulation (De Mao 2011). We depleted BRG1 in SupT1 cells by siRNA nucleofection (Figure 4A) and compared expression of endogenous Wnt target genes in the presence or absence of Wnt stimulation (Figure 4B, second panel). Depletion of BRG1 resulted in decrease of the targets *HK2, BMP4,* and *ZnRD1* while expression of the targets *AXIN2,* and *ROMO1* were not affected. These results suggest that SWI/SNF may be involved in regulation of a subset of, but not all Wnt-regulated genes. In addition, this complex, which has been implicated in both transcriptional activation and repression may be differentially regulating distinct Wnt targets (Figure 4B, second panel).

We also examined the effect of depletion of SMC1 and SMC2, core subunits of cohesin and condensin, respectively, on Wnt target gene regulation. Recent studies on AML have described mutations in genes of the cohesin complex such as SMC1, SMC3, and Rad21 in leukemia (Thol F 2014) suggesting a role for them in transcription regulation. Condensin complex is require for maintenance of nuclear architecture and optimal cell functions. Depletion of SMC1 or SMC2 in the absence of Wnt stimulation (Figure 4A) caused an increase in target gene expression on some targets, consistent with a potentially repressive role for these components in transcription of some targtes. In the presence of Wnt stimulation, depletion of SMC1 or SMC2 caused differential, target gene-dependent increase or decrease

in gene expression. For example, while SMC2 (Figure 4B, fourth panel) appears to be required for Wnt-dependent *HK2* expression, on other genes examined its depletion had no effect. Depletion of SMC1 (Figure 4B, third panel) on the other hand in the presence of Wnt resulted in decreases expression of *ZnRD1*and *ROMO1*, while expression of other targets were unaffected (Figure 4B, third panel). The SMC2 depleted cells showed decrease in expression of *HK2*, and *ZnRD1*, while expression of other targets were unaffected (Figure 4B, fourth panel).

Thus, while MYBBP1a appears to be a necessary Wnt-dependent co-activator for all Wnt target genes examined, BRG1 is only required for activation of some targets, and SMC1 and SMC2 appear to play a regulatory role on expression of Wnt target genes in both the presence and absence of Wnt stimulation.

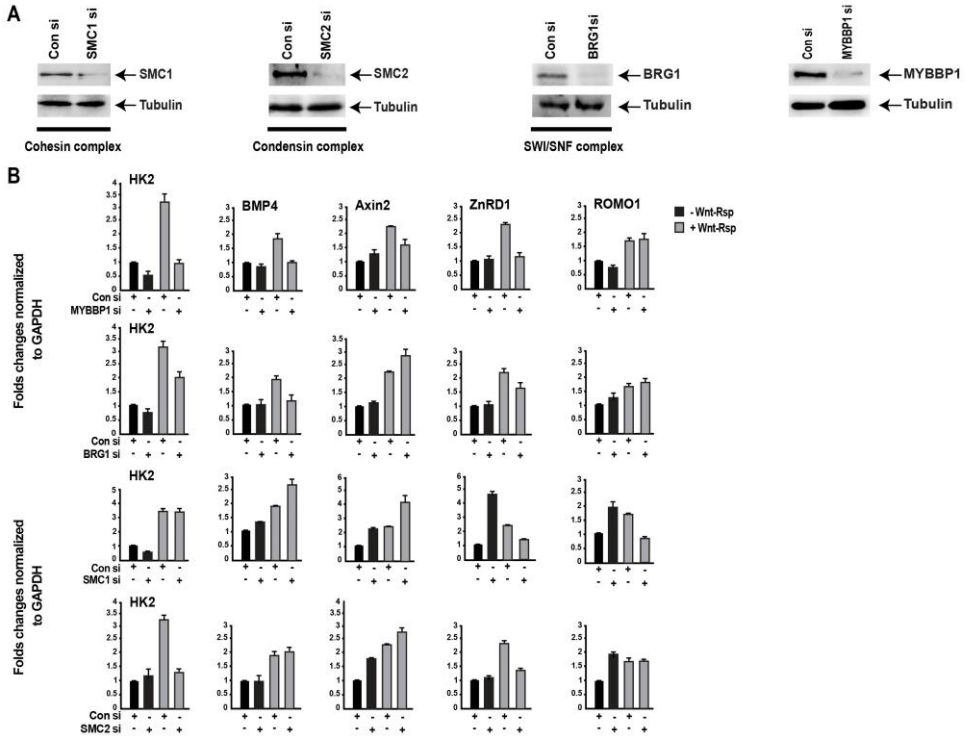

Figure 4. siRNA depletion of novel TCF1 and/or LEF1 cofactors identified by Mass Spec results in change in expression of Wnt target genes. A) Western blot analysis demonstrates depletion of specific TCF1/LEF1 complex subunits MYBBP1, BRG1, SMC1, and SMC2 as indicated in SupT1 cells 48 hours after siRNA nucleofection. B) RT-PCR analysis indicated changes in TCF/LEF target gene activity after depletion of specific protein complex subunits. Total RNA was collected and extracted after 4 hours Wnt3A-Rsp stimulation and used in qRT-PCR analysis of Wnt induced genes *HK2*, *BMP4*, *AXIN2*, *ROMO1*, and *ZnRD1*. Error bars represent the STDV of two independent experiments.

Specific recruitment of LEF1, TCF1, and β-catenin at *BMP4* and *ROMO1* regulatory regions in SupT1 leukemic cells

Our RNA sequencing data demonstrated that, specific Wnt target genes are regulated differentially by TCF1 and LEF1 in response to Wnt stimulation. Of the differentially regulated genes identified, we chose to focus on *BMP4* and *ROMO1* (Figure 5A and E), which are regulated by LEF1 and TCF1 respectively.

We performed chromatin immunoprecipitation (ChIP) followed by qPCR analysis in SupT1 cells in the presence or absence of Wnt3A-Rsp treatment. ChIP assays were conducted using antibodies directed against β-catenin as well as the Wnt-responsive N-terminal β-catenin interaction domains of TCF1 and LEF1 (Figure 5). The immunoprecipitated and extracted DNA was analyzed by qPCR with specific primer pairs spanning the promoter region, TCF/LEF binding sites, gene body and upstream or downstream control regions of *BMP4* and *ROMO1* Wnt target genes (Figure 5). The schematic representation of the human *BMP4* and *ROMO1* loci and amplicons scanned in chromatin immunoprecipitation experiments by qPCR are shown in Figure 5A and E. qPCR analysis of the immunoprecipitated material with primers specific for the *BMP4* and *ROMO1* indicated that, β-catenin was recruited to the promoter region and TCF/LEF sites of both *BMP4* and *ROMO1* specifically upon Wnt stimulation (figure5D and H). Interestingly, while TCF1 was present on the TCF sites on the *BMP4* gene in the absence of Wnt, Wnt signaling caused removal of TCF1 from *BMP4* concomitant with specific recruitment of LEF1 to *BMP4* promoter and regulatory regions. Conversely, the opposite enrichment profile was observed for TCF1 and LEF1 on the regulatory regions of *ROMO1*. While LEF1 was present on *ROMO1* amplicons 2 and 3, Wnt activation caused its removal concomitant with recruitment of TCF1 to those regions. (Figure 5B-C and 5F-G). TCF1, LEF1 and β-catenin enrichment was specific to the TCF/LEF binding regions of these two target genes and not the non-bound upstream and downstream control regions. Together, these results demonstrated that LEF1 and TCF1 are differentially enriched and recruited to the regulatory regions of the Wnt target genes *BMP4* and *ROMO1* in the absence or presence of Wnt stimulation.

Discussion

TCF1 and LEF1, members of the highly conserved TCF/LEF family, which share almost identical HMG box DNA binding domains, have nevertheless been shown to play distinct and specific functions in T cells (Mao and Byers, 2011). In leukemia, TCF1 and LEF1 display both opposite and cooperative roles. While there is some functional redundancy between the two TCF/LEF

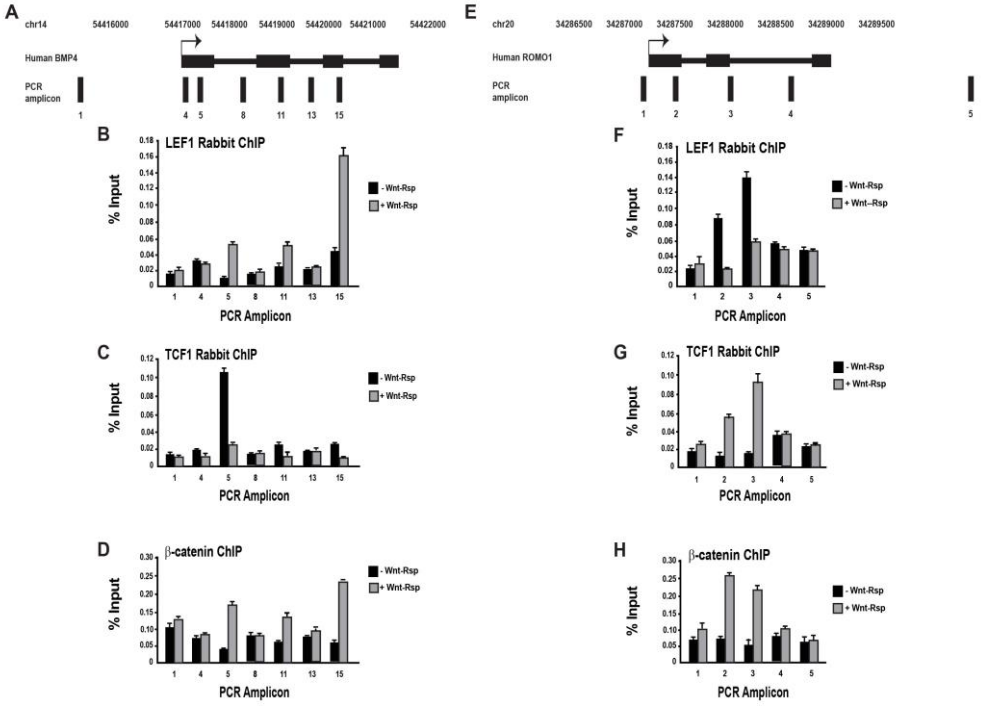

Figure 5. Specific recruitment of LEF1, TCF1 and β-catenin at *BMP4* and *ROMO1* regulatory regions in SupT1 leukemic cells. A) Schematic representation of the human *BMP4* locus and amplicons scanned in Chromatin immunoprecipitation experiments by qPCR. SupT1 cells were stimulated 12 hours with either Wnt3A-Rsp CM or CCM and were subjected to ChIP using antibodies against LEF1 recognizing the β-catenin binding domain of LEF1 (B), TCF1 recognizing the β-catenin binding domain of TCF1 (C), and β-catenin (D). Chromatin was immunoprecipitated with the specified antibodies followed by qPCR using primer pairs spanning the human *BMP4* locus as indicated in (A). E) Schematic representation of the human *ROMO1* locus and amplicons scanned in Chromatin immunoprecipitation experiments by qPCR. SupT1 cells were stimulated 12 hours with either Wnt3A-Rsp CM or CCM and were subjected to ChIP using antibodies against LEF1 recognizing the β-catenin binding domain of LEF1 (F), TCF1 recognizing the β-catenin binding domain of LEF1 (G), and β-catenin (H). Chromatin was immunoprecipitated with the specified antibodies followed by qPCR using primer pairs spanning the human *ROMO1* locus as indicated in (E). Results are presented as percentage immunoprecipitated over input and are representative of qPCR replicates.

family members, TCF1 functions as a tumor suppressor gene, while LEF1 functions as a proto oncogene (Tiemessen et al., 2012; Tiemessen and Staal, 2013). This functional specification between TCF1 an LEF1 in T cells and leukemia, enticed us to probe the mechanistic determinants that lead to the functional specifications of TCF1 and LEF1, which are co-expressed in T cells. One mechanism that could mediate the functionally distinct roles played by LEF1 and TCF1 is that they may bind to their target genes in the context of compositionally and functionally different complexes. To investigate the possibility that LEF1 and TCF1, despite their high homology, bind to distinct transcriptional cofactors, we performed

immunoprecipitation coupled to mass spectrometry analysis of LEF1 and TCF1 in the presence or absence of Wnt signaling. Using this approach we identifed both previously known but also novel putatively unique interaction partners for TCF1 and LEF1 in SupT1 cells. To identify LEF-1 specific and TCF1-specific target genes, we performed RNA sequencing analysis after siRNA depletion of TCF1 or LEF1 in the presence or absence of Wnt signaling. Focusing on the Wnt target genes *BMP4* and *ROMO1*, we demonstrated, using chromatin immunoprecipitation assays, differential recruitment of TCF1 and LEF1 to the regulatory regions of these Wnt targets in response to Wnt stimulation. Thus, our data provide mechanistic evidence for the functional specialization observed in T cell leukemia for TCF1 and LEF1.

We find TCF1 and LEF1 to be present in distinct isoforms in leukemic T cells as well as $CD4^+$ primary T cells, including both the full length Wnt responsive isoform as well as the dominant negative forms lacking the β-catenin interaction domain. Several studies on isoforms expression in different TCF/LEF family members have shown that TCF1 and LEF1 are expressed in a tissue and cell-type specific manner and within the same cell type, different TCF/LEF isoforms are produced and expressed via alternative splicing and promoter usage (Mao and Byers, 2011). We found that LEF1 is expressed predominantly in its Wnt responsive form, while TCF1 is expressed almost entirely in the dominant negative form, although Wnt responsive forms of TCF1 were also present in SupT1 leukemic cells. TCF/LEF factors, in the absence of β-catenin in the nucleus are thought to bind to their consensus sites of the regulatory regions of their target genes and repress transcription. The expression profiles of LEF1, predominantly in its Wnt responsive β-catenin binding form, and TCF1, in its dominant negative Wnt-unresponsive form are consistent with the *in vivo* role for TCF1 as a tumor suppressor gene, and LEF1 as a proto-oncogene.

The many different binding partners identified for LEF1 and TCF1 point to the likely presence of different sub-complexes of LEF1 and TCF1, which may mediate distinct functions for LEF1 or TCF1. In other words, part of the LEF1 in the nucleus maybe in complex with the cohesion complex and mediator/ FACT, facilitating long range enhancer-promoter interaction functions to facilitate transcription, while a different sub-complex of LEF may at the same time, together with MYBBP1 and/or SWI/SNF be recruited to and activate a sub-set of Wnt target genes. Conversely, a sub-complex of TCF1 may consist of TCF1 together with the identified co-repressors SIN3A and/or CHD4/5 performing a repressive role in transcription of a subset of the target genes, while at the same time TCF1 may exist in an activating complex consisting of MYBBP1, or LDB1 which may be recruited to and activating another subset of the TCF target genes. Future studies examining the binding profile/enrichment of some of these co-factors on the TCF/LEF target genes will address their selectivity and target-specificity.

The identification of distinct binding partners for TCF1 and LEF1 also gives support to the notion that cooperative binding, of TCF1 or LEF1 to their consensus sites in regulatory regions of target genes, together with other partner DNA binding protein cofactors can confer additional binding specificity and affinity. Indeed it has been shown in the intestine cooperative binding by CDX2 directs TCF4 binding to specific regulatory regions allowing CDX2 TCF4 co-occupancy (Verzi et al., 2010). In hemato-cardiovascular progenitors, combination of transcription factor binding sites on specific enhancers invites cooperative binding to specific sites by TCF, CDX, GATA, and FOX (Ishitobi et al., 2011). Protein-protein interaction and cooperative DNA binding of Smads and TCF4/β-catenin to their cognate sequences has also been shown to be important in integrating the cooperative effects of the Wnt and BMP pathways at the molecular level (Rodriguez-Carballo et al., 2011). TCF/LEF factors can also interact with other proteins, including ALY (Bruhn et al., 1997), Smad (Labbe et al., 2000; Nishita et al., 2000) and c-Jun (Nateri et al., 2005), allowing the formation of large nuclear complexes that control the expression of genes containing a particular combination of regulatory sequences in their promoter/enhancer. Therefore, cooperative binding to distinct cofactors and DNA elements can result in differential targeting and gene regulation by TCF1 and LEF1.

Among the specific factors identified, which interacted with LEF1 in the presence of Wnt signaling, the SMC1A and SMC3 subunits of the Cohesin complex were intriguing. Aside from its function during replication in sister chromatid cohesion, recently there have been a number of studies, which have described a role for cohesin in facilitation of enhancer promoter interaction (Daniel et al., 2014; Dowen and Young, 2014), and in activation of transcription via its interaction with mediator (Muto et al., 2014; Sharov et al., 2014). Depletion of Cohesin in SupT1 resulted in decreased expression of some but not all targets in the presence of Wnt signaling, consistent with a potentially activating role for cohesion in a subset of Wnt target genes.

Several subunits of the SWI/SNF ATP-dependent chromatin remodeling complex, including its catalytic subunit BRG1 also interacted with LEF1 in a Wnt-dependent manner. Several of the identified Wnt-induced interactors of TCF1, are previously described repressors such as SIN3A, and the ATP dependent chromatin remodeling factors CHD4 and CHD5. Interestingly, both BRG1 and CHD4 have previously been implicated functionally in Wnt-induced gene regulation (Curtis and Griffin, 2012). Mouse genetic studies indicated that CHD4, the catalytic subunit of the repressive NURD complex and BRG1 play antagonistic roles to co-regulate vascular Wnt signaling. Our data, identifying CHD4 as a specific interaction partner of TCF1, while SWI/SNF interacted with LEF1 suggests that the antagonistic functions of CHD4 and BRG1 may be relayed transcriptionally in the context of different TCF/LEF family members and sub-complexes.

Several studies on LIM domain-binding protein (LDB1), a specific TCF1 interaction partner we identified by mass Spec have shown both inhibitory effects on LIM homeodomain transcription factors and synergistic transcriptional activation events (Bach et al., 1997; Dawid et al., 1998). LIM domains are able to bind LDB1, which acts as a negative co-regulator via the recruitment of the SIN3A/histone deacetylase co-repressor complex (Bach et al., 1999). The identification of SIN3A, CHD4 and CHD5, repressors known to function in the context of histone deacetylases, repressive histone methyltransferase and DNA methylases, within the TCF1 and not the LEF1 complex also provide a possible mechanistic basis for the predominant dominant negative expression of TCF1 and its function as a tumor suppressor in Wnt target gene regulation. Importantly, in the hematopoietic system, it has been shown that TCF1 and LEF1 can also function in a β-catenin- independent manner. It will be interesting to examine whether some of the identified novel binding partners for LEF1 or TCF1 are involved in relaying potential Wnt-independent TCF/LEF transcriptional activity.

Our ChIP data indicate a distinct enrichment pattern of LEF1 and TCF1 on two differentially regulated Wnt target genes, *BMP4* and *ROMO1* in vivo. To better characterize the recruitment of TCF1 and LEF1 to their target genes in the presence or absence of the Wnt signal, and to examine this potential differential binding profile on other targets, it is critical that TCF1/LEF1 enrichment is examined on a genome wide scale. It will also be interesting to complement ChIP sequencing studies probing enrichment of TCF1/LEF1 and β-catenin with those of other novel putative cofactors identified to determine their overlap in genome-wide binding profiles, and specificity for Wnt target gen regulation. Future studies combining genome wide gene expression analysis in cells depleted of candidate novel factors in the presence or absence of Wnt signaling will also contribute to clarify the role of not only the co-expressed TCF1 and LEF1 transcription factors but also their putative novel binding partners in T-ALL leukemic cell lines.

Materials and Methods

Cell Lines, Antibodies and Reagents

SupT1 and Jurkat T-leukemic cell lines (ATCC) were grown in RPMI-1640 containing 10% FCS, penicillin (100 units/mL) and streptomycin (100 µg/mL) in 37˚C, 5% CO2 incubator.

Antibodies LEF1 (sc-8591, Santa Cruz), LEF1 (C12A5, cell signalling technology), TCF1 (05-510, Upstate), TCF1 (sc-13025, Santa Cruz), MYBBP1 (NB100-61050 Novus biologicals), BRG1 (kind gift from prof. Verrijzer), SMC1 and SMC3 (kind gift from Dr. Wendt) were used in chromatin immunoprecipitation (ChIP), immunoprecipitation (IP), and Western blotting.

Wnt 3A and control L-cell conditioned media was collected as supernatant from mouse L cells (ATCC CRL-2647 (control) and ATCC CRL-2648 (producing Wnt3a protein) as previously described (Willert et al., 2003). Rspondin conditioned media was kind gift from Prof. Hans Clevers.

Immunoprecipitation (IP) and Western blot analysis

Jurkat and SupT1 cells were lysed in protein lysis buffer (PLB) (1% Triton X-100, 2mM EDTA, 1mM DTT, 5% glycerol in PBS). Proteins were separated on an SDS-PAGE gel and subjected to western blot analysis using antibodies for specific proteins as indicated.

For immunoprecipitation coupled to Mass Spec analysis, 1 billion SupT1 cells were split in two parts, either stimulated with Wnt3A-Rsp CM or control conditioned media (CCM) over night. Cells were collected and lysed in PLB buffer (1% Triton X-100, 2 mM EDTA, 1 mM DTT, 5% glycerol in PBS) supplemented with fresh protease inhibitors (PICs). Cellular lysates were pre-cleared with protein A and G agarose beads overnight at 4˚C. Precleared lysates were subjected to immunoprecipitation using 2-5 µg antibodies specific for TCF1 and LEF1 as indicated for two hours prior to addition of protein A and G-Agarose beads for an additional 3 hours on rotator at 4˚C. IPs were washed 5 times in PLB, and proteins were separated by SDS–PAGE electrophoresis and visualized by colloidal blue staining (Invitrogen). Polypeptides were identified by mass spectrometry on an LTQ-Orbitrap XL mass spectrometer from ThermoFischer.

Mass spectrometric analysis

1D SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide (D4, 98%, Cambridge Isotope Laboratories, Inc.) and digestion with trypsin (Promega, sequencing grade), essentially as described by Wilm et al (van den Berg et al., 2008). Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to either an LTQ-Orbitrap XL mass spectrometer (Thermo) operating in positive mode. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 170 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode by CID. Peak lists were automatically created from raw data files using the Proteome Discoverer (version 1.3; Thermo). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the Uniprot database (release 2014_03, taxonomy: *H. sapiens*). The peptide tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2

missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 65. Individual peptide MS/MS spectra with Mascot scores below 25 were checked manually and either interpreted as valid identifications or discarded.

Isolation of Primary $CD4^+$ T Cells

PBMCs were isolated from buffy coats obtained from healthy donors (Sanquin blood supply, The Netherlands) by Ficol gradient density sedimentation. Purified PBMCs were directly subjected to the CD4+ T-Cell enrichment kit (Stem cell technologies, Cat No. 19052). $CD4^+$ T cells were purified according to the protocol and cultured in RPMI-1640 containing 10% FCS and penicillin: streptomycin in the presence of 5 ng/ml of IL-2 (Sigma) for several days. Cells were collected and lysed in PLB containing fresh PICs and were subjected to Western blot analysis.

Amaxa Nucleofection

Nucleofection of SupT1 cell line was done as previously described (Mahmoudi T 2006, Rafati 2011). Briefly, cells were split to $3X10^5$ cells/ml 24 h prior to Amaxa nucleofection. Five million cells were centrifuged at 1000 rpm for 10 min at room temperature, resuspended in 100 µl of solution R (Amaxa nucleofector kit R, Cat No. VCA1001), and nucleofected with 20 nM siRNA using program O28. Nucleofected cells were resuspended in 500 µl of pre-warmed, serum-free RPMI-1640 lacking antibiotics and allowed to recover at 37˚C in a 5% CO2 incubator for 15 min. Pre-warmed complete RPMI-1640 (2 ml) was then added to the cells.

RNA purification

SupT1 cells were nucleofected with non-targeting control siRNA or siRNA specific for TCF1, LEF1, MYBBP1, BRG1, SMC1, and SMC3 as indicated. 36 to 48 hours after nucleofection cells were either stimulated with Wnt3A-Rsp CM or CCM. Four hours post stimulation cells were collected and RNA was extracted and purified using the Promega kit.

qRT-PCR analysis

c-DNAs were synthesized using the Invitrogen kit and subjected to qRT-PCR analysis using specific primers for Wnt target genes *AXIN2, BMP4, HK2, ROMO1* and *ZnRD1,* and control primers *GAPDH, and β-2 Microglubulin.* Expression data are presented as fold increase normalized to *GAPDH* control. qPCR primer sequences were designed using the program Beacon designer and listed in table 1.

RNA sequencing and data analysis

RNA sequencing was performed at genomics facility of Biomedical Sciences Research Center (B.S.R.C) Alexander Fleming Institute, Greece on the Ion Torrent PROTON sequencer. FPKMs were computed using CuffDiff. Genes were selected based on log2 fold changes. R statistical software was used to generate heat maps with row-scaled option and row clustering was computed using Euclidean distance matrix and hclust complete method.

Chromatin Immunoprecipitation (ChIP) and Real-Time qPCR Analysis

SupT1 cells were subjected to ChIP assays as previously described (Rafati 2011). Briefly, cells were stimulated either with Wnt3A-Rsp CM or CCM overnight and crosslinked by addition of formaldehyde to a final concentration of 1% for 30 min at RT. The crosslinking reaction was quenched with 125 mM glycine, cells were washed with buffer B (0.25%Triton-X 100, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6) and buffer C (150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA,20 mM Hepes, pH 7.6), and resuspended in ChIP incubation buffer (1% SDS, 1% Triton-X 100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6). Chromatin was sheared by sonication to an apparent length of 500-1000 bp using a BioRuptor sonicator (Cosmo Bio Co., Ltd) with 20 X 1 min pulses at maximum setting. Approximately 20 million cells were used per IP. 2-5 µg of the indicated antibody was used in IP reaction with the chromatin and BSA-blocked protein A+G beads overnight at 4˚C. IPs were washed twice with each buffer 1 (0.1% SDS, 0.1% deoxycholate, 1%Triton-X 100, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA,20 mMHepes pH 7.6), buffer 2 (0.1% SDS, 0.1% deoxycholate,1% Triton-X 100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA,20 mM Hepes pH 7.6), buffer 3 (250 mM LiCl, 0.5% deoxycholate,0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH 7.6), and buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6). Immunoprecipitated complexes were eluted in elution buffer (1% SDS, 0.1 M NaHCO3) for 30 min at RT and decrosslinked overnight at 65˚C in presence of 200 mM NaCl2.DNA was phenol: chloroform extracted, chroloform: isoamylalcohol extracted, ethanol precipitated, and resuspended in 100 µl H2O. Input and immunoprecipitated DNA (5 ul) were subjected to qPCR cycles with specific primers spanning distinct regions within the Wnt induced genes *BMP4* and *ROMO1*. Primer sequences are provided in table 1.

ChIP samples were analysed by quantitative PCR in CFX manager real-time PCR detection system (BioRad) using Go Taq qPCR master mix 2x (Promega). ChIP values were normalized as a percentage of input. Sequences of qPCR primer pairs used to amplify are provided in Table 1.

References

F. Albano, A. Zagaria, L. Anelli, N. Coccaro, L. Impera, C.F. Minervini, A. Minervini, A.R. Rossi, G. Tota, P. Casieri and G. Specchia, Gene expression profiling of chronic myeloid leukemia with variant t(9;22) reveals a different signature from cases with classic translocation, *Molecular cancer* 12 (2013), p. 36.

F.A. Atcha, J.E. Munguia, T.W. Li, K. Hovanes and M.L. Waterman, A new beta-catenin-dependent activation domain in T cell factor, *The Journal of biological chemistry* 278 (2003), pp. 16169-16175.

F.A. Atcha, A. Syed, B. Wu, N.P. Hoverter, N.N. Yokoyama, J.H. Ting, J.E. Munguia, H.J. Mangalam, J.L. Marsh and M.L. Waterman, A unique DNA binding domain converts T-cell factors into strong Wnt effectors, *Molecular and cellular biology* 27 (2007), pp. 8352-8363.

Y. Atlasi, R. Noori, C. Gaspar, P. Franken, A. Sacchetti, H. Rafati, T. Mahmoudi, C. Decraene, G.A. Calin, B.J. Merrill and R. Fodde, Wnt signaling regulates the lineage differentiation potential of mouse embryonic stem cells through Tcf3 down-regulation, *PLoS genetics* 9 (2013), p. e1003424.

I. Bach, C. Carriere, H.P. Ostendorff, B. Andersen and M.G. Rosenfeld, A family of LIM domain-associated cofactors confer transcriptional synergism between LIM and Otx homeodomain proteins, *Genes & development* 11 (1997), pp. 1370-1380.

I. Bach, C. Rodriguez-Esteban, C. Carriere, A. Bhushan, A. Krones, D.W. Rose, C.K. Glass, B. Andersen, J.C. Izpisua Belmonte and M.G. Rosenfeld, RLIM inhibits functional activity of LIM homeodomain transcription factors via recruitment of the histone deacetylase complex, *Nature genetics* 22 (1999), pp. 394-399.

M. Bienz, beta-Catenin: a pivot between cell adhesion and Wnt signalling, *Current biology : CB* 15 (2005), pp. R64-67.

M. Bienz and X. He, Biochemistry. A lipid linchpin for Wnt-Fz docking, *Science* 337 (2012), pp. 44-45.

A. Bigas, J. Guiu and L. Gama-Norton, Notch and Wnt signaling in the emergence of hematopoietic stem cells, *Blood cells, molecules & diseases* 51 (2013), pp. 264-270.

L. Bruhn, A. Munnerlyn and R. Grosschedl, ALY, a context-dependent coactivator of LEF-1 and AML-1, is required for TCRalpha enhancer function, *Genes & development* 11 (1997), pp. 640-653.

K.M. Cadigan, TCFs and Wnt/beta-catenin signaling: more than one way to throw the switch, *Current topics in developmental biology* 98 (2012), pp. 1-34.

K.M. Cadigan and M.L. Waterman, TCF/LEFs and Wnt signaling in the nucleus, *Cold Spring Harbor perspectives in biology* 4 (2012).

S.H. Choi, C. Estaras, J.J. Moresco, J.R. Yates, 3rd and K.A. Jones, alpha-Catenin interacts with APC to regulate beta-catenin proteolysis and transcriptional repression of Wnt target genes, *Genes & development* 27 (2013), pp. 2473-2488.

H. Clevers and R. Nusse, Wnt/beta-catenin signaling and disease, *Cell* 149 (2012), pp. 1192-1205.

C.D. Curtis and C.T. Griffin, The chromatin-remodeling enzymes BRG1 and CHD4 antagonistically regulate vascular Wnt signaling, *Molecular and cellular biology* 32 (2012), pp. 1312-1320.

S. Dandekar, E. Romanos-Sirakis, F. Pais, T. Bhatla, C. Jones, W. Bourgeois, S.P. Hunger, E.A. Raetz, M.L. Hermiston, R. Dasgupta, D.J. Morrison and W.L. Carroll, Wnt inhibition leads to improved chemosensitivity in paediatric acute lymphoblastic leukaemia, *British journal of haematology* 167 (2014), pp. 87-99.

B. Daniel, B.L. Balint, Z.S. Nagy and L. Nagy, Mapping the genomic binding sites of the activated retinoid x receptor in murine bone marrow-derived macrophages using chromatin immunoprecipitation sequencing, *Methods in molecular biology* 1204 (2014), pp. 15-24.

I.B. Dawid, J.J. Breen and R. Toyama, LIM domains: multiple roles as adapters and functional modifiers in protein interactions, *Trends in genetics : TIG* 14 (1998), pp. 156-162.

J. de Boer, R. Siddappa, C. Gaspar, A. van Apeldoorn, R. Fodde and C. van Blitterswijk, Wnt signaling inhibits osteogenic differentiation of human mesenchymal stem cells, *Bone* 34 (2004), pp. 818-826.

W. de Lau, W.C. Peng, P. Gros and H. Clevers, The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength, *Genes & development* 28 (2014), pp. 305-316.

P.W. Derksen, E. Tjin, H.P. Meijer, M.D. Klok, H.D. MacGillavry, M.H. van Oers, H.M. Lokhorst, A.C. Bloem, H. Clevers, R. Nusse, R. van der Neut, M. Spaargaren and S.T. Pals, Illegitimate WNT signaling promotes proliferation of multiple

myeloma cells, *Proceedings of the National Academy of Sciences of the United States of America* 101 (2004), pp. 6122-6127.

J.M. Dowen and R.A. Young, SMC complexes link gene expression and genome architecture, *Current opinion in genetics & development* 25 (2014), pp. 131-137.

K.E. Edmaier, K. Stahnke, N. Vegi, M. Mulaw, S. Ihme, A. Scheffold, K.L. Rudolph and C. Buske, Expression of the lymphoid enhancer factor 1 is required for normal hematopoietic stem and progenitor cell function, *Leukemia* 28 (2014), pp. 227-230.

R. Fodde and T. Brabletz, Wnt/beta-catenin signaling in cancer stemness and malignant behavior, *Current opinion in cell biology* 19 (2007), pp. 150-158.

D.M. Gonzalez and D. Medici, Signaling mechanisms of the epithelial-mesenchymal transition, *Science signaling* 7 (2014), p. re8.

D. Gradl, A. Konig and D. Wedlich, Functional diversity of Xenopus lymphoid enhancer factor/T-cell factor transcription factors relies on combinations of activating and repressing elements, *The Journal of biological chemistry* 277 (2002), pp. 14159-14171.

R.W. Groen, M.E. Oud, E.J. Schilder-Tol, M.B. Overdijk, D. ten Berge, R. Nusse, M. Spaargaren and S.T. Pals, Illegitimate WNT pathway activation by beta-catenin mutation or autocrine stimulation in T-cell malignancies, *Cancer research* 68 (2008), pp. 6969-6977.

A. Hecht and M.P. Stemmler, Identification of a promoter-specific transcriptional activation domain at the C terminus of the Wnt effector protein T-cell factor 4, *The Journal of biological chemistry* 278 (2003), pp. 3776-3785.

S. Hoppler and C.L. Kavanagh, Wnt signalling: variety at the core, *Journal of cell science* 120 (2007), pp. 385-393.

K. Hovanes, T.W. Li and M.L. Waterman, The human LEF-1 gene contains a promoter preferentially active in lymphocytes and encodes multiple isoforms derived from alternative splicing, *Nucleic acids research* 28 (2000), pp. 1994-2003.

N.P. Hoverter, J.H. Ting, S. Sundaresh, P. Baldi and M.L. Waterman, A WNT/p21 circuit directed by the C-clamp, a sequence-specific DNA binding domain in TCFs, *Molecular and cellular biology* 32 (2012), pp. 3648-3662.

H. Ishitobi, A. Wakamatsu, F. Liu, T. Azami, M. Hamada, K. Matsumoto, H. Kataoka, M. Kobayashi, K. Choi, S. Nishikawa, S. Takahashi and M. Ema, Molecular basis for Flk1 expression in hemato-cardiovascular progenitors in the mouse, *Development* 138 (2011), pp. 5357-5368.

A. Kuhnl, M. Kaiser, M. Neumann, L. Fransecky, S. Heesch, M. Radmacher, G. Marcucci, C.D. Bloomfield, W.K. Hofmann, E. Thiel and C.D. Baldus, High expression of IGFBP2 is associated with chemoresistance in adult acute myeloid leukemia, *Leukemia research* 35 (2011), pp. 1585-1590.

A. Kuwahara, H. Sakai, Y. Xu, Y. Itoh, Y. Hirabayashi and Y. Gotoh, Tcf3 represses Wnt-beta-catenin signaling and maintains neural stem cell population during neocortical development, *PloS one* 9 (2014), p. e94408.

E. Labbe, A. Letamendia and L. Attisano, Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways, *Proceedings of the National Academy of Sciences of the United States of America* 97 (2000), pp. 8358-8363.

L. Levy, C. Neuveut, C.A. Renard, P. Charneau, S. Branchereau, F. Gauthier, J.T. Van Nhieu, D. Cherqui, A.F. Petit-Bertron, D. Mathieu and M.A. Buendia, Transcriptional activation of interleukin-8 by beta-catenin-Tcf4, *The Journal of biological chemistry* 277 (2002), pp. 42386-42393.

V.S. Li, S.S. Ng, P.J. Boersema, T.Y. Low, W.R. Karthaus, J.P. Gerlach, S. Mohammed, A.J. Heck, M.M. Maurice, T. Mahmoudi and H. Clevers, Wnt signaling through inhibition of beta-catenin degradation in an intact Axin1 complex, *Cell* 149 (2012), pp. 1245-1256.

F. Liu, O. van den Broek, O. Destree and S. Hoppler, Distinct roles for Xenopus Tcf/Lef genes in mediating specific responses to Wnt/beta-catenin signalling in mesoderm development, *Development* 132 (2005), pp. 5375-5385.

W. Liu, L. Wang, W. Zhao, G. Song, R. Xu, G. Wang, F. Wang, W. Li, J. Lian, H. Tian, X. Wang and F. Sun, Phosphorylation of CDK2 at threonine 160 regulates meiotic pachytene and diplotene progression in mice, *Developmental biology* 392 (2014), pp. 108-116.

C.D. Mao and S.W. Byers, Cell-context dependent TCF/LEF expression and function: alternative tales of repression, de-repression and activation potentials, *Critical reviews in eukaryotic gene expression* 21 (2011), pp. 207-236.

S. Moniz, F. Verissimo, P. Matos, R. Brazao, E. Silva, L. Kotelevets, E. Chastre, C. Gespach and P. Jordan, Protein kinase WNK2 inhibits cell proliferation by negatively modulating the activation of MEK1/ERK1/2, *Oncogene* 26 (2007), pp. 6071-6081.

A. Muto, S. Ikeda, M.E. Lopez-Burks, Y. Kikuchi, A.L. Calof, A.D. Lander and T.F. Schilling, Nipbl and mediator cooperatively regulate gene expression to control limb development, *PLoS genetics* 10 (2014), p. e1004671.

A.S. Nateri, B. Spencer-Dene and A. Behrens, Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development, *Nature* 437 (2005), pp. 281-285.

M. Nishita, M.K. Hashimoto, S. Ogata, M.N. Laurent, N. Ueno, H. Shibuya and K.W. Cho, Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer, *Nature* 403 (2000), pp. 781-785.

R.M. Okamura, M. Sigvardsson, J. Galceran, S. Verbeek, H. Clevers and R. Grosschedl, Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1, *Immunity* 8 (1998), pp. 11-20.

M.J. Pazin, P.L. Sheridan, K. Cannon, Z. Cao, J.G. Keck, J.T. Kadonaga and K.A. Jones, NF-kappa B-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer in vitro, *Genes & development* 10 (1996), pp. 37-49.

E. Rodriguez-Carballo, A. Ulsamer, A.R. Susperregui, C. Manzanares-Cespedes, E. Sanchez-Garcia, R. Bartrons, J.L. Rosa and F. Ventura, Conserved regulatory motifs in osteogenic gene promoters integrate cooperative effects of canonical Wnt and BMP pathways, *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 26 (2011), pp. 718-729.

J. Roman-Gomez, L. Cordeu, X. Agirre, A. Jimenez-Velasco, E. San Jose-Eneriz, L. Garate, M.J. Calasanz, A. Heiniger, A. Torres and F. Prosper, Epigenetic regulation of Wnt-signaling pathway in acute lymphoblastic leukemia, *Blood* 109 (2007), pp. 3462-3469.

P.P. Roozen, M.H. Brugman and F.J. Staal, Differential requirements for Wnt and Notch signaling in hematopoietic versus thymic niches, *Annals of the New York Academy of Sciences* 1266 (2012), pp. 78-93.

A.A. Sharov, A. Nishiyama, Y. Qian, D.B. Dudekula, D.L. Longo, D. Schlessinger and M.S. Ko, Chromatin properties of regulatory DNA probed by manipulation of transcription factors, *Journal of computational biology : a journal of computational molecular cell biology* 21 (2014), pp. 569-577.

P.L. Sheridan, C.T. Sheline, K. Cannon, M.L. Voz, M.J. Pazin, J.T. Kadonaga and K.A. Jones, Activation of the HIV-1 enhancer by the LEF-1 HMG protein on nucleosome-assembled DNA in vitro, *Genes & development* 9 (1995), pp. 2090-2104.

J. Shi, L. Wang and L.H. Hu, [Aberrant localization of beta-catenin in leukemia cell lines], *Zhongguo shi yan xue ye xue za zhi / Zhongguo bing li sheng li xue hui = Journal of experimental hematology / Chinese Association of Pathophysiology* 14 (2006), pp. 1096-1100.

M. Shulewitz, I. Soloviev, T. Wu, H. Koeppen, P. Polakis and C. Sakanaka, Repressor roles for TCF-4 and Sfrp1 in Wnt signaling in breast cancer, *Oncogene* 25 (2006), pp. 4361-4369.

F.J. Staal and H. Clevers, Tales of the unexpected: Tcf1 functions as a tumor suppressor for leukemias, *Immunity* 37 (2012), pp. 761-763.

F.J. Staal and A.W. Langerak, Signaling pathways involved in the development of T-cell acute lymphoblastic leukemia, *Haematologica* 93 (2008), pp. 493-497.

F.J. Staal, J.J. van Dongen and A.W. Langerak, Novel insights into the development of T-cell acute lymphoblastic leukemia, *Current hematologic malignancy reports* 2 (2007), pp. 176-182.

F.C. Steinke and H.H. Xue, From inception to output, Tcf1 and Lef1 safeguard development of T cells and innate immune cells, *Immunologic research* 59 (2014), pp. 45-55.

I. Struewing, T. Boyechko, C. Barnett, M. Beildeck, S.W. Byers and C.D. Mao, The balance of TCF7L2 variants with differential activities in Wnt-signaling is regulated by lithium in a GSK3beta-independent manner, *Biochemical and biophysical research communications* 399 (2010), pp. 245-250.

M.M. Tiemessen, M.R. Baert, T. Schonewille, M.H. Brugman, F. Famili, D.C. Salvatori, J.P. Meijerink, U. Ozbek, H. Clevers, J.J. van Dongen and F.J. Staal, The nuclear effector of Wnt-signaling, Tcf1, functions as a T-cell-specific tumor suppressor for development of lymphomas, *PLoS biology* 10 (2012), p. e1001430.

M.M. Tiemessen and F.J. Staal, Wnt signaling in leukemias and myeloma: T-cell factors are in control, *Future oncology* 9 (2013), pp. 1757-1772.

M. van de Wetering, R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Loureiro, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Mortin and H. Clevers, Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF, *Cell* 88 (1997), pp. 789-799.

D.L. van den Berg, W. Zhang, A. Yates, E. Engelen, K. Takacs, K. Bezstarosti, J. Demmers, I. Chambers and R.A. Poot, Estrogen-related receptor beta interacts with Oct4 to positively regulate Nanog gene expression, *Molecular and cellular biology* 28 (2008), pp. 5986-5995.

M.P. Verzi, P. Hatzis, R. Sulahian, J. Philips, J. Schuijers, H. Shin, E. Freed, J.P. Lynch, D.T. Dang, M. Brown, H. Clevers, X.S. Liu and R.A. Shivdasani, TCF4 and CDX2, major transcription factors for intestinal function, converge on the same cis-regulatory regions, *Proceedings of the National Academy of Sciences of the United States of America* 107 (2010), pp. 15157-15162.

M.L. Waterman, Lymphoid enhancer factor/T cell factor expression in colorectal cancer, *Cancer metastasis reviews* 23 (2004), pp. 41-52.

A. Weise, K. Bruser, S. Elfert, B. Wallmen, Y. Wittel, S. Wohrle and A. Hecht, Alternative splicing of Tcf7l2 transcripts generates protein variants with differential promoter-binding and transcriptional activation properties at Wnt/beta-catenin targets, *Nucleic acids research* 38 (2010), pp. 1964-1981.

K. Willert, J.D. Brown, E. Danenberg, A.W. Duncan, I.L. Weissman, T. Reya, J.R. Yates, 3rd and R. Nusse, Wnt proteins are lipid-modified and can act as stem cell growth factors, *Nature* 423 (2003), pp. 448-452.

H.H. Xue and D.M. Zhao, Regulation of mature T cell responses by the Wnt signaling pathway, *Annals of the New York Academy of Sciences* 1247 (2012), pp. 16-33.

Y.Y. Yang, P.C. Lee, Y.T. Huang, W.P. Lee, Y.J. Kuo, K.C. Lee, Y.C. Hsieh, T.Y. Lee and H.C. Lin, Involvement of the HIF-1alpha and Wnt/beta-catenin pathways in the protective effects of losartan on fatty liver graft with ischaemia/reperfusion injury, *Clinical science* 126 (2014), pp. 163-174.

F. Yi, L. Pereira, J.A. Hoffman, B.R. Shy, C.M. Yuen, D.R. Liu and B.J. Merrill, Opposing effects of Tcf3 and Tcf1 control Wnt stimulation of embryonic stem cell self-renewal, *Nature cell biology* 13 (2011), pp. 762-770.

Q. Yu, A. Sharma and J.M. Sen, TCF1 and beta-catenin regulate T cell development and function, *Immunologic research* 47 (2010), pp. 45-55.

T. Zhou, X. He, R. Cheng, B. Zhang, R.R. Zhang, Y. Chen, Y. Takahashi, A.R. Murray, K. Lee, G. Gao and J.X. Ma, Implication of dysregulation of the canonical wingless-type MMTV integration site (WNT) pathway in diabetic nephropathy, *Diabetologia* 55 (2012), pp. 255-266.

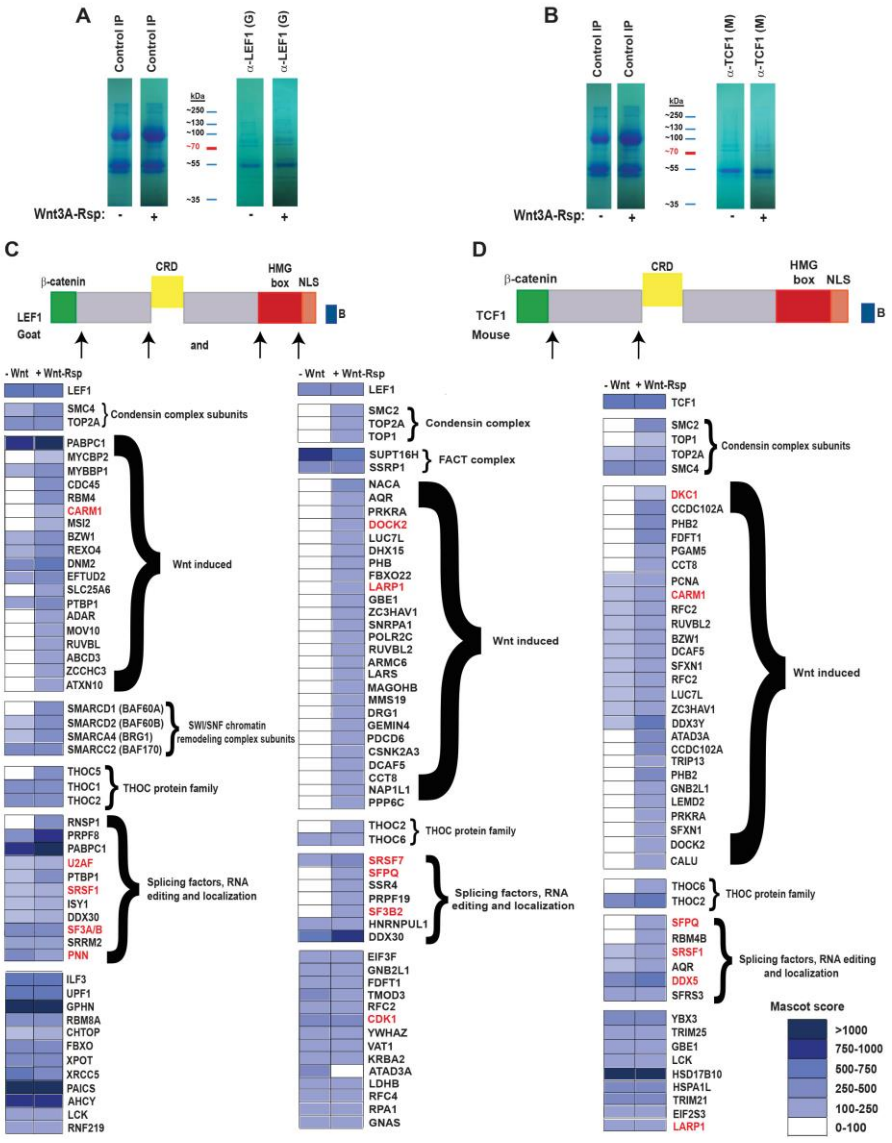

Figure S1. Identification of the Wnt- independent complexes of TCF1 and LEF1 in SupT1 leukemic cells. A) Colloidal blue stained gels of LEF1 containing complexes immunoprecipitated from SupT1 cells using antibodies directed against a region "N-term-CRD" after either Wnt3A-Rsp CM stimulation or CCM. B) Colloidal blue stained gels of TCF1 containing complexes immunoprecipitated from SupT1 cells using antibodies directed against a region "N-term-CRD" after either Wnt3A-Rsp CM stimulation or CCM. C) Mass spectrometry analysis of LEF1 co-factors immunopurified from SupT1 cells either stimulated with Wnt3A-Rsp CM or CCM overnight. A LEF1 Abs recognizing a region "N-term-CRD" of LEF1 (see schematic presentation below) was used to immunoprecipitate the LEF1 complex from pre-cleared SupT1 lysates. A heatmap depicting mascot scores for identified peptides. D) Mass spectrometry analysis of TCF1 co-factors immunopurified from SupT1 cells either stimulated with Wnt3A-Rsp CM or CCM overnight. A TCF1 Ab recognizing a region "N-term-CRD" of TCF1 (see schematic presentation below) was used to immunoprecipitate the TCF1 complex from pre-cleared SupT1 lysates. A heatmap depicting mascot scores for identified peptides.

Chapter 5

HDAC7 Is a Repressor of Myeloid Genes Whose Downregulation Is Required for Transdifferentiation of Pre-B Cells into Macrophages

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Abstract

B lymphopoiesis is the result of several cell-commitment, lineage-choice, and differentiation processes. Every differentiation step is characterized by the activation of a new, lineage-specific, genetic program and the extinction of the previous one. To date, the central role of specific transcription factors in positively regulating these distinct differentiation processes to acquire a B cell–specific genetic program is well established. However, the existence of specific transcriptional repressors responsible for the silencing of lineage inappropriate genes remains elusive. Here we addressed the molecular mechanism behind repression of non-lymphoid genes in B cells. We report that the histone deacetylase HDAC7 was highly expressed in pre-B cells but dramatically down-regulated during cellular lineage conversion to macrophages. Microarray analysis demonstrated that HDAC7 re-expression interfered with the acquisition of the gene transcriptional program characteristic of macrophages during cell transdifferentiation; the presence of HDAC7 blocked the induction of key genes for macrophage function, such as immune, inflammatory, and defense response, cellular response to infections, positive regulation of cytokines production, and phagocytosis. Moreover, re-introduction of HDAC7 suppressed crucial functions of macrophages, such as the ability to phagocytose bacteria and to respond to endotoxin by expressing major pro-inflammatory cytokines. To gain insight into the molecular mechanisms mediating HDAC7 repression in pre-B cells, we undertook coimmunoprecipitation and chromatin immunoprecipitation experimental approaches. We found that HDAC7 specifically interacted with the transcription factor MEF2C in pre-B cells and was recruited to MEF2 binding sites located at the promoters of genes critical for macrophage function. Thus, in B cells HDAC7 is a transcriptional repressor of undesirable genes. Our findings uncover a novel role for HDAC7 in maintaining the identity of a particular cell type by silencing lineageinappropriate genes.

Introduction

The generation of B cells is the result of several cellular transitions that take place in a stepwise manner and comprise cell lineage choices, cell commitment and differentiation. Every differentiation step leads to the activation of specific genes characteristic of the new cellular stage. This is achieved by the action of well defined networks of transcription factors specific to each particular cellular state [1,2]. In the bone marrow, lymphocyte development begins at the lymphoid-primed multipotent progenitor (LMPPs) stage. LMPPs become common lymphoid progenitors (CLPs), which have the potential to differentiate into B and T lymphocytes, as well as natural killer (NK) cells [3]. The transcription factors IKAROS, PU.1 and MEF2C are critical for the cellular commitment of LMPPs to the lymphoid lineage [3–5]. Later, the transcription factors E2A, EBF and FOXO-1 are required for the early specification of CLPs into pro-B cells, whereas PAX5 required to maintain B cell identity along differentiation into mature B cells [6–11]. However, there is an increasing body of evidence indicating that the repression of lineage inappropriate genes is a pivotal mechanism to properly acquire a particular cellular state during B lymphopoiesis. For example, PAX5 not only induces the expression of a B-cell specific genetic program, it also suppresses inappropriate genes of alternative lineages, thereby ensuring its role in maintaining B cell identity and differentiation [12–14]. Recently, it has been reported that the transcription factor MEF2C, by activating lymphoid specific genes and repressing myeloid genes, is involved in the cellular choice towards the lymphoid lineage [5]. These studies suggest that B cell transcription factors must also recruit transcriptional co-repressors to silence undesirable genes. To date, very little is known on the role of transcriptional repressors during B lymphopoiesis.

Histone deacetylases (HDACs) have emerged as crucial transcriptional co-repressors in highly diverse physiological systems. To date, 18 human HDACs have been identified and grouped into four classes. Class I HDACs (HDAC1, 2, 3, and 8), class II HDACs (HDAC4, 5, 6, 7, 9, and 10), class III HDACs, also called sirtuins, (SIRT1, 2, 3, 4, 5, 6, and 7) and class IV HDAC (HDAC11). Class II HDACs are further subdivided into class IIa (HDAC4, 5, 7, 9) and class IIb (HDAC6 and 10) [15,16]. Unlike other HDACs, Class IIa HDACs have three unique features. First, they are expressed in a tissue-specific manner and are involved in development and differentiation processes. They exert their transcriptional repressive function in skeletal, cardiac, and smooth muscle, the bone, the immune system, the vascular system, and the brain among others. Second, they are signaldependent co-repressors which become phosphorylated at conserved serine residues in the regulatory N-terminal domain leading to their nuclear export. Third, they contain a regulatory Nterminal domain that mediates their interactions with tissuespecific transcription factors such as members of the MEF2 family [15,16]. This last feature prompted us to ask whether members of the class IIa HDACs subfamily could be lineage-specific transcriptional repressors crucial to maintain B cell identity and biology.

To address this question we have used a cellular transdifferentiation system that we reported recently [17]. This system consists of a pre-B cell line (HAFTL cells) transduced with a retroviral vector for stable expression of a b-estradiol-inducible form of C/EBPa (C10 and C11 cells). After addition of b-estradiol, C10 and C11 cells are converted into functional macrophage-like cells at 100% efficiency within 48–72 hours. The conversion of pre-B cells into macrophages is direct and does not involve overt retro-differentiation through hematopoietic stem and progenitor cells [18]. Unexpectedly, this cellular transdifferentiation process appears to occur in the absence of significant changes in the DNA methylation status of key lymphoid or myeloid genes, but involves changes in histone modification on both types of genes [19]. Since HAFTL cells are a fetal liver cell line immortalized by Ha-ras transformation we, in parallel, also investigated the involvement of class IIa HDACs in normal primary B cell precursors. Here, we report that during the conversion of pre-B cells into macrophages, HDAC7 expression is down-regulated. In pre-B cells HDAC7 specifically interacts with the transcription factor MEF2C and is recruited to putative MEF2 sites located at the promoters of key macrophage genes. Forced re-expression of HDAC7 interferes with the establishment of the gene transcriptional program and functional characteristics of macrophages. Importantly, HDAC7 depletion in pre-B cells results in the de-repression of macrophage genes.

Results

HDAC7 is down-regulated during the transdifferentiation of pre-B cells into macrophages

In order to study the potential role of class IIa HDACs in B lymphocyte biology we first analyzed their expression levels during the cellular transdifferentiation of pre-B cells into macrophages. RT-qPCR and Western blotting experimental approaches showed that HDAC7 expression was dramatically down-regulated during the conversion of C10 cells into macrophages at 72 hours after bestradiol treatment (Figure 1A and 1B). Notably, no changes in Hdac4, Hdac5 and Hdac9 expression levels were observed during the cellular reprogramming process. As previously described [17], the B cell genes Rag1 and Pax5 became down-regulated, whereas the expression of the myeloid genes C/Ebpb and Csf1r were upregulated during cellular reprogramming (Figure 1A). Consistent with our findings, HDAC7 is not present in RAW264.7 macrophages at neither the RNA nor protein levels (Figure 1A and 1B). As expected, the expression of the B cell transcription factors IKAROS, E2A, EBF and PAX5 were down-regulated during the cellular conversion. In contrast, protein levels of the transcription factors MEF2C and RUNX1 did not significantly change during transdifferentiation (Figure 1B). To further confirm our findings we analyzed the kinetics of

HDAC7 expression during the cellular transdifferentiation in our previously reported microarray experiments [17]. Strikingly, similar to the B cell specific transcription factor PAX5, HDAC7 expression was downregulated during cellular reprogramming (Figure S1). Since HAFTL cells are a fetal-derived pre-B cell line transformed by Ha-ras we wondered whether our findings could be extended to normal primary cells. We analyzed HDAC7 expression in both primary B cell precursors and primary macrophages in a recently reported microarray analysis [18]. Importantly, we found that, similar to PAX5, HDAC7 was highly expressed in B cell precursors compared to primary macrophages (Figure 1C and Figure S1). In agreement with our results with the C10 cell line, C/EBPa-mediated conversion of primary pre-B cells into macrophages also resulted in the down-regulation of HDAC7 (Figure 1C and Figure S1). Our data demonstrate that among the different class IIa HDACs, HDAC7 shows a lymphoid-specific expression pattern and suggest that by repressing lineage inappropriate genes in pre-B cells, such as genes characteristic of macrophages, HDAC7 might be crucial in maintaining B cell functions and identity.

HDAC7 re-expression interferes with the gene transcriptional program of the reprogrammed macrophages

Our observed dramatic down-regulation of HDAC7 upon transdifferentiation of pre-B cells into the macrophage lineage was consistent with its potential role as a transcriptional repressor of macrophage-specific genes in pre-B cells. To test whether the presence of HDAC7 could interfere with the acquisition of a macrophage-specific gene program during transdifferentiation of pre-B cells into macrophages, we performed a gain of function experimental approach followed by genome-wide microarray analysis. We transduced C10 cells with a retroviral vector carrying HDAC7-Flag (C10-HDAC7 cells). As a control, C10 cells were transduced with an empty retroviral vector (C10-MSCV cells). As expected, b-estradiol treatment of C10-MSCV cells resulted in the total down-regulation of endogenous HDAC7 protein levels, while C10-HDAC7 cells expressed the exogenous HDAC7 protein at similar levels as in untreated C10-MSCV cells even at 72 hours after b-estradiol treatment (Figure S2). We then examined the genome-wide effects of HDAC7 re-expression on the gene transcription program of C10 cells induced to transdifferentiate to macrophages. Microarray experiments were conducted in both C10-MSCV and C10-HDAC7 cells un-induced or induced to transdifferentiate for 48 and 72 hours. The addition of b-estradiol to C10-MSCV cells resulted in the up- and down-regulation of 1609 and 1798 genes at 48 hours and of 1531 and 1567 genes at 72 hours after treatment, in agreement with our previous report [17]. Importantly, the exogenous expression of HDAC7 in C10HDAC7 cells treated with b-estradiol totally or partially abrogated the up-regulation of 988 and 866 genes, after 48 and 72 hours respectively. Earlier analyses showed that the vast majority of the up-regulated genes

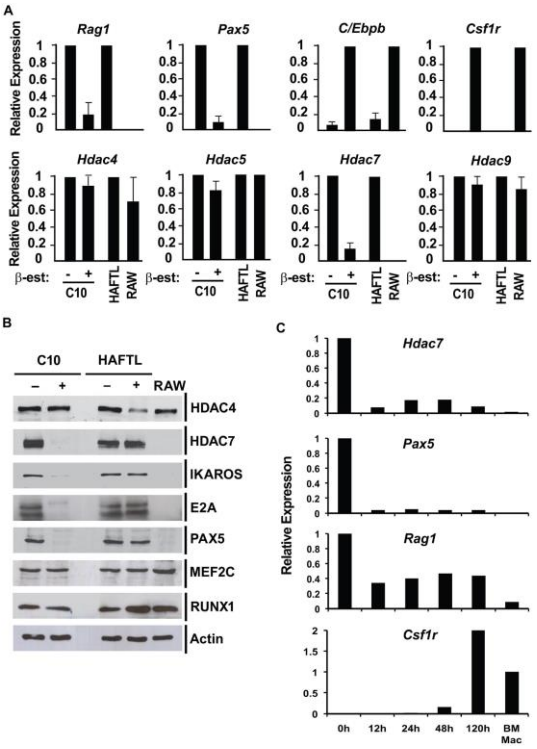

Figure 1. HDAC7 is down-regulated during the transifferentiation of pre-B cells into macrophages. A. RT-qPCR experiments for gene expression changes of class IIa HDACs, B cell and macrophages genes (uninduced cells (2) and b-estradiol induced C10 cells (+) for 72 hours). HAFTL pre-B cells and RAW264.7 cells were used as control. Data are represented as the mean +/2 standard error of the mean (SEM) of three independent experiments. B. Western blot of Class IIa HDACs and B cell transcription factors in the same experimental conditions shown in A. C. RT-qPCR experiments for gene expression changes of Hdac7, Pax5, Rag1 and Csf1r genes (uninduced (0) and b-estradiol induced primary pre-B cells transduced with a retroviral vector for C/EBPa expression for the indicated times. Primary macrophages were used as control.

correspond to key genes for macrophage function, whereas the down-regulated genes are associated with cell cycle processes and with important functions for B cell development and biology [17]. To determine the type of genes whose up-regulation was affected by the presence of HDAC7 we performed a gene set enrichment analysis based on the gene ontology (GO) categories corresponding to Biological Processes, Cellular Components and Molecular Functions, as well as on KEGG pathways. To do so we took advantage of Gitools, a recently developed bioinformatics tool [20]. Gitools allows accessing many available biological databases, performing analysis and visualizing data using interactive heat-maps. Strikingly, the Biological Process enrichment revealed that the set of up-regulated genes affected by HDAC7 belong to GO categories representing key macrophage related functions, such as immune, inflammatory and defense response, cellular response to infections, positive regulation of cytokines production and phagocytosis (Figure 2A). Importantly, we found that

the categories enriched in the KEGG pathways analysis correspond to similar biological processes (Figure S3). Gene ontology (GO) analysis corresponding to Cellular Components and Molecular Functions reinforce the above results (Figure 2B and 2C). Among the genes whose upregulation is impaired by HDAC7 we found several that are involved in phagocytosis (such as Fcgr1, Fcgr2b and Fcgr3), genes related to the immune response (such as the chemokine genes Cxcl10, Cxcl11 and Ccl2), Toll-like receptors (Tlr3, Tlr4, Tlr7, Tlr8 and Tlr9), interleukins (Il18 and Il15) and TNF pathway-related genes (Tnfsf10, Tnfsf11 and Tnfsf13b) (Figure 3). A full list of the genes found in each category is presented in the interactive website http://bg.upf.edu/C10-HDAC7/ (for statistics see Dataset S1). To validate our microarray analysis, we selected several representative genes and tested their mRNA levels by RT-qPCR. We observed that the presence of HDAC7 significantly interfered with the increase in the mRNA levels of Cxcl10, Fcgr1, Ifi35, Ifi47, Tlr3, Tlr9, Il18 and C3 genes after b-estradiol treatment (Figure 4). Importantly, HDAC7 re-introduction did not interfere with the down-regulation of key genes for B cell differentiation and biology such as the transcription factor PAX5, reinforcing the notion that HDAC7 is a repressor of macrophage genes in B cell precursors (Figure S5). During the trasdifferentiation of C10 cells into macrophages the cells stop dividing and many genes related to the cell cycle, such as genes involved in mitosis, become downregulated [17]. We have observed that the presence of HDAC7 also interferes with the down-regulation of these types of genes, corroborating that HDAC7 blocks, at least in part, the cellular transdifferentiation process (Figure S4, Dataset S2, and interactive website http://bg.upf.edu/C10-HDAC7/). Our data clearly demonstrate that HDAC7 represses the expression of central genes for macrophage function, and strongly suggest that HDAC7 acts as a specific transcriptional repressor of lineage inappropriate genes in B cell precursors.

HDAC7 knock down leads to the de-repression of macrophage genes

To determine the physiological function of HDAC7 in B cell precursors and test whether it is involved in the repression of macrophage genes, we performed a loss of function experimental approach. We knocked down HDAC7 by siRNA in both HAFTL cells and primary pre-B cells (Figure 5). Strikingly, we observed that the reduction in HDAC7 mRNA levels resulted in the derepression of key macrophage genes such as Itgam (Mac-1), Fcgr1 and Ccl3 (Figure 5). These data demonstrate that HDAC7 is involved in the repression of lineage inappropriate genes in B cell precursors.

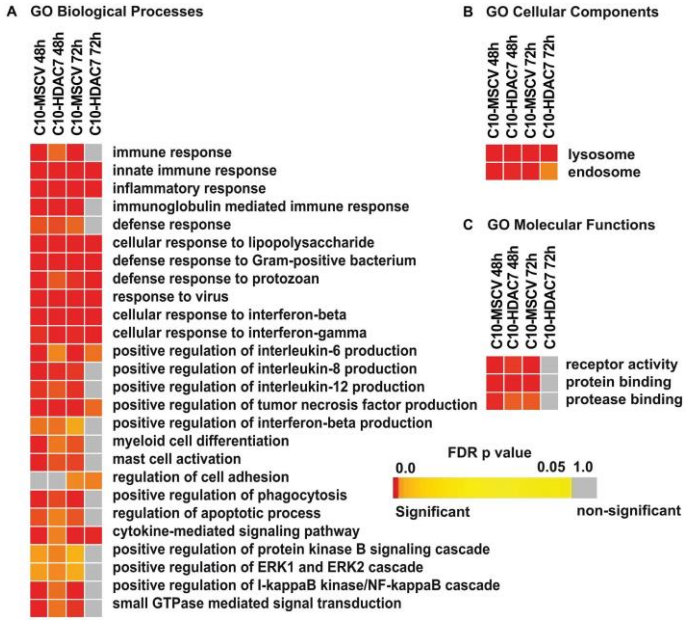

Figure 2. HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. Heat-maps showing significantly (corrected p-value ,0.05) enriched GO Biological Process (A), Cellular Components (B) and Molecular Functions (C) categories among the up-regulated genes affected by the re-expression of HDAC7 during transdifferentiation of pre-B cells into macrophages. Colours toward red indicate high statistic significance, yellow indicates low statistic significance, and gray indicates no statistical significance. The list of genes for the enrichment analysis in each column is as follows: C10-MSCV-48h includes genes up-regulated after b-estradiol treatment for 48 hours. C10-HDAC7-48h includes genes up-regulated after b-estradiol treatment for 48 hours which are down-regulated in the presence of HDAC7 (HDAC7 re-expression). C10-MSCV72h includes genes up-regulated after b-estradiol treatment for 72 hours. C10-HDAC7-72h includes genes up-regulated after b-estradiol treatment for 72 hours which are down-regulated after re-expression of HDAC7.

HDAC7 binds to the transcription factor MEF2C in pre-B cells and is recruited to the promoters of target genes critical for macrophage function

At the mechanistic level, class IIa HDACs exert their actions as transcriptional repressors by interacting with specific transcription factors recruited to the promoters of genes required for development and cell differentiation [15,16]. To address whether HDAC7 specifically interacts with particular sequence-specific transcription factors in pre-B cells, we undertook a candidateapproach and performed co-immunoprecipitation experiments to test for potential interaction between endogenous HDAC7 and the B cell transcription factors IKAROS, E2A, PAX5, and MEF2C in the C10 parental line (HAFTL cells). HDAC7 specifically associated with MEF2C, but not with the other B cell transcription factors tested (Figure 6A). Recently,

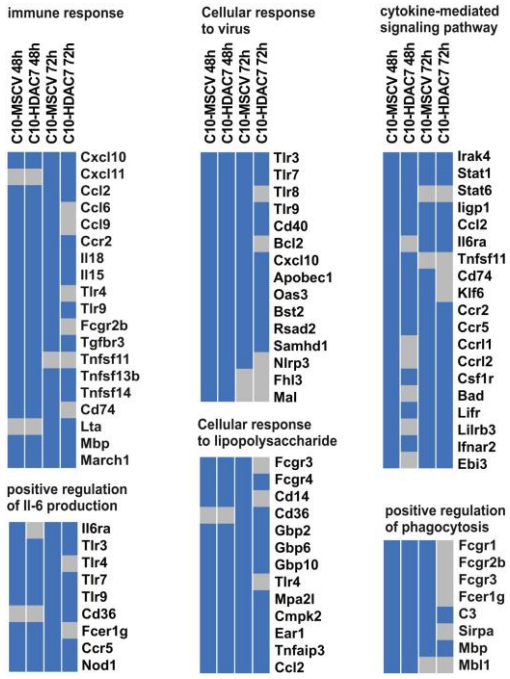

Figure 3. HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. Heat-maps showing observed differentially expressed genes for selected enriched GO categories. Blue colour cell indicates positive events while gray colour indicates that the gene was not observed differentially expressed in that experimental condition.

Camargo and colleagues have reported that MEF2C is crucial in the cellular choice towards the lymphoid versus the myeloid lineage in LMPPs [5]. Microarray analysis of control and MEF2C-deficient LMPPs revealed that the absence of MEF2C resulted in the up-regulation of genes enriched in GO categories related to the immune system, such as genes involved in the inflammatory and defense response of the cells [5]. Comparison of these data with our set of genes whose upregulation was affected by HDAC7 re-expression during cellular transdifferentiation showed a significant overlap by Chi-square test of 46 ($p,10^{26}$) and 30 ($p=0.00044$) genes, respectively (Figure 6B). Strikingly, the phagocytosis-related genes Fcgr1, Fcgr2b and Fcgr3 were found to be targets of both HDAC7 and MEF2C. These data indicate that MEF2C represses genes characteristic of macrophages in hematopoietic progenitors and suggest that HDAC7 may also silence lineage inappropriate genes at earlier stages of lymphocyte development.

To test whether in pre-B cells HDAC7 is recruited to the promoters of macrophage-specific genes whose up-regulation is impaired in the presence of exogenously expressed HDAC7, we performed chromatin immunoprecipitation (ChIP) assays. Notably, using the TFconsite bioinformatic tool we found that promoters of Fcgr1, Cxcl10 and Itgam contain putative MEF2 binding sites. We designed primers spanning the upstream regulatory regions, gene body, and

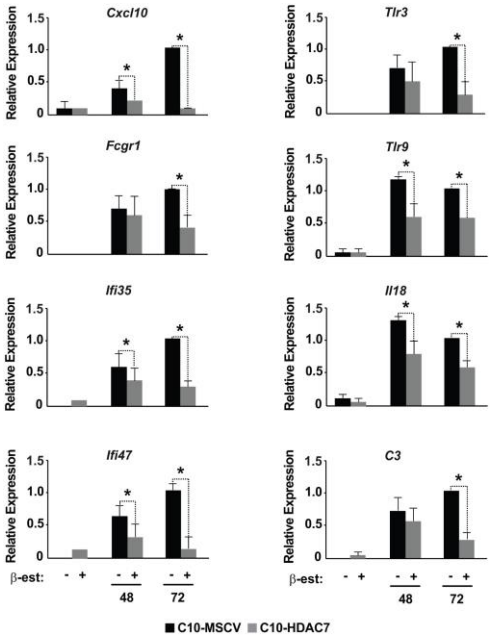

Figure 4. RT–qPCR for microarray validation of selected genes. RT-qPCR experiments for gene expression changes for 8 up-regulated genes, Cxcl10, Fcgr1, Ifi35, Ifi47, Tlr3, Tlr9, Il18 and C3 in the absence or in the presence of HDAC7. Data are represented as the mean +/2 standard error of the mean (SEM) of three independent experiments. The two-way ANOVA test was used to calculate significant levels between the indicated groups.

downstream regulatory regions of mouse Fcgr1, Cxcl10 and Itgam (Figure 6C and 6F and Figure S6A). Chromatin prepared from C10 cells un-induced or induced with b-estradiol for 72 hours was subjected to ChIP with antibodies specific for MEF2C and HDAC7. qPCR analysis of the immunoprecipitated material with primers specific for the Fcgr1, Cxcl10 and Itgam loci indicated that both MEF2C and HDAC7 were specifically and significantly enriched at the identified putative MEF2 binding sites in un-induced C10 cells (Figure 6D, 6E, 6G and 6H and Figure S6B). Importantly, while significant MEF2C enrichment around its putative binding sites was found in both un-induced and induced conditions, HDAC7 enrichment at the target genes was lost upon b-estradiol induction (Figure 6D, 6E, 6G and 6H and Figure S6B). Taken together these data indicate that via interaction with MEF2C, HDAC7 is recruited to the promoters of myeloid genes in B cell precursors, resulting in their transcriptional silencing.

HDAC7 re-expression interferes with the functional properties of the reprogrammed macrophages

We previously reported that during reprogramming of pre-B cells into macrophages there is an increase in the expression of Mac-1 and a down-regulation of CD19 levels, two cell surface

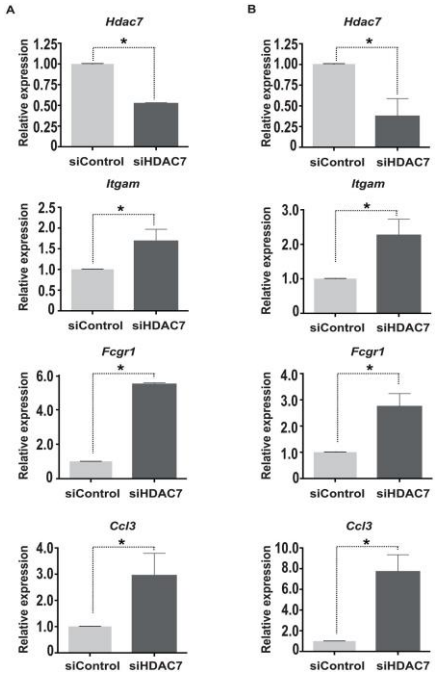

Figure 5. HDAC7 knock down leads to the de-repression of macrophage genes. HAFTL cells (A) and primary B cell precursors (B) were transfected with control siRNA or siRNAs targeting HDAC7. 72 hours after transfection Hdac7, Itgam, Fcgr1 and Ccl3 mRNA levels were determined by RT-qPCR experiments. Data are represented as the mean +/2 standard error of the mean (SEM) of three independent experiments. The two-way ANOVA test was used to calculate significant levels between the indicated groups. *P,0.001.

surface markers characteristic for macrophages pre-B cells, respectively. The reprogrammed macrophages show high phagocytic activity and respond to LPS treatment with cytokine production [17]. To test whether the presence of HDAC7 could interfere with the functional characteristics of the reprogrammed macrophages we undertook three different experimental approaches. First, we followed the expression kinetics of CD19 and Mac-1 (CD11b), two cell surface markers characteristic for pre-B cells and macrophages, respectively by flow cytometry. In both C10-MSCV and C10HDAC7 cells, 100% of the population became CD19 negative and Mac-1 positive 72 hours after addition of b-estradiol (Figure 7A). However, the presence of exogenous HDAC7 resulted in a significant block in the expression levels of Mac-1 (Figure 7A and 7B). In contrast, CD19 protein levels decreased to the same extent regardless of the presence of HDAC7 (Figure 7A and 7B). Exogenous expression of HDAC7 in primary B cell precursors also interfered with the up-regulation of Mac-1 in transdifferentiation experiments (Figure 7C). These results demonstrated that in the presence of HDAC7 the reprogrammed cells are not able to express the levels of Mac-1 protein normally present in macrophages. Second, we tested the phagocytic properties of the transdifferentiated C10 cells exogenously expressing HDAC7. Interestingly, C10-HDAC7 cells treated with b-estradiol showed a significantly reduced capacity to phagocytose red

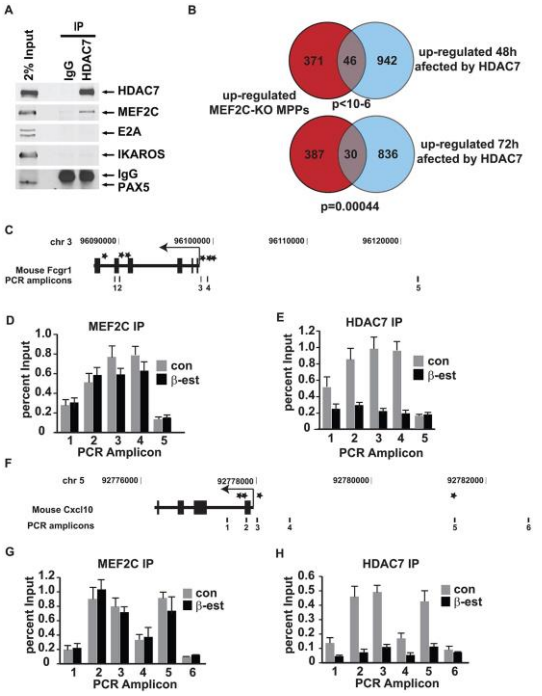

Figure 6. HDAC7 binds to the transcription factor MEF2C and is recruited to the promoter of key genes for macrophage function in pre-B cells. A. Co-immunoprecipitation experiments showing the specific binding of HDAC7 with MEF2C in pre-B cells. HDAC7 does not bind with IKAROS, PAX5 and E2A. B. Venn diagrams showing the total number of genes up-regulated in MEF2C deficient LMPPs, genes up-regulated at 48 and 72 hours of b-estradiol treatmentaffected by HDAC7 and the overlapping between both conditions. C and F. Schematic representation of the mouse Cxcl10 and Fcgr1 locus and amplicons scanned in Chromatin immunoprecipitation experiments by qPCR. Asterisks indicate MEF2 binding sites location. D, E, G and H. Chromatin immunoprecipitation experiments showing the enrichment of HDAC7 and MEF2C to putative MEF2 binding sites on the Cxcl10 and Fcgr1 gene loci in pre-B cells. Results are presented as percentage immunoprecipitated over input and are representative of three independent experiments.

fluorescence protein-expressing bacteria compared to the control C10-MSCV cells (Figure 7D). Lastly, we analyzed the inflammatory response of both cell lines after treatment with LPS in the presence or absence of b-estradiol for 48 hours. Strikingly, expression of HDAC7 resulted in a significant inhibition in the expression of the proinflammatory cytokines, Tnfa, Il-1a and Il-6, by the macrophagelike converted cells in response to LPS (Figure 7E). We next tested whether HDAC7 re-expression results in the reduction of Mac-1 protein levels once the cells have been reprogrammed into macrophages. We first treated C10 cells with b-estradiol for 72 hours and the reprogrammed macrophages were transduced with a retroviral vector carrying HDAC7-Flag. We observed a decrease in the expression levels of Mac-1 24 hours after expression of exogenous HDAC7 (Figure S7A). However, we did not observe any effect on either the expression of macrophage genes or the phagocytic capacity of RAW cells expressing exogenous HDAC7 (Figure S7B, S7C and S7D). We have recently shown that

C/EBPa-mediated reprogramming of pre-B cells into macrophages occurs in the absence of significant changes in the DNA methylation of crucial genes suggesting that the reprogrammed macrophages retained an epigenetic memory characteristic of the cell of origin [19]. Therefore, we speculate that the chromatin structure present in differentiated macrophages is not permissive for the recruitment of HDAC7 to its target genes and that such recruitment is only possible in a chromatin environment characteristic of B cells, in line the notion that HDAC7 is a lymphoid-specific transcriptional repressor. Taken together, these results demonstrate that HDAC7 significantly interferes with key functional characteristics of the transdifferentiated macrophages.

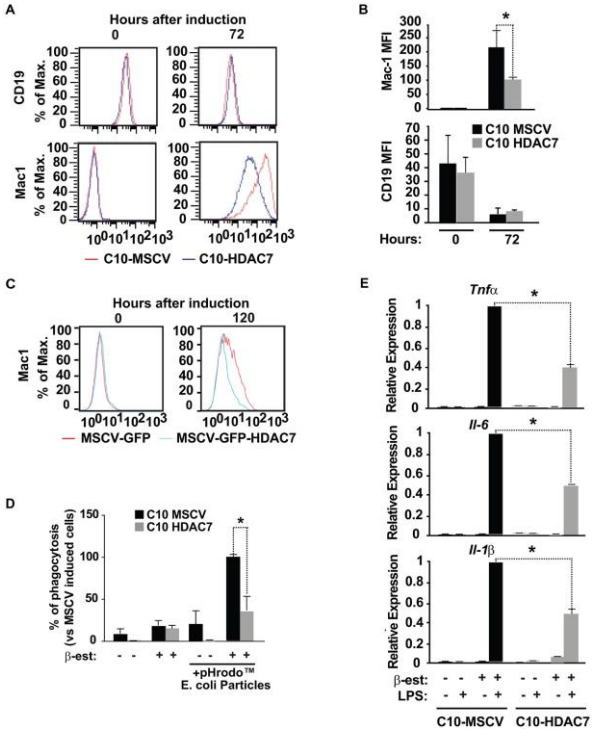

Figure 7. HDAC7 re-expression interferes with the functional properties of the converted macrophages. A. Histograms for Mac-1 and CD19 expression levels in C10-MSCV and C10-HDAC7 cells untreated or treated with b-estradiol for 72 hours. B. Mean fluorescence intensity (MFI) of Mac-1 and CD19 of C10-MSCV and C10-HDAC7 cells untreated or treated with b-estradiol for 72 hours. Data are represented as the mean +/2 standard error of the mean (SEM) of three independent experiments. *P,0.001. C. Primary pre-B cells were transduced with the indicated retroviral vectors and 48 hours after induce to transdifferentiate. Histograms for Mac-1 proteins levels are shown. D. Capacity of C10-MSCV and C10-HDAC7 cells untreated or treated with b-estradiol for 48 hours to phagocytose red fluorescence bacteria. Data are given as mean 6 SEM of values obtained in three independent experiments. Statistical significance was determined by two-way ANOVA followed by Bonferrony multiple comparison test. *P,0.001. E. Effect of HDAC7 expression in LPS-mediated Tnfa, Il-1a and Il-6 gene expression. C10-MSCV and C10-HDAC7 cells were treated or not with b-estradiol for 48 hours. Then, the cells where incuated or not with LPS for 6 hours and RNAs analyzed by RT-qPCR. Data are represented as the mean +/2 standard error of the mean (SEM) of three independent experiments. P values were calculated by the two-way ANOVA test. *P,0.001.

Interaction of HDAC7 with MEF2C and its catalytic activity are essential for repression of Mac-1

Class IIa HDACs interact with MEF2 proteins via a conserved motif of 17 amino acids located in the amino-terminal region of the proteins. To definitively prove that HDAC7 represses macrophage genes through its interaction with MEF2C, we generated retroviral vectors carrying mutants of HDAC7 with either a deletion of the entire 17 amino acids stretch (HDAC7DMEF) or with substitutions of crucial lysine residues (HDAC7K86AK88A). We tested the HDAC7 mutants for their ability to repress Mac-1 during the transdifferentiation of pre-B cells into macrophages. Expression of wild-type HDAC7 resulted in a significant decrease in Mac-1 positive cells and mRNA levels (Figure 8) whereas expression of the HDAC7 mutants defective for MEF2C binding had no significant effect. We next tested whether the enzymatic activity of HDAC7 is necessary for its repressive action on Mac-1 expression during the conversion of pre-B cells into macrophages. We generated a retroviral vector for HDAC7 mutated in its catalytic domain (HDAC7-H657A), a C-terminal truncated construct HDAC7(1–487) that completely lacks the HDAC catalytic domain but contains the MEF2 interacting motif and a N-terminal truncated construct HDAC7(438–915) bearing the enzymatic motif but lacking the MEF2 domain. As shown in Figure 8, forced expression of wild-type HDAC7 interfered with the up-regulation of Mac-1 protein levels 48 hours after induction of cellular reprogramming. In contrast, the HDAC7-H657A, the HDAC7(1–487) and the HDAC7(438–915) constructs did not block the up-regulation of Mac-1 levels. These experiments demonstrate that both the HDAC7-MEF2C interaction, as well as its catalytic activity, are necessary for HDAC7 to repress macrophage genes during cellular reprogramming.

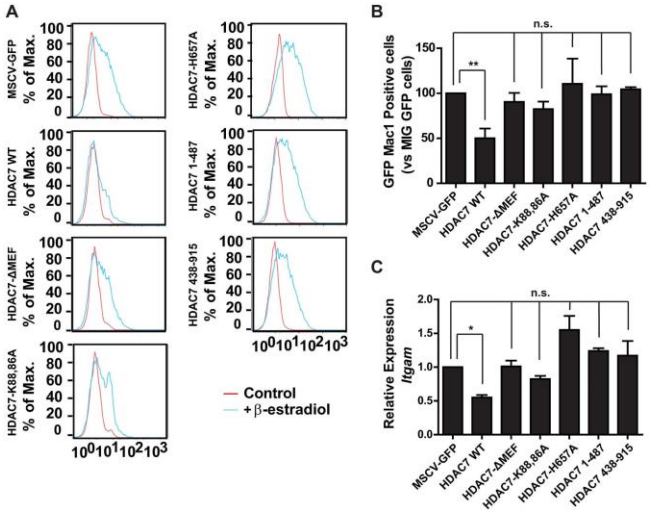

Figure 8. Interaction of HDAC7 with MEF2C and its catalytic activity are essential for repression of Mac-1. C11 cells werinfected with MSCV-GFP, MSCV-GFP-HDAC7, MSCV-GFP-HDAC7(DMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCV-GFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP- HDAC7(438–915) viruses and induced with b-estradiol for 48 hours. A. Cells were stained with a Mac-1 antibody and the GFP-positive fractions were gated and the results plotted. B. Percentage of Mac-1 positive cells treated with b-estradiol for 48 hours. Data are given as mean 6 SEM of values obtained in three independent experiments. Statistical significance was determined by two-way ANOVA followed by Bonferrony multiple comparison test. *P,0.0001. C. RT-qPCR experiments for Itgam gene expression changes in cells treated with b-estradiol for 48 hours. Data are given as mean 6 SEM of values obtained in three independent experiments. Statistical significance was determined by two-way ANOVA followed by Bonferrony multiple comparison test. *P,0.001.

Discussion

Our findings have revealed that HDAC7 is expressed in B cell precursors and not in macrophages (Figure 9). Using a cellular transdifferentiation system we have demonstrated that HDAC7 represses the expression of a large number of macrophage genes during the conversion of pre-B cells into this myeloid cell type. More importantly, depletion of HDAC7 in pre-B cells results in the de-repression of key macrophage genes. Functionally, the presence of HDAC7 interferes with the acquisition of key functional features of the converted macrophages. These aberrant macrophage- converted cells do not express the Mac-1 protein levels normally present in macrophages, are not able to properly phagocytose bacteria and do not respond adequately to endotoxin by expressing major pro-inflammatory cytokines. At the mechanistic level, HDAC7 specifically interacts with the transcription factor MEF2C in pre-B cells and is recruited to MEF2 binding sites located at the promoters of genes critical for macrophage function (Figure 6 and Figure S6). In addition to the interaction with MEF2C, the catalytic activity of HDAC7 is also necessary to repress macrophage genes. Our results

demonstrate that HDAC7 is expressed in fetal and adult pre-B cells suggesting that it might play a role in both types of B lymphopoiesis.

Why is HDAC7 specifically expressed in pre-B cells and not macrophages? Based on our results, we conclude that HDAC7 is a key transcriptional repressor of lineage inappropriate genes in preB cells. Using a ChIP-seq approach Murre and colleagues have identified HDAC7 as a target of the transcription factors E2A, EBF and Foxo1 in B cell precursors (pro-B cells) postulating that it could be an important regulator of B cell development and indicating that HDAC7 function is not restricted to T cells within the hematopoietic system [21]. In addition, our results showing that in pre-B cells HDAC7 interacts with the transcription factor MEF2C, allowing the recruitment to the MEF2 binding sites on promoters of genes characteristic of macrophages reinforces our conclusion. Indeed, in the late nineties, MEF2C was found, among the different MEF2 family members, to be specifically expressed in B cells within the lymphocyte lineage, suggesting that it could have a role in B cell development and function [22]. More recently, it has been reported that MEF2C regulates B cell proliferation and survival after BCR activation and p38 MAPK signaling [23,24]. MEF2C is also expressed at earlier stages of lymphocyte development [5,25]. It has been reported that MEF2C is involved in the cellular choice towards the lymphoid versus the myeloid lineage in lymphoid-primed multipotent progenitors (LMPPs) [25]. At the molecular level MEF2C activates the transcription of lymphoid specific genes and represses myeloid genes [25]. We have observed a significant overlap between MEF2C regulated genes in LMPPs and our identified HDAC7 target genes in pre-B cells. Moreover, HDAC7 mutants that do not interact with MEF2C are unable to suppress the up-regulation of macrophage genes during the reprogramming process. Given this scenario, we propose that HDAC7 is the MEF2C transcriptional co-repressor responsible for the silencing of myeloid genes in B cells and lymphoid precursors.

Finally, in light of previous reports demonstrating the expression and functions of HDAC7 in T cells, our results have uncovered a more general role for HDAC7 in the lymphoid lineage compartment within the hematopoietic system. In addition, the finding that HDAC7 is dramatically down-regulated during the transdifferentiation of pre-B cells into macrophages not only reinforces this notion, but also provides an additional level of complexity to the way its activity and function are regulated. During T cell development in the thymus and later in a differentiated and specialized T cell type, cytotoxic T lymphocytes (CTLs), HDAC7 is regulated in a signal dependent manner responsible for its phosphorylation and for its subsequent nucleo-cytoplasmic distribution [26–31]. In resting thymocytes, HDAC7 is localized in the nucleus, where it represses the expression of a large number of genes involved in both positive (survival) and negative (apoptosis) selection of the cells. However, in response to TCR signaling, HDAC7 becomes phopshorylated and translocates to the cytoplasm where it can no longer repress its target genes [26,27,29,31]. Later in the periphery, HDAC7 is constitutively phosphorylated and found in the cytoplasm of CTLs allowing the expression of key genes for

the function of this specialized T cell type [30]. We could postulate that within a particular hematopoietic cellular lineage, the HDAC7 gene transcriptional repressive function is regulated in a signal-dependent way by controlling its phosphorylation status and altering its cellular distribution. That is, HDAC7 is unphosphorylated or phosphorylated and localized either in the cell nucleus or in the cytoplasm depending on the requirement to silence or activate specific genes at different stages of T cell development. In contrast, at the branching points where cellular lineage choice has to be made, e.g. lymphoid versus myeloid, HDAC7 might be regulated at the expression level. For instance, our results showing that HDAC7 expression is down-regulated during the conversion of pre-B cells into macrophages further support this hypothesis. However, as is the case for T cells, we cannot rule out the possibility that HDAC7 is additionally regulated in a signal dependent manner during B cell development and differentiation. In this regard, we have found that HDAC7 is expressed in the nucleus of pre-B cells (data not shown) probably due to the lack of a fully functional B cell receptor (BCR). However, it is highly probable that HDAC7 function is also regulated via BCR signaling at later cell differentiation stages when B cells develop into mature antibody-secreting cells.

Based on the findings of this study, we therefore propose that in the hematopoietic system, HDAC7 is not only a signal-dependent transcriptional repressor involved in different developmental steps of a particular lymphocyte type, but also a lineage-specific transcriptional repressor responsible for maintaining the identity of lymphocytes by silencing lineage inappropriate genes. We anticipate that other members of the class IIa HDACs subfamily might be specific repressors of undesirable genes in the cellular differentiation and developmental processes where they exert their actions (e.g. skeletal and cardiac muscle, bone formation and brain).

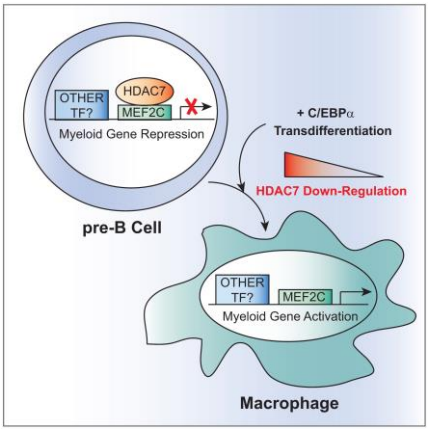

Figure 9. Model for HDAC7-mediated transcriptional repression in pre-B cells. HDAC7 is expressed in pre-B cells and not in macrophages. In pre-B cells, HDAC7 specifically interacts with the transcription factor MEF2C and is recruited to promoters of myeloid genes. During transdifferentiation of pre-B cells into macrophages HDAC7 is down-regulated allowing for expression of macrophages specific genes.

Materials and Methods

Plasmids

MSCV-puro-HDAC7, MSCV-GFP-HDAC7, MSCV-GFPHDAC7(DMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCVGFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP-HDAC7(438–915) construct were generated by cloning the HDAC7-Flag cDNA PCR amplificated from the pCDNA3-HDAC7 wild-type and mutant plasmids previously described [20] into the MSCV-puro or MSCV-GFP vectors
(Clontech).

Antibodies

Anti-HDAC7 (H-273), anti-HDAC7 (C-18), anti-HDAC4 (H92), anti-E2A (V-18), anti-PAX5 (C-20) and anti-RUNX1(DW71) were purchased from Santa Cruz Biotechnology. Anti-IKAROS (ab26083) was purchased from Abcam; anti-MEF2C (D80C1) XP from Cell Signaling Technology; and anti-a-Tubulin (T61999), from Sigma-Aldrich.

Cell culture and b-estradiol treatment

HAFTL cells were grown at 37uC in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 U/ml streptomycin/penicillin. C10 cells (transduced with a MSCVGFP-C/EBPa retroviral vector) and C11 cells (transduced with a MSCV-hCD4-C/EBPa retroviral vector) were cultured at 37uC in RPMI 1640 without phenol red supplemented with 10% of charcoal treated fetal bovine serum, 2 mM glutamine, and 50 U/ ml streptomycin/penicillin. B-cell precursors and macrophages were obtained from mouse bone marrow as described [18]. For transdifferentiation induction, cells were treated with 100 nM bestradiol in the presence of 10 nM of IL-3 and 10 nM of mCSF1 for the indicated periods of time.

Retroviral supernatant generation and cellular transduction

For retrovirus generation the MSCV-puro, MSCV-puroHDAC7, MSCV-GFP, MSCV-GFP-HDAC7, MSCV-GFP- HDAC7(DMEF),MSCV-GFP-HDAC7(K86A/K88A),MSCV-GFPHDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCVGFP-HDAC7(438–915) plasmids were transfected into the packaged cell line Platinum-E and supernatant were collected at 48–72 hours post-transfection. For the generation of C10-MSCV and C10-HDAC7 cells, C10 cells were spin infected and 48 hours after were selected in the presence of 3 ug/ml of puromycin. C11 cells were spin infected with MSCV-GFP, MSCV-GFP-HDAC7, MSCV-GFPHDAC7(DMEF), MSCV-GFP-HDAC7(K86A/K88A), MSCVGFP-HDAC7(H657A), MSCV-GFP-HDAC7(1–487) and MSCV-GFP-HDAC7(438–915) and 48 hours after treated with b-estradiol.

siRNA depletion

Dharmacon siRNA control and on-target smartpools targeting transcript of the mouse HDAC7 gene were used to knockdown its expression HAFTL cells and primary B cell precursors. siRNA were transfected using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). mRNA levels were examined by RT-qPCR experiments 72 hours after siRNA transfection.

RT–qPCR experiments

RNA was extracted Trizol extraction (Qiagen) and cDNA synthesyzed using the High Capacity cDNA Reverse Trancription Kit (AB Applied Biosystems). RT-qPCR were performed in triplicate using SYBR Green I Master (Roche). PCR reactions were run and analyzed using the LightCycler 480 Detection System (Roche). Primers sequences upon request.

Co-immunoprecipitation and Western blot analysis

Total cellular extracts were prepared in PLB buffer (0.5% Triton X-100, 0.5 mM EDTA, 1 mM DTT in PBS) supplemented with protease inhibitors (Complete, Roche Molecular Biochemicals). Immunoprecipitation, SDS-PAGE and Western blot experiments were performed as previously described [32].

Flow cytometry

Cells were un-induced or induced to transdifferentiate. 48 hours later, cells were stained with fluorochrome conjugated antibodies against Mac-1 and CD19 (BD Pharmingen). Mac-1 and CD19 expression were monitored on a Gallios Flow Cytometer (Beckman Coulter) and analyzed by FlowJo software (Tree Star, Inc.).

Phagocytosis assays

C10-MSCV and C10-HDAC7 cells were induced or not for 48 h and subjected to the pHrodo E.coli phagocytosis KiT (Invitrogen) following the manufacturers protocol.

LPS induced inflammatory cytokines

C10-MSCV and C10-HDAC7 cells were induced to transdifferentiate. 48 h after induction they were incubated with LPS (1 mg/mL Sigma) for 6 h. RNA extraction, cDNA synthesis and RT-qPCR were performed as described above. Primers sequences upon request.

Microarray experiments

Biological duplicates of C10-MSCV and C10-HDAC7 cells were un-induced or induced to transdifferentiate for 48 and 72 hours (12 samples in total). Total RNA from cultured cells was extracted by Trizol and then purified. PCR amplified RNAs were hybridized against Affymetrix mouse arrays chip (Mouse Genome 430 PM strip) at the IRB Genomics Facility. Affymetrix raw CEL files and processed (normalized) data have been deposited in GEO database under accession number GSE36827.

Microarray data analysis

Affymetrix CEL files were background corrected, normalized using Bioconductor, package ‘‘affy’’ (version 1.28.1) using ‘expresso’ algorithm [33,34]. Since the Affymetrix chip version used in this study contains only perfect match (pm) probes, for normalization and acquiring raw probe intensities to expression values we used the following parameters: background correction method ‘‘rma’’; normalization method ‘‘constant’’; pm correct method ‘‘pmonly’’; and summary method ‘‘avgdiff’’. Quality of microarray experiment (data not shown) was verified by Bioconductor package ‘‘arrayQualityMetrics’’ (version 3.2.4 under Bioconductor

version 2.7; R version 2.12.1) [35]. To determine genes that are differentially expressed (DE) between two experimental conditions, Bioconductor package Limma was utilized to generate contrast matrices and fit the corresponding linear model [36]. Probe to gene annotation were performed using microarray vendor's annotation data. When more than one probe were annotated to same gene, highest absolute expression value was considered (maximizing). To consider a gene is differentially expressed, besides multiple test corrected, FDR p-value #0.05 as cut off, we also applied Log2 fold change (Log2FC) cut off 0.5 for b-estradiol treatment. We used Log2FC cut off 0.5 for genes that are affected by the expression of HDAC7 in b-estradiol treated cells. Expression data on Mef2c deficient multipotent progenitor cells were obtained from GEO database (accession No. GSE13686) [5]. Data were analyzed using the limma package from Bioconductor. Spots were not background corrected before within array loess normalization. After array normalization using the quantile method log2 ratios (mutant/control) was calculated. To define a gene up-regulated, we used Log2FC $1.0.

Functional and pathway enrichment analysis

Functional annotation of differentially expressed genes is based on Gene Ontology (GO) (http://www.geneontology.org) as extracted from EnsEMBL and KEGG pathway database.
Accordingly, all genes are classified into three ontology categories (i) biological process (BP), (ii) cellular component, (CC) and molecular function (MF) and pathways when possible. We have taken only the GO/pathway categories that have at least 10 genes annotated. We used Gitools for enrichment analysis and heatmap generation (www.gitools.org). Resulting p-values were adjusted for multiple testing using the Benjamin and Hochberg's method of False Discovery Rate (FDR).

Chomatin immunoprecipitations assays

ChIP was performed essentially as previously described [32] on C10 cells un-induced or induced for 72 hours. To shear chromatin to an apparent length of ,500 bp, chromatin was sonicated using a BioRuptor sonicator (Cosmo Bio Co., Ltd) with either 40 45-s pulses (uninduced cells) or 30 45-s pulses (induced cells) at maximum setting. Input and immunoprecipitated DNA were subjected to Sybergreen Q PCR cycles with specific primers (provided upon request).

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References

1. Parra M (2009) Epigenetic events during B lymphocyte development. Epigenetics 4: 462–468.
2. Mercer EM, Lin YC, Murre C (2011) Factors and networks that underpin earlyhematopoiesis. Semin Immunol 23: 317–325.
3. Adolfsson J, Mansson R, Buza-Vidas N, Hultquist A, Liuba K, et al. (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell 121: 295–306.
4. Yoshida T, Ng SY, Zuniga-Pflucker JC, Georgopoulos K (2006) Early hematopoietic lineage restrictions directed by Ikaros. Nat Immunol 7: 382–391.
5. Stehling-Sun S, Dade J, Nutt SL, DeKoter RP, Camargo FD (2009) Regulationof lymphoid versus myeloid fate 'choice' by the transcription factor Mef2c. Nat Immunol 10: 289–296.
6. Bain G, Maandag EC, Izon DJ, Amsen D, Kruisbeek AM, et al. (1994) E2Aproteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. Cell 79: 885–892.
7. Bain G, Robanus Maandag EC, te Riele HP, Feeney AJ, Sheehy A, et al. (1997) Both E12 and E47 allow commitment to the B cell lineage. Immunity 6: 145– 154.
8. Zhuang Y, Soriano P, Weintraub H (1994) The helix-loop-helix gene E2A isrequired for B cell formation. Cell 79: 875–884.
9. Lin H, Grosschedl R (1995) Failure of B-cell differentiation in mice lacking thetranscription factor EBF. Nature 376: 263–267.
10. Dengler HS, Baracho GV, Omori SA, Bruckner S, Arden KC, et al. (2008) Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. Nat Immunol 9: 1388–1398.
11. Urbanek P, Wang ZQ, Fetka I, Wagner EF, Busslinger M (1994) Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. Cell 79: 901–912.
12. Delogu A, Schebesta A, Sun Q, Aschenbrenner K, Perlot T, et al. (2006) Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. Immunity 24: 269–281.
13. Schebesta A, McManus S, Salvagiotto G, Delogu A, Busslinger GA, et al. (2007) Transcription factor Pax5 activates the chromatin of key genes involved in B cell signaling, adhesion, migration, and immune function. Immunity 27: 49–63.
14. Pridans C, Holmes ML, Polli M, Wettenhall JM, Dakic A, et al. (2008) Identification of Pax5 target genes in early B cell differentiation. J Immunol 180: 1719–1728.
15. Parra M, Verdin E (2010) Regulatory signal transduction pathways for class IIa histone deacetylases. Curr Opin Pharmacol 10: 454–460.
16. Martin M, Kettmann R, Dequiedt F (2009) Class IIa histone deacetylases: conducting development and differentiation. Int J Dev Biol 53: 291–301.
17. Bussmann LH, Schubert A, Vu Manh TP, De Andres L, Desbordes SC, et al. (2009) A robust and highly efficient immune cell reprogramming system. Cell Stem Cell 5: 554–566.
18. Di Tullio A, Manh TP, Schubert A, Mansson R, Graf T (2011) CCAAT/ enhancer binding protein alpha (C/EBP(alpha))-induced transdifferentiation of pre-B cells into macrophages involves no overt retrodifferentiation. Proc Natl Acad Sci U S A 108: 17016–17021.
19. Rodriguez-Ubreva J, Ciudad L, Gomez-Cabrero D, Parra M, Bussmann LH, et al. (2011) Pre-B cell to macrophage transdifferentiation without significant promoter DNA methylation changes. Nucleic Acids Res.
20. Perez-Llamas C, Lopez-Bigas N (2011) Gitools: analysis and visualisation of genomic data using interactive heat-maps. PLoS ONE 6: e19541. doi:10.1371/ journal.pone.0019541
21. Lin YC, Jhunjhunwala S, Benner C, Heinz S, Welinder E, et al. (2010) A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. Nat Immunol 11: 635–643.
22. Swanson BJ, Jack HM, Lyons GE (1998) Characterization of myocyte enhancer factor 2 (MEF2) expression in B and T cells: MEF2C is a B cell-restricted transcription factor in lymphocytes. Mol Immunol 35: 445–458.
23. Wilker PR, Kohyama M, Sandau MM, Albring JC, Nakagawa O, et al. (2008) Transcription factor Mef2c is required for B cell proliferation and survival after antigen receptor stimulation. Nat Immunol 9: 603–612.
24. Khiem D, Cyster JG, Schwarz JJ, Black BL (2008) A p38 MAPK-MEF2C pathway regulates B-cell proliferation. Proc Natl Acad Sci U S A 105: 17067– 17072.
25. Gekas C, Rhodes KE, Gereige LM, Helgadottir H, Ferrari R, et al. (2009) Mef2C is a lineage-restricted target of Scl/Tal1 and regulates megakaryopoiesis and B-cell homeostasis. Blood 113: 3461–3471.

26. Dequiedt F, Kasler H, Fischle W, Kiermer V, Weinstein M, et al. (2003) HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. Immunity 18: 687–698.
27. Kasler HG, Verdin E (2007) Histone deacetylase 7 functions as a key regulator of genes involved in both positive and negative selection of thymocytes. Mol Cell Biol 27: 5184–5200.
28. Kasler HG, Young BD, Mottet D, Lim HW, Collins AM, et al. (2011) Histone deacetylase 7 regulates cell survival and TCR signaling in CD4/CD8 doublepositive thymocytes. J Immunol 186: 4782–4793.
29. Dequiedt F, Van Lint J, Lecomte E, Van Duppen V, Seufferlein T, et al. (2005) Phosphorylation of histone deacetylase 7 by protein kinase D mediates T cell receptor-induced Nur77 expression and apoptosis. J Exp Med 201: 793–804.
30. Navarro MN, Goebel J, Feijoo-Carnero C, Morrice N, Cantrell DA (2011) Phosphoproteomic analysis reveals an intrinsic pathway for the regulation of histone deacetylase 7 that controls the function of cytotoxic T lymphocytes. Nat Immunol 12: 352–361.
31. Parra M, Kasler H, McKinsey TA, Olson EN, Verdin E (2005) Protein kinase D1 phosphorylates HDAC7 and induces its nuclear export after T-cell receptor activation. J Biol Chem 280: 13762–13770.
32. Rafati H, Parra M, Hakre S, Moshkin Y, Verdin E, et al. (2011) Repressive LTR nucleosome positioning by the BAF complex is required for HIV latency. PLoS Biol 9: e1001206. doi:10.1371/journal.pbio.1001206
33. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5: R80.
34. Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy–analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20: 307–315.
35. Kauffmann A, Gentleman R, Huber W (2009) arrayQualityMetrics–a bioconductor package for quality assessment of microarray data. Bioinformatics 25: 415–416.
36. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article3.

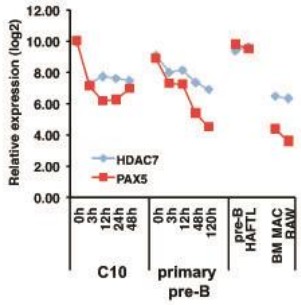

Figure S1. HDAC7 is down-regulated during the transifferentiation of pre-B cells into macrophages. Kinetics of regulation (log2 Affymetrix expression values) of Hdac7 and Pax5 genes in C10 cells and primary pre-B cells transduced with a retroviral vector for inducible expression of C/EBPa. Cells were treated with bestradiol for the times indicated. HAFTL cells, primary pre-B cells, primary bone marrow macrophages and RAW264.7 cells were used as control.

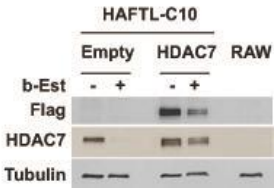

Figure S2. Western blot showing the protein levels of endogenous and exogenously expressed HDAC7 in C10 cells transduced with a untreated or treated with b-estradiol for 72 hours.

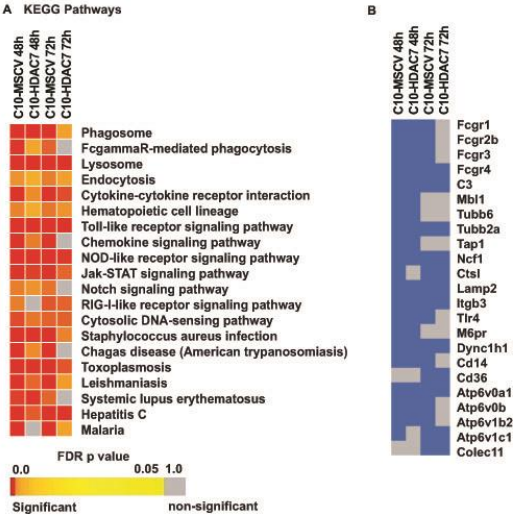

Figure S3. HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. A. Heatmap statistics showing significantly (FDR p-value #0.05) enriched KEGG pathways among the up-regulated genes affected by the reexpression of HDAC7 during transdifferentiation of pre-B cells into macrophages. Colours toward red indicate high statistic significance, yellow indicates low statistic significance, and gray indicates no statistic significance. B. Heat-maps showing observed differentially expressed genes for selected KEGG pathways. Blue colour cell indicates positive events while gray colour indicates that the gene was not observed differentially expressed in that experimental condition.

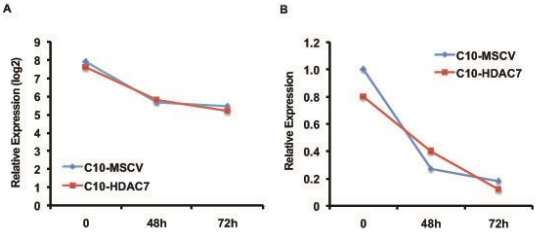

Figure S5. HDAC7 re-expression does not interfere with the down-regulation of Pax5. A. Kinetics of down-regulation (log2 Affymetrix expression values) of Pax5 in C10-MSCV and C10HDAC7 cells un-treated or treated with b-estradiol for the times indicated. B. RT-qPCR validation of the results shown in A.

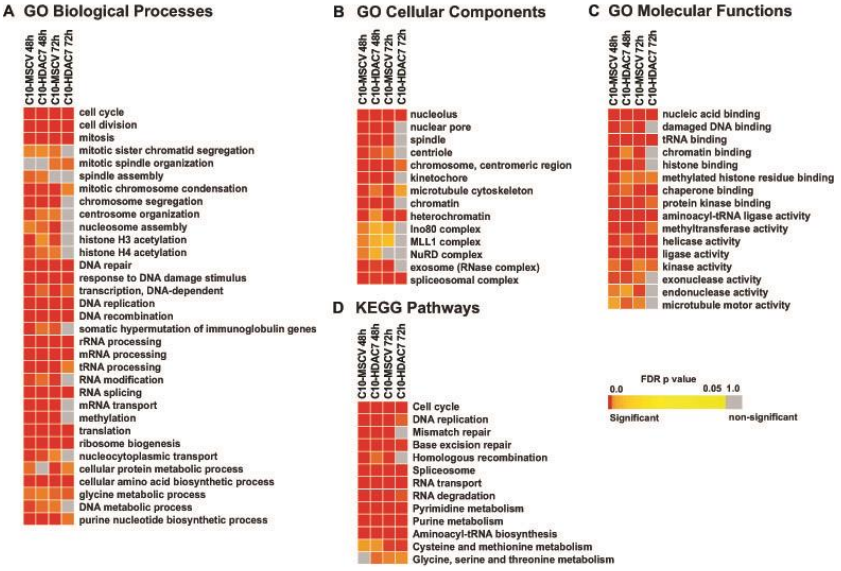

Figure S4. HDAC7 re-expression interferes with the gene transcriptional program of the converted macrophages. Heatmap statistics showing significantly (FDR p-value #0.05) enriched A. GO Biological Processes categories, B. GO Cellular Components categories, C. GO Molecular Functions categories and D. KEGG pathways, among the down-regulated genes affected by the reexpression of HDAC7 during transdifferentiation of pre-B cells into macrophages. Colours toward red indicate high statistic significance, yellow indicates low statistic significance, and gray indicates no statistic significance.

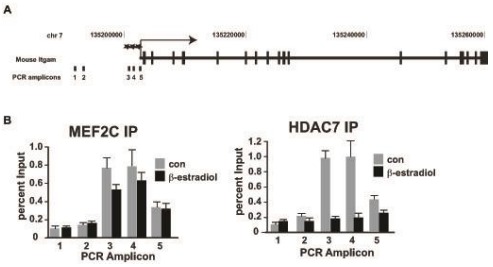

Figure S6. HDAC7 is recruited to the Itgam promoter in pre-B cells. A. Schematic representation of the mouse Itgam locus and amplicons scanned in Chromatin immunoprecipitation experiments by qPCR. Asterisks indicate MEF2 binding sites location. B. Chromatin immunoprecipitation experiments showing the enrichment of HDAC7 and MEF2C to putative MEF2 binding sites on the Itgam gene loci in pre-B cells. Results are presented as percentage immunoprecipitated over input and are representative of three independent experiments.

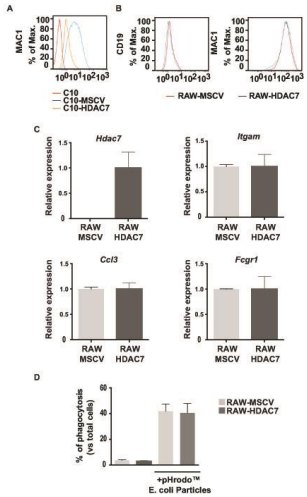

Figure S7. HDAC7 re-expression decreases Mac1 protein levels in the reprogrammed macrophages. A. Histograms for Mac-1 protein levels in reprogrammed machrophages transduced with either an empty vector or a retroviral vector for HDAC7 expression. B. Histograms for Mac-1 and CD19 protein levels in RAW-MSCV and RAW-HDAC7 cells. C. RT-qPCR experiments for gene expression changes for Hdac7, Itgam, Ccl3, and Fcgr1 genes in RAW-MSCV and RAW-HDAC7 cells. D. Capacity of RAW-MSCV and RAW-HDAC7 cells to phagocytose red fluorescence bacteria.

Chapter 6

General Discussion and Future Perspectives

HIV-1 latency represents the main impediments to a cure for HIV-1 infection. The persistence of latent HIV-1 infected cells in patients in whom HIV-1 replication is fully suppressed by c-ART requires that infected patients remain on therapy lifelong. Upon therapy interruption, the activation of a single latently infected cell can result in infection of $CD4^+$ T cells and increase in plasma viremia. The high efficacy of life-long antiviral treatment has converted HIV-1 from a lethal to a chronic disease (Margolis, 2014). However, life-long antiviral therapy can result in side effects, toxicity, and emergence of resistance in infected patients. For example, HIV-1 infected patients on long term c-ART display a higher propensity for cardiovascular problems, increased risk of cardiovascular disease (Mendes et al., 2014), atherosclerosis (Pirs et al., 2014a), dyslipidemia (Leyes et al., 2014), and insulin resistance (Araujo et al., 2014).In addition, life-long antiviral therapy is costly and generates a heavy burden on the medical system. Therefore, efforts are needed not only to manage and treat HIV-1, but too importantly to eradicate the virus from infected patients. Since the persistence of latent HIV-1 reservoir is the main reason that prevents cure, efforts are aimed at targeting and depleting this latent HIV-1 infected reservoir of cells (Johnson et al., 2013; Ruelas and Greene, 2013).

HIV-1 latency results from a block in gene expression of an otherwise replication competent HIV-1 virus. The molecular mechanisms that are involved in transcriptional silencing of the HIV-1 virus resulting in latency are multifactorial and incompletely understood. Mechanisms such as site of genomic integration, orientation of integration with respect to host genes, local activity of host transcription factors which determine HIV-1 basal transcription levels, the switch to TAT mediated transcription elongation, etc., have been implicated in contributing to the establishment and maintenance of HIV-1 latency. Thus, latency establishment and maintenance is a complex multifactorial phenomenon, and the resulting reservoirs need to be targeted and purged using multifactorial strategies.

The current approach in the development of a curative HIV-1 treatment is termed “shock and kill”. The “shock” phase refers to pharmacological attempts aimed at activating latent virus using latency reversing agents (LRAs). Activation of latent HIV-1 infected cells will result in transcription and translation of the viral genome, such that the cell now becomes recognizable. The “kill” phase refers to the elimination of the activated virus by viral cytopathic effects, or the presence of a charged effective immune system or host cytolytic T lymphocytes (CTL) immune responses. Shock and kill in the presence of effective or intensified c-ART also prevents new rounds of infection. Studies exploring shock and kill approaches have shown that monotherapy is not very efficient, for reactivation of latent reservoirs (Siliciano and Siliciano, 2013a). The concept of combination therapy for re-activation purposes seems to have the most potential in reactivation of latent HIV-1 in a substantial proportion of latency infected cells. Targeting different pathways and molecular mechanisms at the same time, with different HIV-1 activators in combination with inhibition

of different HIV-1 repressors would allow for synergistic transcriptional effects to emerge in activation of the latent HIV-1 promoter. Small molecule drugs such as the protein kinase C activators prostratin, and bryostatin, inhibitors of BET proteins such as JQ1, as well as molecules modifying transcriptional repressive chromatin modifications such as DNA methylation, histone methylation, and histone de-acetylation are proposed as putative candidates for inducing the expression of latent HIV (Archin and Margolis, 2014; Siliciano and Siliciano, 2013b). Although different LRAs have been tested for reactivation of latent provirus, it is not clear which of these compounds more efficiently targets latent HIV-1 *in vivo*.

Upon identification and development of a successful combinatorial treatment for re-activation of latently infected cells, the next major issue is whether the cells will be eliminated following reversal of latency. Several studies have shown that, latent HIV-1 infected cells that were re-activated were not cleared because of defects in the HIV-specific CTL response (Siliciano and Siliciano, 2013a). Importantly, these defects could be reversed by *in vitro* stimulation of the $CD8^+$ T cells with GAG peptides, suggesting that it may be necessary to combine latency reversing strategies with therapeutic vaccination (Siliciano and Siliciano, 2013a). In other words, HIV-1 reactivation needs to take place in presence of effective c-ART able to block new rounds of infection and, most importantly, in presence of a functional immune system able to eliminate latently infected cells following reactivation.

The research described in this thesis focuses on the shock phase of shock and kill strategies aimed at HIV-1 eradication. This work aims to delineate the molecular mechanisms involved in the establishment and maintenance of HIV-1 transcriptional latency, and re-activation of latent virus. Specifically the aim is to identify and characterize new factors and signaling pathways involved in HIV-1 transcription regulation. The identification of new molecular targets in HIV-1 transcription would open the possibility for small molecule inhibition and development of therapeutics to re-activate latent HIV-1, a necessary first step, which will be followed by their safe elimination from patients in the presence of c-ART.

In **Chapter 2** we identified and characterized a new mechanism for the repression of HIV-1 transcription at the latent HIV-1 LTR, achieved by the BAF chromatin-remodeling complex. We found that, repression of the latent HIV-1 virus is an active process driven by ATP hydrolysis. The BAF ATP-dependent chromatin remodeling complex positions a repressive nucleosome Nuc-1 (Van Lint et al., 1996; Verdin et al., 1993; Verdin and Van Lint, 1995), over energetically suboptimal DNA sequences immediately downstream of the transcription start site resulting in establishment and maintenance of HIV-1 latency. Although, transcription on the HIV-1 LTR is tightly regulated by its chromatin organization, but also depends on cellular transcription factors and chromatin modifications for either its activation or establishment of latency (Pereira et al., 2000). In the early phase of HIV-1 infection, TAT independent phase, HIV-1 infection is randomly determined by local chromatin microenvironment at the integration

sites of virus, and balance between local concentrations of transcription activators or repressors regulates HIV-1 promoter transcription (Jordan et al., 2001; Schroder et al., 2002; Weinberger et al., 2008).

Upon activation, TAT first recruits acetyltransferases and acetylates itself and histone tails on the HIV-1 LTR (Benkirane et al., 1998; Deng et al., 2000; Kiernan et al., 1999; Marzio et al., 1998; Ott et al., 1999). We find that acetylated TAT recruits PBAF, a biochemically distinct SWI/SNF complex, which shares most common subunits with BAF, but can be distinguished by the presence of PBAF specific subunits, to the HIV-1 promoter *in vivo*. Thus, the two biochemically distinct SWI/SNF chromatin-remodeling complexes BAF and PBAF display function opposing each other on the HIV-1 LTR. BAF is repressive to basal HIV-1 LTR activity by positioning the repressive Nuc-1, and PBAF participates in the TAT mediated transcriptional activation of the HIV-1 promoter.

According to the DNA sequence of the HIV-1 LTR, the sequences of the hypersensistive site (DHS-1) possess the highest histone binding score, or propensity for nucleosome formation. However, this area in known *in vivo* to be largely nucleosome –free and sensitive to digestion by endonucleases. The strict positioning of Nuc-1 over suboptimal sequences raises the question as to the reason behind this energetically costly nucleosomal structure. One possibility is that BAF actively moves the nucleosome away from the hyper sensitive site (DHS-1) on the HIV-1 LTR to allow for binding of cellular transcription factors, whose binding sites are present throughout the DHS-1. For example, consensus sites of NFκB, NFAT and SP1, which are critical for basal HIV-1 LTR transcription, exist on DHS-1 of HIV-1 promoter (Kim et al., 1993). Interestingly our high resolution MNase nucleosomal mapping of the LTR showed that DHS-1 is not devoid of nucleosomes as previously thought, but contains a loosely positioned nucleosome. Upon BAF depletion, nucleosome occupancy over this region was increased, consistent with a role for BAF in actively displacing a preferred nucleosome over this DNA region.

How is the BAF complex recruited to the HIV-1 promoter? The mechanism by which the repressive BAF complex is recruited to the HIV-1 promoter remains unclear. SWI/SNF complexes are thought to be recruited and function in a gene specific manner, recruited via interaction with sequence specific transcription factors during transcription regulation. Studies on HIV-1 transcription have described many binding site for cellular transcription factors including the repressive transcription factors YY1 and LSF1 (He and Margolis, 2002). YY-1 is an attractive candidate transcription repressor that could mediate BAF recruitment to the HIV-1 LTR. It has shown that, YY1 binds to the HIV-1 LTR under basal conditions and is displaced after TAT expression (Mahmoudi et al., 2006). Pleiohomeotic (PHO), the Drosophila homologue of YY1, was shown to physically interact with Drosophila SWI/SNF and direct recruitment of the SWI/SNF complex to target genes (Mohd-Sarip et al., 2002). Future studies

will determine whether YY1 or other LTR transcriptional repressors are responsible for tethering BAF to the latent HIV-1 LTR.

Our work identifies the catalytic subunit of the BAF complex, BRG1, as well as other BAF complex subunits as putative molecular targets in strategies for activation of the latent HIV-1. Depletion of BRG1 or the BAF250 subunit of the BAF complex by siRNA resulted in de-repression of latent HIV-1 and significant increase in HIV-1 expression. Recently, a screen for small molecule inhibitors of the SWI/SNF complex in ES cells has identified a panel of BAF inhibitors. Given the strong role observed for BAF in HIV-1 transcriptional repression, it will be of great interest to test small molecule BAF inhibitors for their potential to activate latent HIV-1, and importantly to synergize with small molecule HDAC inhibitors currently under clinical investigation.

In **Chapter 3** we explored the role of the Wnt signaling pathway in regulation of transcription at the latent HIV-1 LTR. In addition to chromatin remodelers, many signaling pathways and cellular transcription factors and co-factors are known to play a role in the regulation of HIV-1 LTR activity, and could be putative therapeutic targets for activation of latent HIV-1. In this study, we activated the Wnt pathway using three different strategies *in vitro* HIV-1 latent cell models. We treated latently infected cells with the natural Wnt ligands Wnt3A and Rspondin, we exogenously expressed a constitutively active form of β-catenin, and we finally treated the latent cells with small molecule inhibitors of the enzyme GSK3β, an essential component of the β-catenin destruction complex. Using all three strategies, the Wnt pathway could be specifically activated in all *in vitro* latent cell models, and importantly, HIV-1 *GAG* expression was induced in the latent models in response to Wnt activation.

Several studies have indicated a relationship between LEF1/β-catenin/Wnt and HIV-1 replication. While some studies described an activating role for LEF1 and Wnt signaling in HIV-1 transcription regulation, other studies have implicated Wnt signaling as repressive to HIV-1 transcription and replication. Early studies on regulation of HIV-1 expression first identified LEF1 as a transcription factor, which specifically bound to and was purified from the HIV-1 LTR, and activated HIV-1 transcription in *in vitro* reconstituted models (Jones and Peterlin, 1994; Sheridan et al., 1995; Waterman and Jones, 1990). LEF1 activated the HIV-1 enhancer in Jurkat cells and counteract nucleosomal repression *in vitro* (Sheridan et al., 1995). siRNA depletion of AXIN1, the core scaffold component of the β-catenin destruction complex, results in activation of HIV-1 (Kameoka et al., 2009; Kameoka et al., 2007), ascribing a positive role for Wnt signaling in HIV-1 transcription. Although, we and others have shown the role of LEF1/β-catenin/Wnt in HIV-1 activation, several studies have described a repressive role for LEF1/β-catenin/Wnt on HIV-1 transcription and replication mainly in astrocytes (Carroll-Anzinger et al., 2007; Henderson et al., 2012a; Henderson et al., 2012b; Narasipura et al., 2012; Wortman et al., 2002). β-catenin signaling was also found to inhibit HIV-1 replication in

PBMCs (Kumar et al., 2008). This controversy for the role of β-catenin/Wnt signaling in HIV-1 regulation may be explained by the fact that different cell types and different phases of the HIV-1 life cycle were examined. The four distinct TCF/LEF transcription factor family members are expressed in a tissue specific manner and have been shown to be functionally distinct (Mao and Byers, 2011). For example in T cells, TCF1 and LEF1 are co-expressed, while in astrocytes TCF4 is predominantly expressed, something that could potentially explain the observed repressive role for Wnt in HIV-1 transcription is astrocytes. Our results confirmed the specific expression of TCF1 and LEF1 in $CD4^+$ T cell lines and primary memory $CD4^+$ T cells, the main latent HIV reservoirs, using Western blot and mRNA expression analysis. We also fine, using chromatin immunoprecipitation assays, that LEF1, but not TCF1 binds to the HIV-1 LTR Nuc-1 in response to Wnt stimulation. Thus, our data supports the notion that LEF1 is the main TCF/LEF member involved in HIV-1 transcription in $CD4^+$ T cells.

In this study, we focused on Lithium, as a small molecule inhibitor of GSK3b, to induce Wnt activation. Lithium is already in use in clinical practice in treatment of bipolar disorder with known pharmaco-kinetic properties and safety and toxicity profiles (McKnight et al., 2012). Therefore it would potentially be easily incorporated into a therapeutic regimen. We found treatment with Lithium induced significant HIV-1 reactivation, as demonstrated by increase both in GAG mRNA levels and in the expression of LTR reporter genes in latently infected T cell lines. Several studies have reported the limited HIV-1 activation following Lithium treatment alone in chronic HIV-1 patients, which may reflect the inability of chronic HIV patients' immune system to effectively eliminate HIV-1 after activation (Puertas et al., 2014). This finding showed Lithium treatment alone is not sufficient to activate the heterogeneous pool of latently infected cells *in vivo*.

Latency is a complex and multifactorial phenomenon. Multiple genetic and epigenetic mechanisms taking place in different cell types and body compartments are at play to establish latency. The observation that Lithium alone may not be effective or sufficient in reversing latency in patients points to the importance of combinatorial therapy in the context of *shock and kill* strategies. Examination of the regulation of HIV-1 expression reveals that various cellular pathways and transcription complexes participate to regulate activity of the HIV-1 LTR and might serve as molecular targets for compounds to activate latent HIV-1 (Van Lint et al., 2013). We tested functional synergism between Wnt activators and HDAC inhibitors in activation of latent HIV-1. We find that Wnt activation of latently infected cells is significantly and synergistically increased when co-treated with the HDACi's SAHA or VPA. At the molecular level, this effect was demonstrated by synergistic recruitment of the downstream effectors of Wnt, LEF1 and β-catenin to the LTR Nuc-1 as well as synergistic enrichment of acetylated histones at the LTR Nuc-1 region when latent cells were treated with both SAHA and Wnt agonists. As well, we find that Lithium treatment in combination with HDACi's results in dramatic remodeling of the latent HIV-1 LTR leading to re-structuring

the repressive Nuc-1 as shown by high resolution MNase nucleosomal mapping studies. Thus, we provide mechanistic evidence for the functional synergism we see in HIV-1 activation, accomplished by these molecules, which target distinct pathways in HIV-1 transcription.

Our results support a cooperative model for the observed synergism between HDACi's and Wnt activators: Latent HIV-1 is characterized by chromatin repression dependent on sites of integration and chromatin environment, and the latent LTR is characterized by hypo acetylated histones, generated by the enzymatic activity of HDACs. The hypo acetylated histones form a compact and rigid chromatin structure which inhibits gene expression (Pearson et al., 2008; Tyagi et al., 2010). Inhibition of HDACs by HDACi's cause acetylation of histones which resulting in relaxation of chromatin structure allowing DNA access to sequence specific transcription factors and transcription activation. In the latent state, LEF1 because of dense and rigid chromatin structure may be unable to access its consensus sequences. This access is increase in the presence of HDAC inhibition, which increases acetylation at the LTR, allowing easier access of LEF1 to its sites on the HIV-1 LTR. In the presence of Wnt signaling, β-catenin complexes with LEF1, increasing its affinity for DNA and recruits a number of transcriptional co-activators including the acetyl transferases P300/CBP to the HIV-1 LTR resulting in further LTR histone acetylation and chromatin remodeling as demonstrated by removal of the repressive Nuc-1, and synergistic transcriptional activation.

Monotherapy in the context of latency reversal drugs has some limitations, which include toxicity, and lack of specificity, and generation of resistance. Many drugs including HDACi's target cellular proteins with important pleiotropic functions. To increase the target specificity and efficiency of drugs in a future purge and kill therapeutic strategy, it will be essential to target pathways, which when targeted combinatorially would confer specificity for the HIV-1 promoter. Our work identifies a positive role for Wnt pathway agonists in activation of latent HIV. Moreover, our data showed that Lithium and HDACi's are able to synergistically activate HIV-1, via a synergistic mechanism that is detected strongly at the molecular level. More studies are needed to clarify the complex interplay between the Wnt pathway, immune cells and HIV-1. Importantly, future studies will determine the effect of Lithium and other Wnt agonists alone and in combination with HDACi's and other putative LTR activators in activation of latent HIV-1 in HIV-1 infected patients.

In **Chapter 4** we examined the molecular mechanism for the observed in vivo functional specialization displayed by the highly homologous TCF1 and LEF1 proteins in T cell leukemia. Several studies have shown that, while they contain almost identical HMG box DNA binding domains, TCF1 and LEF1 have distinct and specific functions in T cells (Mao and Byers, 2011). Genetic studies on mice have shown opposing and cooperative roles in T cells played by TCF1 and LEF1, which function as a tumor suppressor and proto-oncogene respectively (Staal and Clevers, 2012; Tiemessen et al., 2012; Tiemessen and Staal, 2013).

We first examined whether the distinct roles played by TCF1 and LEF1 is accomplished by their presence in distinct transcriptional complexes, together with functionally distinct binding partners. Using a Mass Spec approach we identified the protein binding partners of TCF1 and LEF in the absence and presence of Wnt stimulation in SupT1 T-ALL cells. Using this approach we identified many novel as well as previously known interactors for TCF1 and LEF1 in the Wnt induced and uninduced conditions. Of these novel interactors, many were shared between TCF1 and LEF1, but others were uniquely present in either the LEF1 or TCF1 complexes.

To identify LEF1-specific and TCF1-specific target gene profiles in SupT1 leukemic cells, we performed RNA sequencing analysis of cells depleted of either TCF1 or LEF1 in the Wnt induced or uninduced condition. Interestingly, we found that depletion of TCF1 or LEF1 in the absence of Wnt stimulation resulted in de-repression of the majority of the Wnt target genes, consistent with a repressive role for these factors in Wnt target gene regulation in the absence of Wnt. This observation also argued against redundancy between TCF1 and LEF1 in repression of Wnt targets as depletion of either gene resulted in de-repression. In the presence of Wnt, depletion of LEF1 abrogated activation of a majority of Wnt target genes, consistent with a role for LEF1 in mediating β-catenin Wnt activation of targets. Interestingly, depletion of TCF1 resulted in abrogation of Wnt induction in a subset of Wnt target genes, distinct from the LEF1 regulated target genes, while expression of other genes were unaffected. Thus, TCF1 and LEF1 display target gene specificity in Wnt-mediated gene activation.

Interestingly, our results showed that TCF1 interacted specifically with several known repressors such as SIN3A, CHD4, and CHD5, while LEF1 was found in complex mainly with proteins with known transcriptional activating functions such as BRG1 and SMC1. The identification of a large number of binding partners for LEF1 and TCF1 points to the likely presence of different sub-complexes of TCF1 and LEF1, together which may be recruited distinctly to target genes, and which may relay distinct functions. For example, TCF1, in the context of a complex containing CHD4 and SIN3A may function as a repressor of some target genes, while when bound to activating partners such as MYBBP1a can play an activating role in transcription of another subset of target genes. The identification of specific partners for TCF1 and LEF1 also support the notion that these factors bind cooperatively with other transcription factors and co-factors to target genes. This cooperativity of the complex in binding to TCF/LEF sites in the context of a combination of binding sites can confer additional binding specificity and affinity for specific TCF1 and LEF1 target genes in leukemic T cells. Future studies examining the genome wide recruitment and gene targets of these binding partners will help define their specificity for Wnt target gene regulation and cooperativity with TCF1/LEF1.

Focusing on two Wnt target genes identified by RNA sequencing analysis, we probed enrichment of TCF1/LEF1 and β-catenin to the regulatory regions of these genes by chromatin immunoprecipitation assays in the presence or absence of Wnt stimulation. We find specific enrichment pattern of LEF1 and TCF1 on regulatory regions of the Wnt target genes, *BMP4* and *ROMO1*. On *ROMO1*, while LEF1 is present in the absence of Wnt, TCF1 enrichment is induced specifically in response to Wnt. On *BMP4* on the other hand the opposite enrichment profile is observed. While TCF1 is enriched on *BMP4* in the absence of Wnt, LEF1 is specifically recruited in response to Wnt stimulation. To better characterize the recruitment of TCF1 and LEF1 to their target genes in the presence or absence of the Wnt signal, and to examine this potential differential binding profile on other targets, it is critical that TCF1/LEF1 enrichment is examined on a genome wide scale. It will also be interesting to complement ChIP-sequencing studies probing enrichment of TCF1/LEF1 and β-catenin with those of other novel putative cofactors identified to determine their overlap in genome-wide binding profiles. Future studies combining genome wide gene expression analysis in cells depleted of candidate novel factors in the presence or absence of Wnt signaling will also contribute to clarify the role of not only the co-expressed TCF1 and LEF1 transcription factors but also their putative novel binding partners in T-ALL leukemic cell lines.

Chapter 5 described the role of HDAC7 as a transcriptional repressor of undesirable genes in B cells. We found that depletion of HDAC7 in pre-B cells cause the de-repression of macrophage target genes. Functionally, the presence of HDAC7 interferes with the acquisition of key functional features of the converted macrophages. These macrophages, lacking expression of Mac-1, are not able to function normally in phagocytosis and responding to endotoxin. The mechanistic future of HDAC7 is interaction with MEF2C transcription factor and recruitment to the MEF2C sites at regulatory regions of macrophage target genes. In addition to the interaction with MEF2C, the catalytic activity of HDAC7 is also necessary to inhibit macrophage target genes. Our results demonstrated that HDAC7 is expressed in both the fetal and adult pre-B cells suggesting that it might play a role in both types of B lymphopoiesis.

A very important question raised from our founding is, why is HDAC7 specifically expressed in pre-B cells and not macrophages? Our results described that HDAC7 is a main transcription inhibitor of unfit genes in pre-B cells. It has been shown, that HDAC7 is a target for important transcription factors in pro-B cells such as FOXO1, E2A, and EBF indicating that HDAC7 is a key regulator of development in B cells as well as T cells (Lin et al., 2010). Our ChIP data showing interaction of HDAC7 with MEF2C and recruitment to the MEF2C binding site on promoters of macrophage target genes is consistent with this notion. MEF2C is a member of the MEF2 family transcription factors, which have a role in B cell development and function (Swanson et al., 1998). Several studies have shown, MEF2C to be involved in regulation of B cell proliferation and survival after activation and p38 MAPK signaling (Khiem et al., 2008;

Wilker et al., 2008), expression at earlier stages of lymphocyte development (Gekas et al., 2009; Stehling-Sun et al., 2009), and in the cellular choice towards the lymphoid versus the myeloid lineage in lymphoid-primed multipotent progenitors (LMPPs) (Gekas et al., 2009). Our results showed overlap between MEF2C target genes in LMPPs and our tested HDAC7 target genes in pre-B cells. Mutations in HDAC7 block interaction with MEF2C and suppress the up-regulation of macrophage genes during the reprogramming process. These data all together, described the role of HDAC7 as a MEF2C transcriptional co-repressor who's responsible for the silencing of myeloid genes in B cells and lymphoid precursors.

Previous studies on role of HDAC7 in T cells have demonstrated that, HDAC7 expression and functions are involved in T cells development and differentiation (Parra et al., 2005; Parra et al., 2007). It has been shown in specific type of T cells, cytotoxic T lymphocytes (CTLs), phosphorylation of HDAC7 and its nucleo-cytoplasmic shuttling is regulated by signal transduction (Dequiedt et al., 2003; Kasler and Verdin, 2007; Kasler et al., 2011; Navarro et al., 2011; Parra et al., 2005). In resting state of T cells, HDAC7 is in the nucleus and represses the expression of genes involved in survival or apoptosis of the cells, while in active state of T cells HDAC7 becomes phosphorylated and translocates to the cytoplasm, and in the nucleus target genes can be activate (Dequiedt et al., 2003; Dequiedt et al., 2005; Kasler and Verdin, 2007; Parra et al., 2005). Phosphorylation and dephosphorylation of HDAC7 and its nucleo-cytoplasm shuttling in T cells happens according to requirement of silencing or activation at different stages of T cell development. In contrast, at the branching points where cellular lineage choice has to be made, e.g. lymphoid versus myeloid, HDAC7 might be regulated at the expression level. In (Dequiedt et al., 2005) ages of lymphocytes development, also function as a lineage-specific transcriptional repressor responsible for maintaining the identity of lymphocytes by silencing lineage inappropriate genes.

Future perspectives in HIV therapy:

Targeting the latent reservoir for activation is one of the major challenges toward eradication of the HIV-1 virus. While highly active antiretroviral therapy (HAART) results in dramatic decrease in HIV-1 replication to below limits of detection and reduces the viral transmission, it does not eliminate the HIV-1 latent reservoirs (Salgado et al., 2011; Shen and Siliciano, 2008; Spivak and Siliciano, 2010). A small fraction of resting $CD4^+$ T cells containing transcriptionally silent virus persist despite HAART and can become activated if HAART is interrupted (Haggerty et al., 2006; Siliciano and Siliciano, 2004). Importantly, HAART is not accessible to many populations in developing countries. Even with access to HAART significant challenges remain in the field, because life-long regimens are expensive and accompanied by side effects, propensity for formation of cardiovascular disease (Mendes et al., 2014), atherosclerosis (Pirs et al., 2014b), dyslipidemia (Leyes et al., 2014) and insulin resistance (Araujo et al., 2014). Therefore, there is strong need for novel therapeutic approaches to

eradicate HIV-1 from infected patients (Katlama et al., 2013; Shan and Siliciano, 2013). While understanding the mechanisms of HIV-1 latency in resting $CD4^+$ T cells is critical to develop approaches to successfully eliminate latent HIV-1, there are still numerous scientific challenges ahead in the path toward developing strategies that can effectively eradicate the latent reservoirs.

1- Model systems to study HIV-1 latency

One of the major challenges in the field is the availability of relevant model systems to study HIV-1 latency and reactivation. Although, *in vitro* cell lines are helpful in the molecular study and characterization of HIV-1 latency (Jordan et al., 2003), they do not recapitulate the quiescent state of primary resting memory cells, the major cell type harboring latent HIV-*1 in vivo*. Development of *ex vivo* primary cell models of HIV-1 latency therefore has significantly advanced the understanding of HIV-1 latency and facilitated the development of techniques to eliminate the viral reservoir. Two types of primary *ex vivo* CD4 model systems for HIV-1 latency have been developed based on experimentally mimicking the two routes by which latent HIV infections are thought to occur *in vivo*. The first model is based on the fact that HIV-1 can directly infect a subset of resting $CD4^+$ T cells (Cameron et al., 2010; Swiggard et al., 2005). In this model HIV-1 can integrate into resting $CD4^+$ T cells in the absence of activating stimuli, and a percentage of resting memory T cells can produce HIV-1 when stimulated. In the "pre-activation latency" model latent HIV-1 infection occurs in resting $CD4^+$ T cells, and may provide a model for HIV-1 latency. The second model is based on infection of partially activated $CD4^+$ T cells that revert to a resting state (Bosque and Planelles, 2009). In the "post-activation latency" model, analysis of the T cell–signaling pathways and characterization of the HIV-1 LTR elements that are involved in reactivation of HIV-1 in memory $CD4^+$ T cells would be possible. Other primary cell models were generated to improve the infection efficiency and half-life of primary cultures in these systems (Lassen et al., 2012; Saleh et al., 2007; Yang et al., 2009). Recent studies on reactivation efficiency in the different primary systems indicated that, they respond differently to reactivation agents (Shan and Siliciano, 2013; Spina et al., 2013). Therefore, future model systems are needed that mimic all the *in vivo* relevant cell types and sub-populations which are involved in HIV-1 latency.

2- Detection of latent reservoir

Another major challenge in the field concerns current technical limitations toward detection of the latent reservoir. The frequency of resting $CD4^+$ T cells that become latently infected is very low *in vivo* and varies between individuals. Only one per million $CD4^+$ T cells is estimated to carry latent provirus (Chun et al., 1997). Because of the rarity of latently infected cells in patients on HAART, large amounts of patients' material is needed to detect the latent reservoir (Siliciano and Siliciano, 2005). Highly sensitive PCR assays for HIV-1 DNA have already been generated. Some of these assays can distinguish between integrated proviruses and unintegrated HIV DNA (O'Doherty et al., 2002; Yu et al., 2008). A main problem with DNA

PCR-based assays is that they cannot distinguish between replication competent and either defective (Sanchez et al., 1997), or hypermutated (Kieffer et al., 2005) or silenced forms of the viral genome. Therefore, measurement of the viral RNA is a better way to quantitate reactivated functional virus. The first method to quantitate reactivation of the latent viral reservoirs is co-culture assay (Laird et al., 2013; Siliciano and Siliciano, 2005). In this method, PBMCs are collected from HIV-1 infected individuals, resting $CD4^+$ T cells are purified, and are plated in serial dilutions. Then they are activated in the presence of donor $CD4^+$ T cells, the targets for HIV-1 infection, to allow outgrowth of replication competent HIV-1 released from infected cells in which latency has been reversed. The viral RNA or proteins such as P24 are detected in cell supernatant. Increase in HIV-1 RNA in plasma or cell associated HIV-1 RNA (CA), is also an indication of activation of transcription of HIV-1 which is believed to directly reflect the reactivation of latent HIV-1 reservoir *in vivo* (Archin et al., 2012; Fischer et al., 2004; Lewin et al., 1999; Pasternak et al., 2009). However, the increased RNA may be the result of transcription of an otherwise defective (replication incompetent) virus, which expresses GAG, but perhaps is defective in replication. The quantification of viral RNA is performed by real-time reverse transcription PCR (RT-PCR) coupled to nested PCR (Pasternak et al., 2008) or droplet digital PCR assays (White et al., 2012). The PCR techniques have limitations due to small differences at the lower quantification ranges, and the false positive signals. Recently, a novel RT-PCR assay were developed using a reverse primer that hybridizes with the poly(A) tail of HIV-1 mRNAs. This assay quantifies spliced HIV-1 RNA and not read-through transcripts with high specificity and sensitivity (Shan and Siliciano, 2013). Given the limitations of the described assays it is unclear which assay should be used to monitor HIV-1 reservoirs in clinical trials or eradication strategies. Therefore, better more specific techniques are needed in order to quantitate and detect the latent reservoir and its response to LRAs.

3- Activation of latent reservoir

Another major challenge in the field is identification and testing of potent and effective LRAs. One of the most promising approaches to cure HIV-1 is to purge its latent cellular reservoirs by activating the transcriptional activity of latent HIV-1 provirus in infected $CD4^+$ T cells. Reactivation results in the generation of activated cells that either die from the direct cytopathic effects of the virus or are cleared by host mechanisms. Complex molecular mechanisms regulate expression of provirus in latently infected cells. Therefore, it is necessary to target responsible mechanisms, the signaling pathways and the molecules involved in silencing the HIV-1 transcription to develop strategies to reverse HIV-1 latency. Pharmacological agents that stimulate the protein kinase C pathway have been shown to induce HIV-1 virus replication. Protstratin and Bryostatin 1, PKC–NF-κB pathway activators can synergize with other drugs to reactivate virus, and promote cellular apoptosis (DeChristopher et al., 2012; Perez et al., 2010). Recent studies have identified small molecules capable of reactivating HIV-1 gene expression (Korin et al., 2002; Xing et al., 2012; Xing et al., 2011). Interest has also been focused on inhibiting histone deacetylases (HDAC), chromatin

modifying complexes, which block HIV-1 DNA transcription by preventing acetylation of chromatin, resulting in a compact repressive structure not permissive to transcription. Two candidate HDAC inhibitors (HDACi), Valproic acid (VPA) and Vorinostat (SAHA), have already been under clinical investigations. Despite initial promising results, further studies on VPA failed to find any benefit on the reactivation and decay of the latent reservoir (Archin et al., 2010; Routy et al., 2012; Siliciano et al., 2007). Recent studies showed more benefit in using SAHA in reactivating virus in *in vitro* models of latency. Other studies using HDAC inhibitors such as FK288 (romidepsin), LBH589 (panobinostat), ITF2357 (givinostat), and PXD101 (belinostat) showed greater potency than Vorinostat/SAHA in reactivating virus in latently infected cell lines (Sahu and Cloyd, 2011). Although, each of these LRAs can reverse latency, effective reactivation of latent reservoir will likely require multi-drug combination. Combination therapy, using HIV-1 transcription activators and inhibition of HIV-1 repressors at the same time has the potential to more efficiently and potently activate the latent HIV-1 provirus than monotherapy (Boehm et al., 2013; Pandelo Jose et al., 2014; Wortman et al., 2002; Ying et al., 2012). Several studies have indicated that the use of multiple latency reversing compounds can result in synergistic activation of HIV-1 transcription which would allow complete activation of the latent reservoir (Abreu et al., 2014; Micheva-Viteva et al., 2011; Pandelo Jose et al., 2014; Ying et al., 2012). In synergistic conditions, lower concentrations of each compound will be necessary to induce HIV-1 activation, which is an important therapeutic consideration. Under synergistic conditions, the toxic side effects and pleiotropic consequence of each molecule on gene expression will be limited and can provide a level of specificity for activation of the HIV-1 LTR.

In this thesis we identified potential new molecular targets for therapeutic approaches aimed at latency reversal; we identified and characterized the BAF chromatin remodeling complex **(Chapter 2)** which is a large multiprotein complex containing the ATP-dependent BRG1 component as a repressor of HIV-1. Small molecule inhibition of the activity of this complex is an attractive approach in HIV-1 latency reversal. We also demonstrated that the Wnt pathway has an activating role in HIV-1 transcription **(Chapter 3)**. Various cellular proteins, from the Wnt receptors to components of the destruction complex, to the activating transcriptional complexes are putative candidates for small molecule regulation to activate the Wnt pathway and thus latent HIV-1. Future studies thus will be critical in the identification and testing of candidate molecules which inhibit BAF and activate the Wnt pathways.

4- Elimination of latent reservoir

Upon identification and development of a successful combinatorial treatment for re-activation of latently infected cells, the next major issue is whether the cells will be eliminated following reversal of latency. Several studies have shown that, latent HIV-1 infected cells that were re-activated were not cleared because of defects in the HIV-specific CTL response (Siliciano and Siliciano, 2013b). Importantly, these defects could be reversed by *in vitro* stimulation of the $CD8^+$ T cells with GAG peptides, suggesting that it may be necessary to

combine latency reversing strategies with therapeutic vaccination (Siliciano and Siliciano, 2013b). Therapeutic vaccines can restimulate CD8 cytotoxic T lymphocyte in order to prevent or control virus rebound and the re-establishment of the latent infection in $CD4^+$ T cells after treatment interruptions (Lichterfeld et al., 2012; Vanham and Van Gulck, 2012). In other words, HIV-1 reactivation needs to take place in presence of effective c-ART, which would block new rounds of infection and, most importantly, in presence of a functional effective immune system able to eliminate latently infected cells following reactivation. Finally, several studies have shown that gene therapy might be a promising approach to delete the virus from infected cells or generate cells resistant to HIV-1 infection (Mitsuyasu et al., 2009; van Lunzen et al., 2007; Wheeler et al., 2011). However, the safety and, efficacy of gene delivery to specific cells and tissues, and access to these treatments are major limitations for these approaches to eradicate HIV-1 infection, which needs further investigation.

Refrences:

C.M. Abreu, S.L. Price, E.N. Shirk, R.D. Cunha, L.F. Pianowski, J.E. Clements, A. Tanuri and L. Gama, Dual role of novel ingenol derivatives from Euphorbia tirucalli in HIV replication: inhibition of de novo infection and activation of viral LTR, *PloS one* 9 (2014), p. e97257.

S. Araujo, S. Banon, I. Machuca, A. Moreno, M.J. Perez-Elias and J.L. Casado, Prevalence of insulin resistance and risk of diabetes mellitus in HIV-infected patients receiving current antiretroviral drugs, *European journal of endocrinology / European Federation of Endocrine Societies* 171 (2014), pp. 545-554.

N.M. Archin, M. Cheema, D. Parker, A. Wiegand, R.J. Bosch, J.M. Coffin, J. Eron, M. Cohen and D.M. Margolis, Antiretroviral intensification and valproic acid lack sustained effect on residual HIV-1 viremia or resting CD4+ cell infection, *PloS one* 5 (2010), p. e9390.

N.M. Archin, A.L. Liberty, A.D. Kashuba, S.K. Choudhary, J.D. Kuruc, A.M. Crooks, D.C. Parker, E.M. Anderson, M.F. Kearney, M.C. Strain, D.D. Richman, M.G. Hudgens, R.J. Bosch, J.M. Coffin, J.J. Eron, D.J. Hazuda and D.M. Margolis, Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy, *Nature* 487 (2012), pp. 482-485.

N.M. Archin and D.M. Margolis, Emerging strategies to deplete the HIV reservoir, *Current opinion in infectious diseases* 27 (2014), pp. 29-35.

M. Benkirane, R.F. Chun, H. Xiao, V.V. Ogryzko, B.H. Howard, Y. Nakatani and K.T. Jeang, Activation of integrated provirus requires histone acetyltransferase. p300 and P/CAF are coactivators for HIV-1 Tat, *The Journal of biological chemistry* 273 (1998), pp. 24898-24905.

D. Boehm, V. Calvanese, R.D. Dar, S. Xing, S. Schroeder, L. Martins, K. Aull, P.C. Li, V. Planelles, J.E. Bradner, M.M. Zhou, R.F. Siliciano, L. Weinberger, E. Verdin and M. Ott, BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism, *Cell cycle* 12 (2013), pp. 452-462.

A. Bosque and V. Planelles, Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells, *Blood* 113 (2009), pp. 58-65.

P.U. Cameron, S. Saleh, G. Sallmann, A. Solomon, F. Wightman, V.A. Evans, G. Boucher, E.K. Haddad, R.P. Sekaly, A.N. Harman, J.L. Anderson, K.L. Jones, J. Mak, A.L. Cunningham, A. Jaworowski and S.R. Lewin, Establishment of HIV-1 latency in resting CD4+ T cells depends on chemokine-induced changes in the actin cytoskeleton, *Proceedings of the National Academy of Sciences of the United States of America* 107 (2010), pp. 16934-16939.

D. Carroll-Anzinger, A. Kumar, V. Adarichev, F. Kashanchi and L. Al-Harthi, Human immunodeficiency virus-restricted replication in astrocytes and the ability of gamma interferon to modulate this restriction are regulated by a downstream effector of the Wnt signaling pathway, *Journal of virology* 81 (2007), pp. 5864-5871.

T.W. Chun, L. Carruth, D. Finzi, X. Shen, J.A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T.C. Quinn, Y.H. Kuo, R. Brookmeyer, M.A. Zeiger, P. Barditch-Crovo and R.F. Siliciano, Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection, *Nature* 387 (1997), pp. 183-188.

B.A. DeChristopher, B.A. Loy, M.D. Marsden, A.J. Schrier, J.A. Zack and P.A. Wender, Designed, synthetically accessible bryostatin analogues potently induce activation of latent HIV reservoirs in vitro, *Nature chemistry* 4 (2012), pp. 705-710.

L. Deng, C. de la Fuente, P. Fu, L. Wang, R. Donnelly, J.D. Wade, P. Lambert, H. Li, C.G. Lee and F. Kashanchi, Acetylation of HIV-1 Tat by CBP/P300 increases transcription of integrated HIV-1 genome and enhances binding to core histones, *Virology* 277 (2000), pp. 278-295.

F. Dequiedt, H. Kasler, W. Fischle, V. Kiermer, M. Weinstein, B.G. Herndier and E. Verdin, HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis, *Immunity* 18 (2003), pp. 687-698.

F. Dequiedt, J. Van Lint, E. Lecomte, V. Van Duppen, T. Seufferlein, J.R. Vandenheede, R. Wattiez and R. Kettmann, Phosphorylation of histone deacetylase 7 by protein kinase D mediates T cell receptor-induced Nur77 expression and apoptosis, *The Journal of experimental medicine* 201 (2005), pp. 793-804.

M. Fischer, B. Joos, B. Hirschel, G. Bleiber, R. Weber, H.F. Gunthard and H.I.V.C.S. Swiss, Cellular viral rebound after cessation of potent antiretroviral therapy predicted by levels of multiply spliced HIV-1 RNA encoding nef, *The Journal of infectious diseases* 190 (2004), pp. 1979-1988.

C. Gekas, K.E. Rhodes, L.M. Gereige, H. Helgadottir, R. Ferrari, S.K. Kurdistani, E. Montecino-Rodriguez, R. Bassel-Duby, E. Olson, A.V. Krivtsov, S. Armstrong, S.H. Orkin, M. Pellegrini and H.K. Mikkola, Mef2C is a lineage-restricted target of Scl/Tal1 and regulates megakaryopoiesis and B-cell homeostasis, *Blood* 113 (2009), pp. 3461-3471.

C.M. Haggerty, E. Pitt and R.F. Siliciano, The latent reservoir for HIV-1 in resting CD4+ T cells and other viral reservoirs during chronic infection: insights from treatment and treatment-interruption trials, *Current opinion in HIV and AIDS* 1 (2006), pp. 62-68.

G. He and D.M. Margolis, Counterregulation of chromatin deacetylation and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) by the HIV-1 repressor YY1 and HIV-1 activator Tat, *Molecular and cellular biology* 22 (2002), pp. 2965-2973.
L.J. Henderson, S.D. Narasipura, V. Adarichev, F. Kashanchi and L. Al-Harthi, Identification of novel T cell factor 4 (TCF-4) binding sites on the HIV long terminal repeat which associate with TCF-4, beta-catenin, and SMAR1 to repress HIV transcription, *Journal of virology* 86 (2012a), pp. 9495-9503.
L.J. Henderson, A. Sharma, M.C. Monaco, E.O. Major and L. Al-Harthi, Human immunodeficiency virus type 1 (HIV-1) transactivator of transcription through its intact core and cysteine-rich domains inhibits Wnt/beta-catenin signaling in astrocytes: relevance to HIV neuropathogenesis, *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32 (2012b), pp. 16306-16313.
L.F. Johnson, J. Mossong, R.E. Dorrington, M. Schomaker, C.J. Hoffmann, O. Keiser, M.P. Fox, R. Wood, H. Prozesky, J. Giddy, D.B. Garone, M. Cornell, M. Egger, A. Boulle and A.S.A.C. International Epidemiologic Databases to Evaluate, Life expectancies of South African adults starting antiretroviral treatment: collaborative analysis of cohort studies, *PLoS medicine* 10 (2013), p. e1001418.
K.A. Jones and B.M. Peterlin, Control of RNA initiation and elongation at the HIV-1 promoter, *Annu Rev Biochem* 63 (1994), pp. 717-743.
A. Jordan, D. Bisgrove and E. Verdin, HIV reproducibly establishes a latent infection after acute infection of T cells in vitro, *The EMBO journal* 22 (2003), pp. 1868-1877.
A. Jordan, P. Defechereux and E. Verdin, The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation, *The EMBO journal* 20 (2001), pp. 1726-1738.
M. Kameoka, Y. Kameoka, P. Utachee, T. Kurosu, P. Sawanpanyalert, K. Ikuta and W. Auwanit, Short communication: RNA interference directed against Axin1 upregulates human immunodeficiency virus type 1 gene expression by activating the Wnt signaling pathway in HeLa-derived J111 cells, *AIDS research and human retroviruses* 25 (2009), pp. 1005-1011.
M. Kameoka, Y. Kitagawa, P. Utachee, P. Jinnopat, P. Dhepakson, P. Isarangkura-na-ayuthaya, K. Tokunaga, H. Sato, J. Komano, N. Yamamoto, S. Oguchi, Y. Natori and K. Ikuta, Identification of the suppressive factors for human immunodeficiency virus type-1 replication using the siRNA mini-library directed against host cellular genes, *Biochem Biophys Res Commun* 359 (2007), pp. 729-734.
H.G. Kasler and E. Verdin, Histone deacetylase 7 functions as a key regulator of genes involved in both positive and negative selection of thymocytes, *Molecular and cellular biology* 27 (2007), pp. 5184-5200.
H.G. Kasler, B.D. Young, D. Mottet, H.W. Lim, A.M. Collins, E.N. Olson and E. Verdin, Histone deacetylase 7 regulates cell survival and TCR signaling in CD4/CD8 double-positive thymocytes, *Journal of immunology* 186 (2011), pp. 4782-4793.
C. Katlama, S.G. Deeks, B. Autran, J. Martinez-Picado, J. van Lunzen, C. Rouzioux, M. Miller, S. Vella, J.E. Schmitz, J. Ahlers, D.D. Richman and R.P. Sekaly, Barriers to a cure for HIV: new ways to target and eradicate HIV-1 reservoirs, *Lancet* 381 (2013), pp. 2109-2117.
D. Khiem, J.G. Cyster, J.J. Schwarz and B.L. Black, A p38 MAPK-MEF2C pathway regulates B-cell proliferation, *Proceedings of the National Academy of Sciences of the United States of America* 105 (2008), pp. 17067-17072.
T.L. Kieffer, P. Kwon, R.E. Nettles, Y. Han, S.C. Ray and R.F. Siliciano, G-->A hypermutation in protease and reverse transcriptase regions of human immunodeficiency virus type 1 residing in resting CD4+ T cells in vivo, *Journal of virology* 79 (2005), pp. 1975-1980.
R.E. Kiernan, C. Vanhulle, L. Schiltz, E. Adam, H. Xiao, F. Maudoux, C. Calomme, A. Burny, Y. Nakatani, K.T. Jeang, M. Benkirane and C. Van Lint, HIV-1 tat transcriptional activity is regulated by acetylation, *The EMBO journal* 18 (1999), pp. 6106-6118.
J.Y. Kim, F. Gonzalez-Scarano, S.L. Zeichner and J.C. Alwine, Replication of type 1 human immunodeficiency viruses containing linker substitution mutations in the -201 to -130 region of the long terminal repeat, *Journal of virology* 67 (1993), pp. 1658-1662.
Y.D. Korin, D.G. Brooks, S. Brown, A. Korotzer and J.A. Zack, Effects of prostratin on T-cell activation and human immunodeficiency virus latency, *Journal of virology* 76 (2002), pp. 8118-8123.
A. Kumar, A. Zloza, R.T. Moon, J. Watts, A.R. Tenorio and L. Al-Harthi, Active beta-catenin signaling is an inhibitory pathway for human immunodeficiency virus replication in peripheral blood mononuclear cells, *Journal of virology* 82 (2008), pp. 2813-2820.
G.M. Laird, E.E. Eisele, S.A. Rabi, J. Lai, S. Chioma, J.N. Blankson, J.D. Siliciano and R.F. Siliciano, Rapid quantification of the latent reservoir for HIV-1 using a viral outgrowth assay, *PLoS pathogens* 9 (2013), p. e1003398.
K.G. Lassen, A.M. Hebbeler, D. Bhattacharyya, M.A. Lobritz and W.C. Greene, A flexible model of HIV-1 latency permitting evaluation of many primary CD4 T-cell reservoirs, *PloS one* 7 (2012), p. e30176.

S.R. Lewin, M. Vesanen, L. Kostrikis, A. Hurley, M. Duran, L. Zhang, D.D. Ho and M. Markowitz, Use of real-time PCR and molecular beacons to detect virus replication in human immunodeficiency virus type 1-infected individuals on prolonged effective antiretroviral therapy, *Journal of virology* 73 (1999), pp. 6099-6103.
P. Leyes, E. Martinez, M. Larrousse, M. Cofan, J. Trabal, A.M. Perez-Heras, M.T. Forga and E. Ros, Effects of ezetimibe on cholesterol metabolism in HIV-infected patients with protease inhibitor-associated dyslipidemia: a single-arm intervention trial, *BMC infectious diseases* 14 (2014), p. 497.
M. Lichterfeld, R.T. Gandhi, R.P. Simmons, T. Flynn, A. Sbrolla, X.G. Yu, N. Basgoz, S. Mui, K. Williams, H. Streeck, N. Burgett-Yandow, G. Roy, M. Janssens, L. Pedneault, P. Vandepapeliere, M. Koutsoukos, M.A. Demoitie, P. Bourguignon, L. McNally, G. Voss and M. Altfeld, Induction of strong HIV-1-specific CD4+ T-cell responses using an HIV-1 gp120/NefTat vaccine adjuvanted with AS02A in antiretroviral-treated HIV-1-infected individuals, *Journal of acquired immune deficiency syndromes* 59 (2012), pp. 1-9.
Y.C. Lin, S. Jhunjhunwala, C. Benner, S. Heinz, E. Welinder, R. Mansson, M. Sigvardsson, J. Hagman, C.A. Espinoza, J. Dutkowski, T. Ideker, C.K. Glass and C. Murre, A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate, *Nature immunology* 11 (2010), pp. 635-643.
T. Mahmoudi, M. Parra, R.G. Vries, S.E. Kauder, C.P. Verrijzer, M. Ott and E. Verdin, The SWI/SNF chromatin-remodeling complex is a cofactor for Tat transactivation of the HIV promoter, *The Journal of biological chemistry* 281 (2006), pp. 19960-19968.
C.D. Mao and S.W. Byers, Cell-context dependent TCF/LEF expression and function: alternative tales of repression, de-repression and activation potentials, *Crit Rev Eukaryot Gene Expr* 21 (2011), pp. 207-236.
D.M. Margolis, How Might We Cure HIV?, *Current infectious disease reports* 16 (2014), p. 392.
G. Marzio, M. Tyagi, M.I. Gutierrez and M. Giacca, HIV-1 tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter, *Proceedings of the National Academy of Sciences of the United States of America* 95 (1998), pp. 13519-13524.
R.F. McKnight, M. Adida, K. Budge, S. Stockton, G.M. Goodwin and J.R. Geddes, Lithium toxicity profile: a systematic review and meta-analysis, *Lancet* 379 (2012), pp. 721-728.
L. Mendes, D. Silva, C. Miranda, J. Sa, L. Duque, N. Duarte, P. Brito, L. Bernardino and J. Pocas, Impact of HIV infection on cardiac deformation, *Revista portuguesa de cardiologia : orgao oficial da Sociedade Portuguesa de Cardiologia = Portuguese journal of cardiology : an official journal of the Portuguese Society of Cardiology* 33 (2014), pp. 501-509.
S. Micheva-Viteva, Y. Kobayashi, L.C. Edelstein, A.L. Pacchia, H.L. Lee, J.D. Graci, J. Breslin, B.D. Phelan, L.K. Miller, J.M. Colacino, Z. Gu, Y. Ron, S.W. Peltz and J.P. Dougherty, High-throughput screening uncovers a compound that activates latent HIV-1 and acts cooperatively with a histone deacetylase (HDAC) inhibitor, *The Journal of biological chemistry* 286 (2011), pp. 21083-21091.
R.T. Mitsuyasu, T.C. Merigan, A. Carr, J.A. Zack, M.A. Winters, C. Workman, M. Bloch, J. Lalezari, S. Becker, L. Thornton, B. Akil, H. Khanlou, R. Finlayson, R. McFarlane, D.E. Smith, R. Garsia, D. Ma, M. Law, J.M. Murray, C. von Kalle, J.A. Ely, S.M. Patino, A.E. Knop, P. Wong, A.V. Todd, M. Haughton, C. Fuery, J.L. Macpherson, G.P. Symonds, L.A. Evans, S.M. Pond and D.A. Cooper, Phase 2 gene therapy trial of an anti-HIV ribozyme in autologous CD34+ cells, *Nature medicine* 15 (2009), pp. 285-292.
A. Mohd-Sarip, F. Venturini, G.E. Chalkley and C.P. Verrijzer, Pleiohomeotic can link polycomb to DNA and mediate transcriptional repression, *Molecular and cellular biology* 22 (2002), pp. 7473-7483.
S.D. Narasipura, L.J. Henderson, S.W. Fu, L. Chen, F. Kashanchi and L. Al-Harthi, Role of beta-catenin and TCF/LEF family members in transcriptional activity of HIV in astrocytes, *Journal of virology* 86 (2012), pp. 1911-1921.
M.N. Navarro, J. Goebel, C. Feijoo-Carnero, N. Morrice and D.A. Cantrell, Phosphoproteomic analysis reveals an intrinsic pathway for the regulation of histone deacetylase 7 that controls the function of cytotoxic T lymphocytes, *Nature immunology* 12 (2011), pp. 352-361.
U. O'Doherty, W.J. Swiggard, D. Jeyakumar, D. McGain and M.H. Malim, A sensitive, quantitative assay for human immunodeficiency virus type 1 integration, *Journal of virology* 76 (2002), pp. 10942-10950.
M. Ott, M. Schnolzer, J. Garnica, W. Fischle, S. Emiliani, H.R. Rackwitz and E. Verdin, Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity, *Current biology : CB* 9 (1999), pp. 1489-1492.
D. Pandelo Jose, K. Bartholomeeusen, R.D. da Cunha, C.M. Abreu, J. Glinski, T.B. da Costa, A.F. Bacchi Rabay, L.F. Pianowski Filho, L.W. Dudycz, U. Ranga, B.M. Peterlin, L.F. Pianowski, A. Tanuri and R.S. Aguiar, Reactivation of latent HIV-1 by new semi-synthetic ingenol esters, *Virology* 462-463 (2014), pp. 328-339.
M. Parra, H. Kasler, T.A. McKinsey, E.N. Olson and E. Verdin, Protein kinase D1 phosphorylates HDAC7 and induces its nuclear export after T-cell receptor activation, *The Journal of biological chemistry* 280 (2005), pp. 13762-13770.
M. Parra, T. Mahmoudi and E. Verdin, Myosin phosphatase dephosphorylates HDAC7, controls its nucleocytoplasmic shuttling, and inhibits apoptosis in thymocytes, *Genes & development* 21 (2007), pp. 638-643.
A.O. Pasternak, K.W. Adema, M. Bakker, S. Jurriaans, B. Berkhout, M. Cornelissen and V.V. Lukashov, Highly sensitive methods based on seminested real-time reverse transcription-PCR for quantitation of human immunodeficiency virus type 1 unspliced and multiply spliced RNA and proviral DNA, *Journal of clinical microbiology* 46 (2008), pp. 2206-2211.

A.O. Pasternak, S. Jurriaans, M. Bakker, J.M. Prins, B. Berkhout and V.V. Lukashov, Cellular levels of HIV unspliced RNA from patients on combination antiretroviral therapy with undetectable plasma viremia predict the therapy outcome, *PloS one* 4 (2009), p. e8490.
R. Pearson, Y.K. Kim, J. Hokello, K. Lassen, J. Friedman, M. Tyagi and J. Karn, Epigenetic silencing of human immunodeficiency virus (HIV) transcription by formation of restrictive chromatin structures at the viral long terminal repeat drives the progressive entry of HIV into latency, *J Virol* 82 (2008), pp. 12291-12303.
L.A. Pereira, K. Bentley, A. Peeters, M.J. Churchill and N.J. Deacon, A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter, *Nucleic acids research* 28 (2000), pp. 663-668.
M. Perez, A.G. de Vinuesa, G. Sanchez-Duffhues, N. Marquez, M.L. Bellido, M.A. Munoz-Fernandez, S. Moreno, T.P. Castor, M.A. Calzado and E. Munoz, Bryostatin-1 synergizes with histone deacetylase inhibitors to reactivate HIV-1 from latency, *Current HIV research* 8 (2010), pp. 418-429.
M. Pirs, B. Erzen, M. Sabovic, P. Karner, L. Vidmar, M. Poljak, B. Jug, M. Mikac and J. Tomazic, Early atherosclerosis in HIV-infected patients below the age of 55 years: Slovenian national study, *Wiener klinische Wochenschrift* 126 (2014a), pp. 263-269.
M. Pirs, B. Jug, B. Erzen, M. Sabovic, P. Karner, M. Poljak and J. Tomazic, Cardiovascular risk assessment in HIV-infected male patients: a comparison of Framingham, SCORE, PROCAM and DAD risk equations, *Acta dermatovenerologica Alpina, Pannonica, et Adriatica* 23 (2014b), pp. 43-47.
M.C. Puertas, M. Salgado, S. Moron-Lopez, D. Ouchi, J.A. Munoz-Moreno, J. Molto, B. Clotet and J. Martinez-Picado, Effect of lithium on HIV-1 expression and proviral reservoir size in the CD4+ T cells of antiretroviral therapy suppressed patients, *Aids* 28 (2014), pp. 2157-2159.
J.P. Routy, C.L. Tremblay, J.B. Angel, B. Trottier, D. Rouleau, J.G. Baril, M. Harris, S. Trottier, J. Singer, N. Chomont, R.P. Sekaly and M.R. Boulassel, Valproic acid in association with highly active antiretroviral therapy for reducing systemic HIV-1 reservoirs: results from a multicentre randomized clinical study, *HIV medicine* 13 (2012), pp. 291-296.
D.S. Ruelas and W.C. Greene, An integrated overview of HIV-1 latency, *Cell* 155 (2013), pp. 519-529.
G.K. Sahu and M.W. Cloyd, Latent HIV in primary T lymphocytes is unresponsive to histone deacetylase inhibitors, *Virology journal* 8 (2011), p. 400.
S. Saleh, A. Solomon, F. Wightman, M. Xhilaga, P.U. Cameron and S.R. Lewin, CCR7 ligands CCL19 and CCL21 increase permissiveness of resting memory CD4+ T cells to HIV-1 infection: a novel model of HIV-1 latency, *Blood* 110 (2007), pp. 4161-4164.
M. Salgado, P. Lopez-Romero, S. Callejas, M. Lopez, P. Labarga, A. Dopazo, V. Soriano and B. Rodes, Characterization of host genetic expression patterns in HIV-infected individuals with divergent disease progression, *Virology* 411 (2011), pp. 103-112.
G. Sanchez, X. Xu, J.C. Chermann and I. Hirsch, Accumulation of defective viral genomes in peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected individuals, *Journal of virology* 71 (1997), pp. 2233-2240.
A.R. Schroder, P. Shinn, H. Chen, C. Berry, J.R. Ecker and F. Bushman, HIV-1 integration in the human genome favors active genes and local hotspots, *Cell* 110 (2002), pp. 521-529.
L. Shan and R.F. Siliciano, From reactivation of latent HIV-1 to elimination of the latent reservoir: the presence of multiple barriers to viral eradication, *BioEssays : news and reviews in molecular, cellular and developmental biology* 35 (2013), pp. 544-552.
L. Shen and R.F. Siliciano, Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection, *The Journal of allergy and clinical immunology* 122 (2008), pp. 22-28.
P.L. Sheridan, C.T. Sheline, K. Cannon, M.L. Voz, M.J. Pazin, J.T. Kadonaga and K.A. Jones, Activation of the HIV-1 enhancer by the LEF-1 HMG protein on nucleosome-assembled DNA in vitro, *Genes & development* 9 (1995), pp. 2090-2104.
J.D. Siliciano, J. Lai, M. Callender, E. Pitt, H. Zhang, J.B. Margolick, J.E. Gallant, J. Cofrancesco, Jr., R.D. Moore, S.J. Gange and R.F. Siliciano, Stability of the latent reservoir for HIV-1 in patients receiving valproic acid, *The Journal of infectious diseases* 195 (2007), pp. 833-836.
J.D. Siliciano and R.F. Siliciano, Enhanced culture assay for detection and quantitation of latently infected, resting CD4+ T-cells carrying replication-competent virus in HIV-1-infected individuals, *Methods in molecular biology* 304 (2005), pp. 3-15.
J.D. Siliciano and R.F. Siliciano, HIV-1 eradication strategies: design and assessment, *Current opinion in HIV and AIDS* 8 (2013a), pp. 318-325.
J.D. Siliciano and R.F. Siliciano, A long-term latent reservoir for HIV-1: discovery and clinical implications, *The Journal of antimicrobial chemotherapy* 54 (2004), pp. 6-9.
J.D. Siliciano and R.F. Siliciano, Recent trends in HIV-1 drug resistance, *Current opinion in virology* 3 (2013b), pp. 487-494.

C.A. Spina, J. Anderson, N.M. Archin, A. Bosque, J. Chan, M. Famiglietti, W.C. Greene, A. Kashuba, S.R. Lewin, D.M. Margolis, M. Mau, D. Ruelas, S. Saleh, K. Shirakawa, R.F. Siliciano, A. Singhania, P.C. Soto, V.H. Terry, E. Verdin, C. Woelk, S. Wooden, S. Xing and V. Planelles, An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4+ T cells from aviremic patients, *PLoS pathogens* 9 (2013), p. e1003834.
A.M. Spivak and R.F. Siliciano, Using highly active antiretroviral therapy to decrease perioperative HIV-1 transmission risk, *Aids* 24 (2010), pp. 1603-1404; author reply 1604.
F.J. Staal and H. Clevers, Tales of the unexpected: Tcf1 functions as a tumor suppressor for leukemias, *Immunity* 37 (2012), pp. 761-763.
S. Stehling-Sun, J. Dade, S.L. Nutt, R.P. DeKoter and F.D. Camargo, Regulation of lymphoid versus myeloid fate 'choice' by the transcription factor Mef2c, *Nature immunology* 10 (2009), pp. 289-296.
B.J. Swanson, H.M. Jack and G.E. Lyons, Characterization of myocyte enhancer factor 2 (MEF2) expression in B and T cells: MEF2C is a B cell-restricted transcription factor in lymphocytes, *Molecular immunology* 35 (1998), pp. 445-458.
W.J. Swiggard, C. Baytop, J.J. Yu, J. Dai, C. Li, R. Schretzenmair, T. Theodosopoulos and U. O'Doherty, Human immunodeficiency virus type 1 can establish latent infection in resting CD4+ T cells in the absence of activating stimuli, *Journal of virology* 79 (2005), pp. 14179-14188.
M.M. Tiemessen, M.R. Baert, T. Schonewille, M.H. Brugman, F. Famili, D.C. Salvatori, J.P. Meijerink, U. Ozbek, H. Clevers, J.J. van Dongen and F.J. Staal, The nuclear effector of Wnt-signaling, Tcf1, functions as a T-cell-specific tumor suppressor for development of lymphomas, *PLoS biology* 10 (2012), p. e1001430.
M.M. Tiemessen and F.J. Staal, Wnt signaling in leukemias and myeloma: T-cell factors are in control, *Future oncology* 9 (2013), pp. 1757-1772.
M. Tyagi, R.J. Pearson and J. Karn, Establishment of HIV latency in primary CD4+ cells is due to epigenetic transcriptional silencing and P-TEFb restriction, *Journal of virology* 84 (2010), pp. 6425-6437.
C. Van Lint, S. Bouchat and A. Marcello, HIV-1 transcription and latency: an update, *Retrovirology* 10 (2013), p. 67.
C. Van Lint, S. Emiliani, M. Ott and E. Verdin, Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation, *The EMBO journal* 15 (1996), pp. 1112-1120.
J. van Lunzen, T. Glaunsinger, I. Stahmer, V. von Baehr, C. Baum, A. Schilz, K. Kuehlcke, S. Naundorf, H. Martinius, F. Hermann, T. Giroglou, S. Newrzela, I. Muller, F. Brauer, G. Brandenburg, A. Alexandrov and D. von Laer, Transfer of autologous gene-modified T cells in HIV-infected patients with advanced immunodeficiency and drug-resistant virus, *Molecular therapy : the journal of the American Society of Gene Therapy* 15 (2007), pp. 1024-1033.
G. Vanham and E. Van Gulck, Can immunotherapy be useful as a "functional cure" for infection with Human Immunodeficiency Virus-1?, *Retrovirology* 9 (2012), p. 72.
E. Verdin, P. Paras, Jr. and C. Van Lint, Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation, *The EMBO journal* 12 (1993), pp. 3249-3259.
E. Verdin and C. Van Lint, Internal transcriptional regulatory elements in HIV-1 and other retroviruses, *Cellular and molecular biology* 41 (1995), pp. 365-369.
M.L. Waterman and K.A. Jones, Purification of TCF-1 alpha, a T-cell-specific transcription factor that activates the T-cell receptor C alpha gene enhancer in a context-dependent manner, *New Biol* 2 (1990), pp. 621-636.
L.S. Weinberger, R.D. Dar and M.L. Simpson, Transient-mediated fate determination in a transcriptional circuit of HIV, *Nature genetics* 40 (2008), pp. 466-470.
L.A. Wheeler, R. Trifonova, V. Vrbanac, E. Basar, S. McKernan, Z. Xu, E. Seung, M. Deruaz, T. Dudek, J.I. Einarsson, L. Yang, T.M. Allen, A.D. Luster, A.M. Tager, D.M. Dykxhoorn and J. Lieberman, Inhibition of HIV transmission in human cervicovaginal explants and humanized mice using CD4 aptamer-siRNA chimeras, *The Journal of clinical investigation* 121 (2011), pp. 2401-2412.
R.A. White, 3rd, S.R. Quake and K. Curr, Digital PCR provides absolute quantitation of viral load for an occult RNA virus, *Journal of virological methods* 179 (2012), pp. 45-50.
P.R. Wilker, M. Kohyama, M.M. Sandau, J.C. Albring, O. Nakagawa, J.J. Schwarz and K.M. Murphy, Transcription factor Mef2c is required for B cell proliferation and survival after antigen receptor stimulation, *Nature immunology* 9 (2008), pp. 603-612.
B. Wortman, N. Darbinian, B.E. Sawaya, K. Khalili and S. Amini, Evidence for regulation of long terminal repeat transcription by Wnt transcription factor TCF-4 in human astrocytic cells, *Journal of virology* 76 (2002), pp. 11159-11165.
S. Xing, S. Bhat, N.S. Shroff, H. Zhang, J.A. Lopez, J.B. Margolick, J.O. Liu and R.F. Siliciano, Novel structurally related compounds reactivate latent HIV-1 in a bcl-2-transduced primary CD4+ T cell model without inducing global T cell activation, *The Journal of antimicrobial chemotherapy* 67 (2012), pp. 398-403.
S. Xing, C.K. Bullen, N.S. Shroff, L. Shan, H.C. Yang, J.L. Manucci, S. Bhat, H. Zhang, J.B. Margolick, T.C. Quinn, D.M. Margolis, J.D. Siliciano and R.F. Siliciano, Disulfiram reactivates latent HIV-1 in a Bcl-2-transduced primary CD4+ T cell model without inducing global T cell activation, *Journal of virology* 85 (2011), pp. 6060-6064.

H.C. Yang, S. Xing, L. Shan, K. O'Connell, J. Dinoso, A. Shen, Y. Zhou, C.K. Shrum, Y. Han, J.O. Liu, H. Zhang, J.B. Margolick and R.F. Siliciano, Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation, *The Journal of clinical investigation* 119 (2009), pp. 3473-3486.
H. Ying, Y. Zhang, X. Zhou, X. Qu, P. Wang, S. Liu, D. Lu and H. Zhu, Selective histonedeacetylase inhibitor M344 intervenes in HIV-1 latency through increasing histone acetylation and activation of NF-kappaB, *PloS one* 7 (2012), p. e48832.
J.J. Yu, T.L. Wu, M.K. Liszewski, J. Dai, W.J. Swiggard, C. Baytop, I. Frank, B.L. Levine, W. Yang, T. Theodosopoulos and U. O'Doherty, A more precise HIV integration assay designed to detect small differences finds lower levels of integrated DNA in HAART treated patients, *Virology* 379 (2008), pp. 78-86.

Summary in English

Finding a cure for the human immunodeficiency virus type 1 (HIV-1) is extremely challenging. Development of highly active anti-retroviral therapy (HAART), transformed HIV-1 infection from an acute syndrome into chronic disease. Although using HAART results in suppression of viral replication, it is not curative. High rates of HIV-1 replication return in infected patients when HAART is interrupted. The presence of a long-lived latent reservoir for HIV-1 in resting $CD4^+$ T cells allows the persistence of the virus despite therapy. This reservoir is composed of replication competent but transcriptionally silent virus, which is the major barrier toward HIV-1 eradication. Understanding the mechanisms that are involved in HIV-1 latency, and developing *in vitro* and *in vivo* models of latency have allowed the design and testing of therapies that target the latent reservoir. The current approach in the development a curative treatment for HIV-1 is called "*Shock and Kill*". This approach entails reactivation of latent HIV-1 using latency reversing agents (LRAs) followed by elimination of the activated virus by an effective host immune system, cytolytic T lymphocytes (CTL) or viral cytopathic effects. Therefore, the first and promising step in eradication of viral reservoirs, is reactivation of latent HIV-1. This process constitutes pharmacological approaches to target different mechanisms and pathways that are involved in maintenance of HIV-1 latency and its reactivation. In this thesis I focus on the *shock* part of *shock and kill* strategies. This research aims to investigate the molecular mechanisms involved in HIV-1 post-integration latency establishment and maintenance, and reactivation of latent HIV-1.

In **Chapter 2** we identified the mechanistic role of SWI/SNF in the establishment and maintenance of HIV-1 latency and its re-activation in latent HIV-1 infected cell models. SWI/SNF is one of the four ATP-dependent chromatin remodeling complex in mammals which contains two functionally distinct sub classes referred to as BAF and PBAF. We determined the nucleosome affinity of the HIV-1 LTR DNA sequence, and found a reverse correlation compared to the nucleosome positioning *in vivo*. Hypersensitive sites (DHS-1) displayed the highest nucleosome affinity, while the positioned nucleosomes displayed lower affinity for nucleosome formation. We examined the role of SWI/SNF in the nucleosome structure of the latent HIV-1 LTR. We found that establishment and maintenance of HIV-1 latency requires BAF, which removes a preferred nucleosome from DHS-1 to position the repressive nucleosome-1 over energetically unfavorable sequences. BAF depletion resulted in activation of the latent HIV-1. Our high resolution MNase nucleosomal mapping analysis showed a dramatic alteration in the LTR nucleosome profile after BAF depletion. Upon activation, BAF was lost from the HIV-1 promoter, while PBAF was specifically recruited by the HIV-1 transactivator protein TAT to facilitate HIV-1 transcription. Thus BAF and PBAF, recruited during different stages of the HIV-1 life cycle, display opposing function on the HIV-1 promoter. As a repressor of HIV-1, BAF may be an attractive therapeutic target to activate the latent reservoir in patients.

In **Chapter 3** we explored the role of the Wnt signaling pathway in activation of transcription of latent HIV-1. We found that activation of the Wnt pathway via different mechanisms including treatment with natural Wnt ligand or small molecule inhibitors resulted

in activation of latent HIV-1. Chromatin immunoprecipitation (ChIP) analysis showed that the Wnt molecular effectors LEF1 and β-catenin are recruited to the HIV-1 LTR after Wnt activation. Upon activation, we demonstrate that, nucleosomal structure on the latent HIV-1 LTR is dramatically restructured using high resolution MNase nucleosomal mapping. Lithium, one of the Wnt agonists in clinical use for treatment bipolar disease, activated HIV-1 transcription in latent HIV-1 infected primary $CD4^+$ T cells. Using HDAC inhibitors such as Valporoic acid (VPA) and Vorinostat (SAHA), currently under clinical investigation, in combination with Wnt activators synergistically enhanced the activation of the latent HIV-1 LTR. Thus, targeting the Wnt pathway by small molecules and Wnt agonists may be an attractive strategy in a combinatorial therapy aimed at activation of latent HIV-1.

In **Chapter 4** we investigated the mechanistic determinants of the functional specialization between the two TCF/LEF members, T cell factor1 (TCF1) and lymphoid enhancing factor 1 (LEF1), two molecular effectors of the Wnt signaling pathway, which are co-expressed in T cells. De-regulation of Wnt signaling is involved in formation of T cell leukemia. We found distinct isoform expression of TCF1 and LEF1 in $CD4^+$ T-ALL leukemic cell lines, including both the Wnt responsive isoform as well as the dominant negative forms. We used immunoprecipitation-mass spectrometry analysis to find novel interacting partners for LEF1 and TCF1 in the presence or absence of Wnt signaling in leukemic cell lines. Using RNA sequencing analysis, we determined target genes which are regulated by TCF1 and LEF1 by siRNA depletion and examined differentially expressed genes in the presence or absence of Wnt signaling. ChIP assays on two novel target genes from RNA sequencing analysis, showed differential recruitment of Wnt molecular effectors to their regulatory regions on the targets in response to Wnt stimulation. Thus, our results provide a mechanistic basis to the *in vivo* observed functional specialization for TCF1 and LEF1 in T cell leukemia.

In **Chapter 5** we addressed the molecular mechanism behind repression of non-lymphoid genes in B cells. We showed that the histone deacetylase HDAC7 was highly expressed in pre-B cells but dramatically down-regulated during differentiation to macrophages. Our microarray analysis demonstrated that expression of HDAC7 interfered with the gene transcriptional program characteristic of macrophages during cell differentiation. We described that presence of HDAC7 blocked the expression of key genes for macrophage-mediated specific functions. Our co-immunoprecipitation and chromatin immunoprecipitation analysis gave insight into the molecular mechanisms mediating HDAC7 repression in pre-B cells. We found specific interaction of HDAC7 with the transcription factor MEF2C in pre-B cells. HDAC7 was recruited to MEF2 binding sites located at the promoters of macrophage target genes. Thus, in B cells HDAC7 is a transcriptional repressor of undesirable genes.

Summary in Dutch / *Samenvatting in het Nederlands*

Het vinden van een remedie voor de humaan immunodeficiëntie virus type 1 (HIV-1) is zeer uitdagend. De ontwikkeling van hoog actieve anti-retrovirale therapie (HAART), getransformeerd HIV-1 infectie vanuit een moeilijke syndroom naar chronische ziekte. Hoewel het toedienen van HAART resulteert in de onderdrukking van virale replicatie, is het niet curatief. Hoge niveaus van HIV-1 replicatie keren terug in geïnfecteerde patiënten wanneer HAART wordt onderbroken. De aanwezigheid van een langlevend, latent reservoir voor HIV-1 in rustende $CD4^+$ T cellen zorgt dat het virus overleeft ondanks therapie. Dit reservoir bestaat uit replicatie-competent maar transcriptioneel onderdrukt virussen is de belangrijkste barrière voor het uitroeien van HIV-1. Inzicht in de mechanismen die betrokken zijn bij HIV-1 latentie en de ontwikkeling van *in vitro* en *in vivo* modellen van latentie hebben het mogelijk gemaakt therapieën die zich op het latente reservoir richten te ontwerpen en te testen. De huidige aanpak bij de ontwikkeling van een curatieve behandeling voor HIV-1 heet "*Shock and Kill*". Deze benadering behelst de re-activatie van het latent virus door gebruik te maken van latentie reactiverende middelen of wel Latency Reversing Agents (LRAs), gevolgd door eliminatie van het geactiveerde virus door een effectief gastheerimmuunsysteem, cytolytische T-lymfocyten (CTL) of virale cytopathische effecten. De eerste veelbelovende stap in het uitroeiing van virale reservoirs, is daarom re-activatie van latent HIV-1. Dit proces omvat farmacologische benaderingen waarmee verschillende mechanismen en routes die betrokken zijn bij het onderhoud van HIV-1 latentie en re-activatie gericht kunnen worden aangepakt. In dit proefschrift richt ik me op de "*Shock*" gedeelte van "*Shock and Kill*" strategie. Dit onderzoek heeft tot doel de moleculaire mechanismen die betrokken zijn bij bewerkstelligen en in stand houden van post-integratie HIV-1 latentie en de re-activatie van latent HIV-1.

In **hoofdstuk 2** identificeerden we de mechanistische rol van SWI / SNF in het opzetten en onderhouden van HIV-1 latentie en de re-activatie in latente HIV-1 geïnfecteerde cel modellen. SWI / SNF is één van de vier ATP-afhankelijke chromatine vervormings complexen in zoogdieren die twee functioneel verschillende subklassen bevat, genaamd BAF en PBAF. We bepaalden de nucleosoom affiniteit van de HIV-1 LTR DNA-sequentie, en vonden een omgekeerde correlatie tegenover de nucleosoom positionering *in vivo*. DNase overgevoelige gebieden (DNase Hypersensitivity Sites; DHS-1) laten de hoogste nucleosomen affiniteit zien, terwijl de gepositioneerde nucleosomen lagere affiniteit voor nucleosome formatie hebben. We onderzochten de rol van SWI / SNF in de nucleosoom structuur van de latente HIV-1 LTR. We vonden dat de oprichting en het onderhoud van HIV-1 latentie BAF vereist, die een voorkeur heeft om het nucleosoom van DHS-1 te verwijderen en daarmee het repressieve nucleosoom-1 op energetisch ongunstig sequenties te positioneren. Het verwijderen van BAF resulteerde in activatie van het latente HIV-1. Het hoge resolutie in kaart brengen van nucleosomale positionering door mideel van MNase behandeling toonde een dramatische verandering in het LTR nucleosoom profiel na BAF verwijdering. Na activatie, verdween BAF van de HIV-1 promotor, terwijl PBAF specifiek door de HIV-1 transactivator eiwit TAT werd aangetrokken om HIV-1 transcriptie vergemakkelijken. Dus BAF en PBAF, aangetrokken

tijdens verschillende stadia van de levenscyclus van HIV-1, tonen tegengestelde functie op de HIV-1 promoter. Als repressor van HIV-1, kan BAF een aantrekkelijk therapeutisch doelwit om het latent reservoir patiënten activeren.

In **hoofdstuk 3** wordt de rol van de Wnt-signaleringsroute onderzocht in de activering van de transcriptie van latente HIV-1. We vonden dat de activatie van de Wnt-signaleringsroute via verschillende methodes, waaronder de behandeling met natuurlijk Wnt ligand of kleine molecuul remmers leidt tot activering van latent HIV-1. Chromatine immunoprecipitatie (chip) analyse toonde aan dat de moleculaire effectoren van Wnt, LEF1 en β-catenine, worden gerekruteerd op de HIV-1 LTR na Wnt activering. We laten met behulp van hoge resolutie MNase nucleosomale mapping zien dat na activatie de nucleosomale structuur op de latente HIV-1 LTR drastisch wordt geherstructureerd. Lithium, een van de Wnt agonisten die klinisch toegepast bij de behandeling van bipolaire ziekte, activeert HIV-1 transcriptie in latent HIV-1 geïnfecteerde primaire $CD4^+$ T-cellen. Het gebruik van Histone deacetylerase (HDAC) inhibitoren zoals Valporoic acid (VPA) en Vorinostat (SAHA), die momenteel worden onderzocht voor klinische toepassing, in combinatie met Wnt activatoren heeft een synergistisch effect op de activering van de latente HIV-1 LTR. Daarom is het aangrijpen op de Wnt signalerings-route door kleine moleculen en Wnt agonisten een aantrekkelijke strategie in een combinatoriële therapie gericht op activering van latent HIV-1.

In **hoofdstuk 4** onderzochten we de mechanistische determinanten van de functionele specialisatie tussen de twee TCF / LEF-leden, T-cel factor1 (TCF1) en lymfoïde verbeteren factor 1 (LEF1), twee moleculaire effectoren van de Wnt signaleringsroute, die gelijktijdig worden geëxpresseerd in T-cellen. Deregulering van Wnt signalering is betrokken bij de vorming van T-cel leukemie. We hebben gezien dat er uitgesproken verschillende isovormen van TCF1 en LEF1 expressie in $CD4^+$ T-ALL leukemische cellijnen, waaronder zowel de Wnt responsieve isovorm en de dominant negatieve vormen. We hebben immunoprecipitatie-massa-spectrometrie-analyse toegepast om nieuwe interactie partners voor LEF1 en TCF1 in de aan- of afwezigheid van Wnt signalering in leukemische cellijnen te vinden. Met behulp van siRNA gemediëerde verwijdering hebben we met RNA-sequencing analyse vastgesteld dat target genen worden gereguleerd door TCF1 en LEF1 en onderzochten we differentiëel tot expressie gebrachte genen in de aanwezigheid of afwezigheid van Wnt-signalering. ChIP assays op twee nieuwe target genen die waren gevonden in de RNA sequencing analyse toonde differentiële werving van Wnt moleculaire effectoren op hun regulerende regionen op de target genen in reactie op Wnt stimulatie. Aldus geven onze resultaten een mechanistische basis voor de *in vivo* waargenomen functionele specialisatie TCF1 en LEF1 in T-cell leukemie. In **hoofdstuk 5** richten we ons op de moleculaire mechanismes achter onderdrukking van niet-lymfoïde genen in B cellen. We toonden aan dat de histondeacetylaseac HDAC7 zeer hoog tot expressie komt in pre-B-cellen, maar dramatisch neer-gereguleerd tijdens differentiatie naar macrofagen. Onze microarray analyse toonde aan dat de expressie van HDAC7 het gen transcriptieprogramma kenmerkend voor macrofagen tijdens cel-differentiatie belemmert. We beschreven dat de aanwezigheid van HDAC7 de expressie van

belangrijke genen voor macrofaag gemediëerde specifieke functies blokkeert. Onze co-immunoprecipitatie en chromatine immunoprecipitatie analyse gaf inzicht in de moleculaire mechanismen die repressie door HDAC7 in pre-B-cellen reguleren. We hebben specifieke interactie van HDAC7 met de transcriptiefactor MEF2C in pre-B-cellen gevonden. HDAC7 werd gerecruteerd naar MEF2 bindingsplaatsen gelegen in de promotor-regionen van macrofagen target genen. Dus in B cellen is HDAC7 een transcriptionele repressor van ongewenste genen.

Scientific Portfolio

Curriculum Vitae

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Education (Academic Preparation):

-2010-Present. PhD. Biomedical Sciences. ErasmusMC. Rotterdam, The Netherlands. Thesis: Molecular Mechanisms in Activation of Latent HIV-1. Promotor: Prof.dr. C.P. Verrijzer, Copromotor: Dr. T. Mahmoudi

-2000-2003. M.Sc. Biology, Genetics. Islamic Azad University, Sciences & Researches Branch, Tehran, Iran. Thesis: Molecular comparative study of UL8 and UL30 genes of native Herpes Simplex Virus Type1 (HSV-1) with KOS foreign strain. Supervisor: Dr. Majid Sadeghizadeh

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Professional History:

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-2004-2005. Academic lecturer in Genetic. Faculty of Basic Sciences, Islamic Azad University, Saveh Branch, Iran.

-2003-2004. Scientific Board of Tuba-Negin Co. Ltd,Tehran – Iran.

PhD portfolio

Courses (educational, training, research and management):

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-2005. Principles of Quality Control (SOP) in Laboratory. Day General Hospital Laboratory, Tehran-Iran

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-2001. Research Methodology (Statistics and SPSS). Faculty of Basic Sciences, Islamic Azad University, Sciences &Researches Branch, Tehran-Iran

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-**2013. Nov 19**. 7th NCHIV conference, Amsterdam, The Netherlnads. **Poster presentation:** New Transcription Regulatory Mechanisms of Latent HIV-1 LTR

-2013. Sep 19. 23rd Medical Genetic Cluster-Symposium, Rotterdam, The Netherlands. **Oral presentation:** Activation of Wnt signaling pathway by natural ligands and small molecule inhibitors activates the latent HIV.

-2011. July 14-17. 18th Work shop of Medical Genetics Cluster, Maastricht, The Netherlands. **Poster presentation**: Repressive LTR nucleosome positioning by the BAF complex is required for HIV latency.

-2004. Feb 13-15. The 2nd Congress of Virology of the Iranian Society of Virology, Tehran, Iran. **Poster presentation:** Molecular comparative study of UL8 and UL30 genes of native Herpes Simplex Virus Type1 (HSV-1) with KOS foreign strain.

List of Publications

-2014. Rafati H, de Crignis E, LeMasters E, Boucher C, Vries R.G.J, Mahmoudi, T. Targeting the Wnt pathway for activation activates latent HIV-1 and is synergistically enhanced upon concomitant inhibition of histone deacetylation. (Submitted)

-2014. Rafati T, Ne E, Solaimani-Kartalaei P, Bezstarosti K, Demmers J, Mahmoudi T. Functional specialization and differential gene regulation of TCF1 and LEF1 in T-ALL SupT1 leukemic cells. (Submitted)

-2013. Atlasi Y, Noori R, Gaspar C, Franken P, Sacchetti A, **Rafati H,** Mahmoudi T, Decraene C, Calin GA, Merrill BJ, Fodde R. Wnt signaling regulates the lineage differentiation potential of mouse embryonic stem cells through Tcf3 down-regulation. PLoS Genet, 9(5), e1003424.

-2013. Barneda-Zahonero B, Román-González L, Collazo O, **Rafati H**, Islam AB, Bussmann LH, di Tullio A, De Andres L, Graf T, López-Bigas N, Mahmoudi T, Parra M.. HDAC7 is a repressor of myeloid genes whose downregulation is required for transdifferentiation of pre-B cells into macrophages. PLoS Genet, 9(5), e1003503

-2011. Rafati H, Parra M, Hakre S, Moshkin Y, Verdin E, Mahmoudi T. Repressive LTR nucleosome positioning by the BAF complex is required for HIV latency. PLoS biology, 9(11), e1001206

-2006. Abolhassani, N, Sadeghi Zadeh, M , Javadi, Gh, **Rafati, H.** Comparative Molecular PCR-RFLP Study of Native Herpes Simplex Virus Type 1 (HSV-1) with KOS Strain Iranian Biomedical Journal, 10 (3), 157-161

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Hale

Molecular mechanisms in activation of Latent HIV-1

1- Components of BAF complex are a putative therapeutic targets to deplete the latent reservoir in HIV infected patients (*This thesis*).

2- Targeting the Wnt pathway by small molecules and Wnt agonists is an attractive strategy in the HIV combinatorial therapy (*This thesis*).

3- Functional synergism between Wnt activators and HDAC inhibitors results in activation of the latent HIV (*This thesis*).

4- Mechanistic evidence does exist behind the functional specialization observed for TCF1 and LEF1 (*This thesis*).

5- In B cells HDAC7 is a key transcriptional repressor (*This thesis*).

6- Chromatin remodeling complexes have well-established roles in a wide range of chromosomal processes, including transcriptional regulation and chromatin assembly. (*Wang, Curr Top Microbiol Immunol., 2003, 274:143-69*)

7- The combined action of transcription factors located on the HIV- long terminal repeat, in concert with the viral transregulatory protein, TAT determines the degree of viral expression at the transcriptional level. (*Verdin, et al., Proc. Natl. Acad. Sci. USA 1990, 87:4874-4878*)

8- Reactivation of latent HIV-1 gene expression is a promising strategy for the depletion of latent viral reservoirs. (*Chunn and Fauci, Proc Natl Acad Sci USA. 1999; 96(20): 10958–10961*)

9- “The first step toward success will be the biggest one.” (*Dennis Waitley*)

10- “The more you learn, the more you realize how little you know.” (*Socrates*)

11- “Life is a balance of holding on and letting go.” (*Rumi*)

Haleh Rafati, Rotterdam, 04-12-2014